Characterization of drought stress regulator *CBF/DREB* genes in *Hordeum vulgare*: Expression analysis in ten different barley cultivars

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DECLARATION

I hereby declare that this submission is my own work and effort, except where explicitly otherwise in the text or in the bibliography.

Bonn, März 2013

Jian Shen

DEDICATION AND ACKNOWLEDGEMENTS

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ABBREVIATIONS

A	Adenine
ABA	Abscisic acid
ABF	ABA-binding factor
ABRE	ABA-responsive element
Alix	ALG-2 interacting protein X,
AP2	APETALA2
bp	Nucleotide base pair
bZIP	Basic leucine zipper
C	Cytosine
CAM	Crassulacean acid metabolism
CaMV	Cauliflower mosaic virus
CBF	C-repeat-binding factor
ChIP	Chromatin immunoprecipitation
cDNA	Complementary DNA
CRT	C-repeat
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration responsive element
DREB	DRE-binding protein
EREBP	Ethylene-responsive element binding protein
ERF	Ethylene-responsive element binding factor
EDTA	Ethylenediaminetetraacetate
FAO	Food and Agriculture Organization of the United Nations
fwd	Forward
G	Guanine
g	Gram
g	Acceleration
h	Hour
HELP-assay	*Hpa*II tiny fragment Enrichment by Ligation-mediated PCR
Hsps	Heat shock proteins
IPCC	Intergovernmental Panel on Climate Change
kb	Kilobase
LB	Luria and Bertani medium
LEA	Late Embryogenesis Abundant
LTRE	Low temperature-responsive element
MBD	Methylated-DNA binding domain
MeDIP	Methylated-DNA immunoprecipitation
min	Minute

ml	Milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
MYB	Myeloblastosis
MYC	Myelocytomatosis
mRNA	Messenger RNA
MS	Murashige and Skoog
NASS	National Agricultural Statistics Service
NCDC	National Climatic Data Center
nm	Nanometers
OD	Optical density
Oligo (dT)	Oligodeoxythymidylic acid
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
QTL	Quantitative Loci Trait
rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Rounds per minute
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
RT	Room temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
T	Thymine
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TE	Tris (10mM)-EDTA (1 mM)
TMV	Tobacco mosaic virus
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume

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ABSTRACT

Improving crop yield under water deficit environments is an urgent task for crop breeding worldwide. Many efforts have been made to identify key regulators of plant drought stress responses. Most of these studies have been performed with the model plant *Arabidopsis thaliana* under controlled laboratory conditions. Little work has been done to investigate the functions of these key regulators in the improvement of drought tolerance in crops, especially under field conditions. In the present study, ten barley *CBF/DREB* gene transcripts (*CBF1*, *CBF2*, *CBF3*, *CBF4*, *CBF6*, *CBF11*, *DREB1*, *DRF1.1*, *DRF1.3* and *DRF2*) were isolated from different barley cultivars by using gene-specific primers. Phylogenetic analysis showed that all candidate *CBF/DREB* genes can be grouped into three phylogenetic subgroups, designated *HvCBF1*, *HvCBF4* and *HvDREB1*. The phylogenetic distribution of barley *CBF/DREB* genes in the reconciled tree coincides with their responses to drought. A strongly constitutive expression of the *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes upon drought has been identified in this study, which is contrary to the published data in the literature. This may be due to the age and physiological state of the plants. By analyzing the *CBF* expression patterns upon drought in leaf tissues of plants grown in the field, we found that barley cultivars respond to drought at different time points. Monitoring the expression of *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* transcripts upon drought in field grown material revealed that rapid dehydration triggered the reduction of the above-mentioned transcripts. This was further validated by experiments performed under controlled laboratory conditions. Furthermore, tissue- and age-dependent expression has been identified in barley *HvCBF1*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes, indicating that the *CBF* transcription factors are developmentally regulated. Importantly, in contrast to the common thought, our analyses of methylation patterns in the *HvCBF1* promoter showed that DNA methylation within the promoter does not always lead to down-regulation of the corresponding gene. Finally, a more efficient and non-invasive method to analyze the barley root architecture and development has been initiated with help and permissions from Prof. Dr. Philip Benfey, Duke University, Durham NC, USA.

1. INTRODUCTION

1.1 Impact of climate change on crop yield

One of the major challenges in agriculture and food security today is the global water shortage caused by population growth and climate change. Climate change, especially global warming caused by increasing concentrations of atmospheric carbon dioxide produced by burning fossil fuels and deforestation, leads to increases in aridity and desertification in many areas worldwide (Petit *et al.*, 1999; Lu *et al.*, 2007). The Intergovernmental Panel on Climate Change (IPCC) projects indicated that atmospheric carbon dioxide concentration has risen from ~260 parts per million (ppm) to ~380 ppm over the past 150 years (Griggs and Noguer, 2002). Correspondingly, earth's mean surface temperature has increased by approximately 0.8 ℃ over the past 100 years, with about two-thirds of the increase occurring over just the past three decades (Carnesale *et al.*, 2011). Previous studies revealed that the global crop productivity declines with the increasing earth ̓s mean surface temperature. The global barley yield decreases 10% with every 1 ℃ increase in earth ̓s mean surface temperature (Adams *et al.*, 1998). Moreover, extreme weather events caused by global warming become more frequent, including extremely dry periods and more heavy rainfall events. According to a special report about extreme weather events by IPCC, global warming exacerbates drought in many areas worldwide, although it is hard to say how much (Field *et al.*, 2012). In 2012, the United States was engulfed in drought. A special report released by the National Climatic Data Center (NCDC) in July indicated that the drought in 2012 was among the ten worst drought disasters over the past century and the worst over the past 50 years (www.ncdc.noaa.gov/sotc/national/2012/7/supplemental/page-5). Approximately 80% of agricultural land in the USA was experiencing drought in 2012. Correspondingly, the crop yields in 2012 declined dramatically in the USA. According to a report released by USDA's National Agricultural Statistics Service (NASS), a massive reduction in corn yields account for 27.5%, while production estimates for soybeans and sorghums fell 7% and 24%, respectively. The global grain supplies were looking therefore tighter-than-expected and prices for some grain crops even exceeded historical record levels over the past several months (www.ers.usda.gov/topics/in-the-news/us-drought-2012-farm-and-food-impacts.aspx#crop). The rapid population growth results in dramatically increased demand for food. Food and Agriculture Organization of the United Nations (FAO) predicted that the global grain production and consumption are projected to increase by approximately 70% by 2050 and to almost double in developing countries. Agriculture activities account for 70% of global water

consumption for irrigation. Irrigated agriculture represents 20% of the total cultivated land but contributes 40% of the total food produced worldwide (Turral *et al.*, 2009). By 2025, 1.8 billion people will be living in countries or regions with absolute water scarcity, and two-thirds of the world's population could be living under water stressed conditions (www.unwater.org/ worldwaterday/faqs.html). Therefore, to understand how crop plants cope with drought stress in conjunction with increases in crop yields under drought conditions plays a crucial role in the increased food demand and in the food security.

1.2 Responses of plants to drought stress

Plants are sessile. This requires plants to recognize the adverse environmental changes rapidly and respond accordingly to minimize damages under extreme conditions. Abiotic stresses such as drought, high salinity and cold adversely affect plant growth and yield. To survive, plants are thought to develop common mechanisms to respond and to adapt these stresses, not only at molecular and cellular levels, but also at physiological and biochemical levels (Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). Due to its high magnitude and wide distribution, drought is one of the greatest environmental constraints worldwide, to which the plants are subjected (see 1.1). The susceptibility of plants to water deficit depends on severity and duration of the stress, interactions between different stress factors, plant genotypes and stages of plant growth and development (Kozlowski, 1968; Hsiao, 1973; Bartels and Souer, 2004). Drought triggers a wide range of plant responses, which can be grouped into three categories, namely physiological responses and morphological adpatations, biochemical responses and molecular responses.

1.2.1 Physiological responses and morphological adaptations of plants to drought

Water deficit can affect plants in different ways. Mild drought triggers the biosynthesis and the accumulation of phytohormone abscisic acid (ABA) in roots. The *de novo* synthesized ABA is then transferred to leaves through the transpiration stream (drought induced root-to-leaf signaling) (Ren *et al.*, 2007; Zhang and Outlaw, 2008). In leaf tissues, ABA promotes stomatal closure by reducing the turgor pressure of the guard cells, which is mediated by the efflux of K^+ and anions from the guard cells (Fan *et al.*, 2004; Nilson and Assmann, 2007). Stomatal closure limits CO_2 uptake and declines the internal CO_2 concentration in the stressed plants, which causes the inhibition of photosynthesis (Farquhar and Sharkey, 1982; Schulze, 1986; Cornic, 2000). Stomatal limitation of photosynthesis has been identified as an early event in plant response to water deficit. Under severe drought stress, photosynthesis is more limited by the chloroplast capacity to fix CO_2 than by the increased diffusive resistance

(Matthews and Boyer, 1984). The non-stomatal limitations of photosynthesis refer to decreases in photosynthetic capacity, including reductions in chlorophyll content, structural damage and functional disturbances of chloroplasts and metabolic impairments (Haupt-Herting and Fock, 2002; Lawlor and Cornic, 2002; Noctor *et al.*, 2002; Li *et al.*, 2006b).

In nature, plants have developed numerous strategies to cope with water deficit. Stomatal transpiration accounts for most of the water loss by plants. Transpiration occurs during photosynthesis, when stomata open for diffusion of carbon dioxide into leaves (Cowan *et al.*, 1978; Jones, 1998). C4 or CAM plants have developed a strategy to fix carbon dioxide with minimal water loss. CAM photosynthesis is evolved in some species as an adaptation to arid conditions (Ranson and Thomas, 1960; Herrera, 2009). At night, the stomata of CAM plants are open, which allow carbon dioxide to enter and be fixed as PEP (Phosphoenolpyruvate) by PEP carboxylase and then stored in the vacuoles. To avoid the transpiration during hot periods, the stomata are closed at daytime and the carbon dioxide is released to the Calvin cycle so that photosynthesis may take place. Unlike CAM plants, C4 plants concentrate carbon dioxide in space but not in time. C4 plants have developed a particular leaf anatomy with bundle sheath cells containing chloroplasts. Instead of direct fixation of RuBisCO in the Calvin cycle, carbon dioxide is incorporated into oxaloacetic acid during daytime in the mesophyll and then transferred as malate/aspartate to bundle sheath cells. In the chloroplast of the bundle sheath cells, the C4 organic acid is decarboxylated and the regenerated carbon dioxide is assimilated by the conventional C3 pathway (Slack and Hatch, 1967).

Anatomical adaptations to water deficit are further observed in many xerophytic plants, such as waxy stomata in prickly pear, spines and succulent stems in cacti, hairy leaves in *Tillandsia* (Kozlowski, 1968). Adaptations to water deficit are observed in the whole plant kingdom. Many annual herbaceous plants complete their life cycles before the annual drought period. Perennials develop efficient organs for water reserve such as succulent leaves or change their physiological mechanisms, e.g. Ocotillo utilizes dormancy to evade drought (Dorantes and Sánchez, 2006). Resurrection plants are able to diminish their metabolic functions, when they are suffering from severe drought stress. The metabolic functions are resumed once water potential increases (Bartels and Salamini, 2001; Bartels, 2005). In many bromeliads, a specific morphological structure is developed for rainwater impounding by tightly overlapping of their leaf bases. Some ferns store humid earth in concave stem (*Polypodium*) (Monneveux and Belhassen, 1996).

One crucial aspect for drought adaptation in plants are morphological changes in plant root systems. Since plants obtain their water and mineral nutrients from roots and the availability of these resources are often restraint, deep and vigorous root systems have been employed in drought-tolerant plants to maximize water uptake (Taylor *et al.*, 1979; Ober and Sharp, 2003; Pinheiro *et al.*, 2005; Xiong *et al.*, 2006). Previous studies have demonstrated how plant roots adapted to cope with edaphic environmental factors, such as water and temperature (Huck *et al.*, 1983; Tsuji *et al.*, 2005; Xiong *et al.*, 2006; Suralta *et al.*, 2008; Luo *et al.*, 2009). Drought inhibits lateral root growth. But the influence of drought stress on root length is complex. It has been reported in maize that severe water stress reduces corn root mass and length in the greenhouse. Moderate water stress in the field, however, strongly increased root length (Eghball and Maranville, 1993). Intraspecific differences in root shape and size have been identified in crop plants (O'Brien, 1979; Grando and Ceccarelli, 1995). Furthermore, genotypic variations for root size have been found in sorghum, maize, barley, wheat, rice, rye and oat (Blum *et al.*, 1977; Murphy *et al.*, 1982; Ekanayake *et al.*, 1985; O Toole and Bland, 1987). Understanding of adaptations of roots to cope with drought helps to improve crop breeding techniques.

1.2.2 Biochemical responses of plants to drought at the cellular level

Cellular water deficit causes loss of turgor and changes in plasma membrane fluidity and compositions, cell volume, solute concentration, protein-protein and protein-lipid interactions. Cellular responses to water deficit are altered depending on the severity and duration of water limitation and plant species. Furthermore, specific cellular responses to water deficit depend on the organ and cell type, the age and stage of development and the subcellular compartment (Bray, 1997). One important cellular response to water deficit is the accumulation of osmotically active compounds and protective proteins including mainly the LEA (Late Embryogenesis Abundant) and LEA-like proteins, heat shock proteins, detoxification proteins and compatible solutes. These protective molecules play a crucial role in stabilizing enzymes, protecting membrane integrity and cell structure, reestablishing turgor and extracting additional water from soil.

1.2.2.1 LEA proteins

LEA proteins have been first identified and characterized in cotton and accumulate to high levels during late stages of seed maturation (Dure and Chlan, 1981). LEA proteins encompass a large group of proteins that are widely spread from bacteria to plants and lower animals (Close and Lammers, 1993; Battista *et al.*, 2001; Browne *et al.*, 2002; Gal *et al.*, 2004;

Tunnacliffe *et al.*, 2005; Kikawada *et al.*, 2006; Hand *et al.*, 2007; Pouchkina-Stantcheva *et al.*, 2007). According to different sequence motifs or biased amino acid composition, plant LEA proteins have been divided into seven different groups (Battaglia *et al.*, 2008). LEA proteins are rich in hydrophilic amino acids and thermal stable. Accumulation of LEA proteins during embryogenesis is in conjunction with increased levels of the phytohormone ABA and confers desiccation tolerance to plant embryos. The expression of LEA proteins is not normal in vegetative tissues, but the expression is induced by osmotic stress or exogenous application of ABA (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002). LEA proteins play important roles in plasma and organellar membrane stabilization by interacting specifically with negatively charged phosphate groups in dehydrated phospholipids so that the mobility of the fatty acyl chain is increased (Tolleter *et al.*, 2010). This promotes the stability of plasma/organellar membranes during dehydration and rehydration, but also upon freezing. The randomly coiled moieties of some LEA proteins are capable of sequestering ions, which help plants maintain the minimum cellular water requirement. Moreover, some LEA proteins could essentially be considered as compatible solutes, which support the likely roles of sugars in maintaining cytoplasmic structures during dehydration (Ingram and Bartels, 1996).

1.2.2.2 Heat shock proteins (Hsps)

Heat shock proteins (Hsps) are ubiquitous and form the most ancient defense system both in prokaryotes and eukaryotes. According to their molecular weights, Hsp proteins are categorized into different families. The major heat shock protein families in plants are referred to as Hsp60, Hsp70, Hsp90, Hsp100 and small Hsps (Schlesinger, 1990; Kotak *et al.*, 2007). The heat shock protein family encompasses many molecular chaperones, which help in folding and assembly of proteins during synthesis and assist in their elimination if they are irreversibly damaged. Hsps play important roles in cytoprotection by interacting with other Hsps and various kinases (Sreedhar and Csermely, 2004). Hsp proteins are usually undetectable in vegetative tissues under normal growth conditions. However, their expression is induced by environmental stresses or developmental stimuli. Accumulation of Hsps confers enhanced tolerance to abiotic stresses to plants, which has been extensively reviewed (Vierling, 1991; Waters *et al.*, 1996; Feder and Hofmann, 1999; Wang *et al.*, 2004; Kotak *et al.*, 2007; Timperio *et al.*, 2008).

1.2.2.3 Compatible solutes

Compatible solutes, also termed osmoprotectants, are cytoprotetive molecules, which are widely spred both in prokaryotes and eukaryotes. Compatible solutes are highly soluble non-

toxic compounds with low-molecular-weight, including amino acids, glycine betaine, sugars or sugar alcohols (Chen and Murata, 2002; Bartels and Sunkar, 2005). Most of the compatible solutes are non-charged at physiological pH, although some bacterial or archaeal osmoprotectants are anionic, e.g. diglycerol phosphate. The neutrality is achieved by binding potassium ions (Roeßler and Müller, 2002; Roberts, 2004; Yancey, 2005). The accumulation of compatible solutes in the cytoplasm does not interfere with normal cellular metabolism (Brown and Simpson, 1972). According to their cytoprotective properties, compatible solutes fall into two broad categories, namely metabolic protection and counteraction of destabilizing forces on macromolecules (Yancey, 2005). The primary role of compatible solutes is to maintain cell volume. Moreover, compatible solutes are hydrophilic and able to replace water at the surface of proteins, protein complexes or membranes under drought stress. This helps to stabilize proteins and cell membranes from damaging effects of desiccation (Yancey *et al.*, 1982; Ingram and Bartels, 1996). Furthermore, some compatible solutes (e.g. sugar alcohol) are functionally interchangeable. Sugar alcohols may act as free radical scavenger during abiotic stresses (Orthen *et al.*, 1994; Shen *et al.*, 1997; Shen *et al.*, 1999; Bolouri-Moghaddam *et al.*, 2010). Similar to LEA proteins and Hsps, accumulation of compatible solutes promotes tolerance to abiotic stresses in plants (Sakamoto and Murata, 2002; Verslues *et al.*, 2006; Urano *et al.*, 2010).

1.2.3 Responses of plants to drought at the molecular level

In recent years, our knowledge of drought responses at the molecular level has been increased. Plants respond to drought by induction of a series of regulatory genes that form the signaling network (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002; Bartels, 2005). The simplest signal transduction system is mediated by phosphotransfer between two types of signal transducers: a ‘‘sensor’’, which is often located in the cytoplasmic membrane and monitors environmental stimuli; and a ‘‘response regulator’’, that mediates changes in gene expression or locomotion in response to sensor signals. This signal transduction system is therefore referred to as the ‘‘two-component system’’ (Parkinson and Kofoid, 1992; Urao *et al.*, 2000). However, the perception of drought signals is not yet concluded. No plant molecule has been identified, which acts as sensor for drought stress (Urao *et al.*, 1999; Wohlbach *et al.*, 2008; Fujii and Zhu, 2012; Kumar *et al.*, 2012). In contrast to signal perception, many key components such as transcription factors and their downstream target genes involved in the signaling cascades have been identified, although their interactions as well as their exact positions in the signaling network deserve investigations. Moreover, additional *cis*- and *trans*-acting elements need to be identified to better understand how plants

cope with environmental stresses. Presently, transcription factors such as DRE-binding protein (DREB)/C-repeat-binding factor (CBF), ABA-binding factor (ABF), MYB and MYC together with the corresponding stress-responsive *cis*-acting regulatory elements dehydration responsive element (DRE), ABA-responsive element (ABRE) and MYB-/MYC-recognition sequence (MYBR/MYCR) have been intensively studied and well characterized in the model plant *Arabidopsis thaliana* and some crop plants e.g. maize and rice (Urao *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; Liu *et al.*, 1998; Nakashima *et al.*, 2000; Ramanjulu and Bartels, 2002; Abe *et al.*, 2003; Dubouzet *et al.*, 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Sakuma *et al.*, 2006; Qin *et al.*, 2007a). Depending on whether the phytohormone ABA is involved, the stress signaling cascades have been distinguished into ABA-dependent and ABA-independent signal transduction pathways (Shinozaki and Yamaguchi-Shinozaki, 1997; 2000; Shinozaki and Yamaguchi-Shinozaki, 2007). In general, ABRE- and MYBR/MYCR-*cis*-elements and their corresponding transcription factors form the ABA-dependent signal transduction pathways, while DREB1/CBF and DREB2 regulons function in ABA-independent gene expression. However, ABA-mediated responses have been identified in *DREB1D/CBF4* in *Arabidopsis* indicating the existence of a cross talk between the ABA-dependent and ABA-independent signal transduction pathways (Haake *et al.*, 2002; Agarwal *et al.*, 2006b). Furthermore, the function of second messengers involved in the signaling pathways e.g. Ca^{2+} and ROS have also been well characterized (Apel and Hirt, 2004; Bartels and Sunkar, 2005; McAinsh *et al.*, 2006). These regulatory mechanisms help plants make corresponding responses and adjustments to drought stress by establishing new cellular homeostasis that leads to enhanced tolerance to dehydration.

1.3 Identification and involvement of *DREB/CBF* genes in drought stress

The *CBF/DREB* transcription factors were first isolated in *Arabidopsis thaliana* using yeast one-hybrid screening. They play important signaling roles in plant stress responses (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Yamaguchi-Shinozaki and Shinozaki, 2005). The *CBF/DREB* transcription factors share a highly conserved AP2/ERF DNA-binding domain and specifically bind to the DRE/CRT elements in the promoter of stress-responsive genes. The dehydration-responsive element (DRE) contains the core sequence A/GCCGAC and is involved in response to drought, high salinity and low temperature in plants (Yamaguchi-Shinozaki and Shinozaki, 1994; Nakashima *et al.*, 2009). The DRE core motif was also identified in C-repeat (CRT) and hereafter referred to as low temperature-responsive element (LTRE) that is present in the promoters of cold-inducible genes (Baker *et al.*, 1994; Lata and

Prasad, 2011). Six *CBF/DREB1* and eight *DREB2* genes were identified in *Arabidopsis* (Sakuma *et al.*, 2002; Nakashima *et al.*, 2009). However, cereal plants are characterized by a larger CBF gene family (Badawi *et al.*, 2007). At least 20 and up to 25 CBF genes have been identified in barley and wheat, respectively (Skinner *et al.*, 2005; Badawi *et al.*, 2007). Compared to the larger CBF gene family, only three *Arabidopsis DREB2* homologues have presently been identified in barley (Xue and Loveridge, 2004; Nayak *et al.*, 2009; Xu *et al.*, 2009). Amino acid alignment of different *CBF/DREB* proteins demonstrates high conservation in the AP2/ERF domain and high sequence similarity in the nuclear localization signal (NLS) at the N-termial region as well as some sequence similarity in the C-terminal acidic region (Medina *et al.*, 1999). In the AP2/ERF domain, the 14th valine and the 19th glutamic acid are crucial for DNA binding specificity. Competitive DNA binding assays revealed that *AtDREB1A*, *AtDREB2A* and *OsDREB2A* proteins have the same binding specificity to two variants of the DRE core sequences ACCGAC and GCCGAC, while *OsDREB1A* demonstrated higher preference for GCCGAC to ACCGAC. This is because of the replacement of glutamic acid by valine at position 19 in the AP2/ERF domain of the *OsDREB1A* gene in rice (Liu *et al.*, 1998; Sakuma *et al.*, 2002; Dubouzet *et al.*, 2003). Furthermore, two signature motifs PKK/RPAGRxKFxETRHP and DSAWR flanked by the AP2/ERF domain were identified in *Arabidopsis*, rapeseed, wheat, rye and tomato *CBFs* (Jaglo *et al.*, 2001).

The function of *CBF/DREBs* in response to drought is well-studied in the model plant *Arabidopsis*. In *Arabidopsis*, the *CBF/DREB1* genes are rapidly and transiently induced by cold, but not by drought and salinity stress (Liu *et al.*, 1998; Nakashima *et al.*, 2009; Lata and Prasad, 2011). However, overexpression of *AtDREB1A/CBF3* gene driven by the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter or the stress inducible *rd29A* promoter led to increased expression of stress-inducible target genes and conferred enhanced tolerance to drought, high salinity and low temperature to the transgenic plants. It should be noted that using the constitutive CaMV 35S promoter for *AtDREB1A/CBF3* overexpression resulted in dwarfed phenotypes under normal growth conditions. Instead of CaMV 35S promoter, the utilization of the stress inducible promoter *rd29A* for *AtDREB1A/CBF3* overexpression minimized the negative effects on plant growth and provided even greater tolerance to stress conditions (Kasuga *et al.*, 1999). Similar effects of *AtDREB1A/CBF3* overepxression were observed in transgenic tobacco, tall fescue and rice plants (Kasuga *et al.*, 2004; Oh *et al.*, 2005; Ito *et al.*, 2006; Zhao *et al.*, 2007). Furthermore, tansgenic tomato plants overexpressing *AtDREB1B/CBF1* genes showed improved tolerance to water deficit

and oxidative stress (Hsieh *et al.*, 2002). In contrast to another three well-studied *AtDREB1/CBF* homologues, *AtDREB1D/CBF4* is induced by drought, but not by low temperature. Overexpression of *AtDREB1D/CBF4* in transgenic *Arabidopsis* plants led to enhanced tolerance to drought and freezing (Haake *et al.*, 2002). In rice, overexpression of barley *CBF4* genes conferred increased tolerance to drought, high salinity and low temperature to transgenic plants without growth retardation (Oh *et al.*, 2007). Transgenic wheat plants overexpressing the soybean *DREB1* gene showed improved tolerance to drought and high salinity (Gao *et al.*, 2005). More than 40 target genes of *DREB1/CBF*s have been identified in *Arabidopsis* using cDNA Affymetrix GeneChip microarrays (Seki *et al.*, 2001; Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Nakashima *et al.*, 2009). Many of these gene products including LEA proteins, osmoprotectant biosynthesis proteins and transcription factors are involved in drought stress tolerance and likely responsible for the improved stress tolerance of the transgenic plants. These data indicate that *DREB1/CBF*s can be used to improve multi-stress tolerance in economically important crops using transgenic approaches.

The *DREB2A* and *DREB1A* transcription factors were simultaneously isolated in *Arabidopsis* (Liu *et al.*, 1998). In contrast to *DREB1A*, the *DREB2A* transcription factors are induced by drought and high salinity, but not by low temperature. Furthermore, overexpression of full-length *AtDREB2A* cDNA did not induce any expression of downstream target genes under normal growth conditions and showed few phenotypic changes in transgenic plants (Liu *et al.*, 1998; Sakuma *et al.*, 2006). Domain analysis using *Arabidopsis* protoplasts identified a transcriptional activation domain between amino acid residues 254 and 335 in the C-terminal region of the *AtDREB2A* genes and a negative regulatory domain (NRD), a Ser- and Thr-rich 30-amino acid region adjacent to the AP2/ERF domain. Deletion of this negative regulatory domain leads to a constitutive active form of *AtDREB2A*, namely *AtDREB2A-CA*. In contrast to overexpression of full-length *AtDREB2A* cDNA, transgenic plants overexpressing *AtDREB2A-CA* (constitutive active form of *AtDREB2A*) showed enhanced tolerance to drought and slightly freezing tolerance with dwarfed phynotypes (Sakuma *et al.*, 2006). Similar to *AtDREB2A*, the expression of *AtDREB2B* is mainly induced in roots under high salinity and in roots and stems under dehydration. Timings of expression of *AtDREB2A* and *AtDREB2B* were similar. But *AtDREB2A* expression was more than ten times stronger than that of *AtDREB2B*, suggesting a more important role of *AtDREB2A* in activation of downstream target genes under drought stress (Nakashima *et al.*, 2000). Furthermore, expression of three *AtDREB2A* related genes (*AtDREB2C*, *AtDREB2D* and *AtDREB2F*) was

induced slightly by high salinity in leaves but not in roots. The expression of *AtDREB2E* was only slightly induced by ABA treatment in roots (Sakuma *et al.*, 2002). Interestingly, transgenic plants overexpressing *AtDREB2C* displayed altered stress responses. They were drought sensitive but freezing and heat tolerant (Lee *et al.*, 2010). Altogether, these data indicate that *AtDREB2* genes play an important role in drought, high salinity and heat stresses.

AtDREB2 homologous genes were isolated in many economically important crops such as barley, rice, maize, wheat, sorghum, soybean, chickpea and pearl millert (Xue and Loveridge, 2004; Egawa *et al.*, 2006; Agarwal *et al.*, 2007; Qin *et al.*, 2007a; Nayak *et al.*, 2009; Bihani *et al.*, 2010; Matsukura *et al.*, 2010; Morran *et al.*, 2010; Mallikarjuna *et al.*, 2011; Mizoi *et al.*, 2013). But little work has been done to generate stress-tolerant grasses overexpressing *DREB2* genes. Similar to overexpression of *AtDREB2A-CA*, transgenic wheat and barley plants overexpressing *TaDREB2* and *TaDREB3* transcription factors under control of the constitutive (double 35S) or drought-inducible (maize *Rab17*) promoters displayed improved survival under severe drought conditions. However, transgenic plants with constitutive overexpression showed growth retardation, delayed flowering and lower grain yields. These negative impacts on plant growth and development could be alleviated by utilizing a stress-inducible maize *Rab17* promoter (Morran *et al.*, 2010). These data indicate that *DREB2* is crucial for improving drought stress tolerance in economically important crops using transgenic approaches.

1.4 DNA methylation and involvement in plant stress responses

DNA methylation occurs both in prokaryotes and eukaryotes. It is an important covalent modification of DNA in the genome involved in response to developmental and environmental stimuli. DNA methylation refers to the addition of a methyl group from S-adenosylmethionine (SAM) by DNA methyltransferase (DNMT) to the 5-carbon position of cytosine (5-methylcytosine, 5mC). In both mammals and plants, methylation is extensively enriched in heterochromatin at centromeric, pericentromeric and subtelomeric repeats as well as other repetitive elements including transposons, retrotransponsons and inverted or tandem repeats (Bender, 2004; Fukuda *et al.*, 2004; Weisenberger *et al.*, 2005; Zhang *et al.*, 2006; Zhang *et al.*, 2008; Vining *et al.*, 2012). While most vertebrates have less than 10% 5mC residues, over 30% of the cytosine residues are methylated in certain plant varieties (Shapiro, 1976; Gruenbaum *et al.*, 1981). In plants, DNA methylation occurs at defined target sequences CG, CHG and CHH, where H represents any nucleotide but not guanine (Chan *et al.*, 2005; Lister *et al.*, 2008). In contrast, DNA methylation occurs almost exclusively in CG

sites in mammalian genomes, although non-CG methylation is prevalent in embryo stem cells (Ramsahoye *et al.*, 2000; Goll and Bestor, 2005).

Presently, many methods have been developed for DNA methylation analysis and have been intensively reviewed (Fraga and Esteller, 2002; Laird, 2003; Shames *et al.*, 2007; Zilberman and Henikoff, 2007; Laird, 2010). These approaches can be grouped into three categories, namely methylation-sensitive restriction endonucleases, bisulfite conversion and affinity purification of methylated DNA. Methylation-sensitive restriction endonucleases are classical tools of DNA methylation analysis using isochizomeric restriction enzymes with different sensitivities to recognition site methylation (Bird and Southern, 1978; Lindsay and Bird, 1987). DNA fragments digested with restriction enzymes are analyzed by Southern blotting or ligated to restriction site specific adaptor and analyzed by PCR (*Hpa*II tiny fragment Enrichment by Ligation-mediated PCR, HELP-assay) (Khulan *et al.*, 2006). The most commonly used restriction enzymes are the isoschizomers *Hpa*II and *Msp*I, which recognize the sequence in the context of CCGG. *Hpa*II is blocked by methylation of any cytosine, while *Msp*I is blocked only by methylation of the outer cytosine (Waalwijk and Flavell, 1978; Zilberman and Henikoff, 2007). Bisulfite conversion provides a convenient and rapid method to identify the methylation status in genomes. Since methylated and unmethylated cytosines possess the same base-pairing characteristics, it is impossible to distinguish them from each other by standard sequencing approaches. This problem can be solved by treating genomic DNAs with sodium bisulfate, which converts cytosines, but not 5-methylcytosines into uracil under denaturing conditions. PCR amplification of converted DNA replaces the uracil with thymine. Bisulfite-treated DNA is analyzed by PCR or DNA sequencing (Xiong and Laird, 1997; Grunau *et al.*, 2001; Sasaki *et al.*, 2003; Ogino *et al.*, 2006; Egger *et al.*, 2012). Affinity purification of methylated DNA accounts for the simplest way to enrich methylated DNA. The most widely used approach is methylated-DNA immunoprecipitation (MeDIP). Genomic DNAs are fragmented and precipitated with 5-methylcytosine-specific antibodies. The precipitated DNA is then analyzed by PCR or whole genome tilling microarrays (Zhang *et al.*, 2006; Zilberman *et al.*, 2007).

Recent evidence reveals that DNA cytosine methylation plays an important role in regulation of gene expression in plant responses to environmental stresses. In maize roots, the transcriptional activation of the *ZmMI1*gene depends on cold-mediated DNA demethylation. *ZmMI1* is only transcribed in response to cold. The gene contains parts of a putative protein coding region and part of a retrotransponson-like sequence. Upon exposure to cold, the

transcriptional activation of *ZmMI1* gene correlates with cold-induced root-specific demethylation in the *AcDs* transposon regions (Steward *et al.*, 2002). In tobacco, abiotic stress induced DNA demethylation in the coding region of the *NtGPDL* gene, which encodes a glycerophosphodiesterase-like protein and is not expressed in healthy leaves under non-stressed growth conditions, correlated with its gene expression (Choi and Sano, 2007). DNA methylation is necessary for the induction of *NtAlix1* (ALG-2 interacting protein X, Alix) under pathogen infection by TMV (tobacco mosaic virus) in tobacco (Wada *et al.*, 2004). Moreover, Wada *et al.* (2004) have demonstrated that hypomethylation of MET1 in transgenic tobacco plants results in specific expression of 31 genes. Ten of these genes are related to biotic and abiotic stresses and six of them are involved in cellular functions. Furthermore, osmotic stress induced hypermethylation of tobacco heterochromatic loci, HRS60 and GRS (Kovarik *et al.*, 1997). DNA cytosine methylation in the promoter regions is proven to result in a transcriptional repression of the corresponding gene by directly interfering with the binding of transcription factors to DNA or by binding with MBD (methylated-DNA binding domain) containing proteins, thereby blocking access to other factors for gene transcription (Eden and Cedar, 1994; Ng *et al.*, 1999; Newell-Price *et al.*, 2000; Bird, 2002; Berdasco *et al.*, 2008). DNA hypermethylation induced by osmotic stress was also identified in *M. cristallinum* plants, indicating changes in DNA methylation are necessary for plant stress protections (Dyachenko *et al.*, 2006; Labra *et al.*, 2008). It should be noted that interactions exist between stress-induced histone modifications and DNA methylation. In soybean, salinity affected the promoter methylation status of three salinity-inducible transcription factors, including two *GmDREB* (*Glyma16g27950* and *Glyma20g30840*) and one *MYB* (*Glyma11g02400*) transcription factors. ChIP (chromatin immunoprecipitation) analysis indicated that the activation of these three DNA methylated transcription factors was correlated with an increased level of histone H3K4 trimethylation and H3K9 acetylation in various parts of the promoter or coding regions. These data suggested that the activation of some transcription factors by DNA methylation and histone modifications play an important role in soybean salinity tolerance (Song *et al.*, 2012).

1.5 A model crop plant: barley

Barley (*Hordeum vulgare* L.) is one of the earliest global domesticated crop plants in the Neolithic Near East (Salamini *et al.*, 2002). Barley belongs to the grass family *Poaceae*, the tribe *Hordeae* and the genus *Hordeum*. Barley is an annual grain crop which reaches 60-120 cm in height. It is a self-pollinating, diploid species with 14 chromosomes. Barley has a large genome of 5.1 gigabases, which was completely sequenced in 2012 (Mayer *et al.*, 2012).

Today, barley represents the fourth most abundant cereal both in area of cultivation and in grain output. Among barley global yield, approximately 75% is used as feed, 20% as raw material for beverages and 5% as food (Sreenivasulu *et al.*, 2008). Barley is widely adapted to adverse environmental conditions and displays much higher tolerance to different environmental stresses than its close relative wheat (Nevo *et al.*, 2012). In recent years, barley attracts broad attentions as a model crop plant because of its broad natural diversity including geographically diverse elite varieties, landrace and wild accessions and tremendous achievements in barley genomics such as rapid accumulation of EST sequences, extensively characterized genome-wide knockout collections (http://barleygenomics.wsu.edu) and efficient transformation techniques and great efforts on barley genome sequencing and physical mapping (Freialdenhoven *et al.*, 1994; Close *et al.*, 2004; Varshney *et al.*, 2007; Harwood *et al.*, 2009; Mayer *et al.*, 2012). Furthermore, large collections of geographical diverse barley cultivars facilitate to understand the effects of climate changes on crop yield and plant adaptations to environmental stresses.

Like wheat and oats, barley presents also two kinds of root systems, namely seminal and adventitious root system. The seminal rootlets occur from germination to the tillering stage and form a fibrous branched mass of roots, whereas the adventitious root system arises from the crown at tillering. In comparison to the seminal roots the adventitious roots tend to be thicker and less branched. Under certain conditions such as drought, adventitious roots do not develop at all (Gregory, 1987) or their growth is limited (Górny, 1992). In other cases, the seminal roots cease functioning during the life of the plant (Briggs, 1978). Seminal roots are responsible for the initial absorption of water and nutrient, whereas adventitious roots serve to anchor the plant besides water and nutrient uptake. Barley can be grown on many soil types including well drained, fertile loams and lighter clay soils. In general, barley grows best in coarse-textured and well-drained soils. The architecture or morphology of the barley roots will depend on the water content and aeration of the soil, its texture and structure, external and internal temperatures and the genetic composition of the variety (Hackett and Bartlett, 1971).

Root characteristics such as the root depth, root length density and root branching are critical for drought tolerance. Pot experiments showed a good relation between root system size and drought tolerance among different barley varieties (Chloupek *et al.*, 2010). However, using the traditional analysis methods for root system architecture such as excavation or washing soil cores destroys the topology of the root system. Recently, some new technologies have

been developed for non-destructively imaging root systems including X-ray computed tomography, NMR and magnet resonance imaging (Van Der Weerd *et al.*, 2001; Gregory *et al.*, 2003; Perret *et al.*, 2007; Van As, 2007; Jahnke *et al.*, 2009). But such technologies are very costly and time-consuming and the equipment is not easily accessible. Researchers in Prof. Benfey ́s laboratory (Duke University, Durham NC, USA) utilize a non-invasive method to study root system architecture. They have developed a virtual root system using the latest mathematical modeling techniques. With these techniques, researchers could reconstruct or remodel the root system in 2D or 3D form without destroying the topology of the roots. It facilitates to observe what is happening at every stage from the molecular scale to visible phenotype, which exactly mimics the biological progress (Iyer-Pascuzzi *et al.*, 2010).

1.6 Objectives of the study

This study is one of the research projects of CROP.SENSe.net network. CROP.SENSe.net is an interdisciplinary research network, with partners working together to non-destructively and quantitatively analyze and screen plant phenotypes throughout plant ́s life cycles. The aim of the project is early, non-biased and faster assessment of traits in the laboratories and field to enable greater efficiency in crop breeding and to optimize decision making in crop management (http://www.cropsense.uni-bonn.de/).

Our project is part of the barley subproject Gs2: Sensor data – Characteristics – Marker association of the genetic Co/localization of plant stress responses, in the CROP.SENSe.net network under direction of Prof. Dr. Jens Léon and Prof. Dr. Dorothea Bartels. The objective is to characterize the regulatory *CBF/DREB* genes from the phenotypic level to the molecular level in barley by using a defined collection of barley genotypes. The so-called barley ``core set ´´consists of 16 different German barley spring cultivars including *Barke*, *Beatrix*, *Djamila*, *Eunova*, *Streif*, *Ursa*, *Victoriana*, *Wiebke*, *Ack Bavaria*, *Apex*, *Heils Franken*, *Isaria*, *Perun*, *Pflugs Intensiv*, *Sissy* and *Trumpf*. The first eight cultivars are modern German barley cultivars, while the last eight cultivars are old German barley cultivars. In this study, the cultivars *Barke*, *Eunova*, *Victoriana*, *Wiebke*, *Apex*, *Heils Franken*, *Isaria*, *Pflugs Intensiv*, and *Trumpf* were utilized.

To identify changes in regulatory genes involved in response to drought is a challenge for the modern breeding programs. Our knowledge of the *CBF/DREB* transcription factors in plant drought responses at the molecular level is mainly based on the functional analysis of these genes in the model plant *Arabidopsis* under controlled laboratory conditions. Little work has been done to investigate the function of *CBF/DREB* transcription factors in drought stress in

crops, especially under field conditions. In this study, the functions of *CBF/DREB* transcription factors in barley drought responses are analyzed both under controlled laboratory conditions and field conditions. Furthermore, more and more evidence indicates that stress-associated gene expressions are regulated by epigenetic mechanisms e.g. DNA methylation (see 1.6). *CBF/DREB* transcription factors play crucial roles in plant abiotic stresses, such as drought, high salinity and low temperature by transcriptional activation of downstream stress-responsive target genes (see 1.4). One purpose of this study was to investigate whether DNA methylation of the *CBF/DREB* transcription factors affects drought stress tolerance among different barley cultivars. Finally, numerous studies have revealed that plant root systems play an important role in plant drought responses (see 1.2). However, how to non-destructively analyze plant root systems is a big challenge. Prof. Dr. Philip Benfey ́s and coworkers have developed a virtual root system using the latest mathematical modeling techniques to rapidly non-destructively analyze plant root systems in rice and maize. This method should be also further developed to analyze barley root systems.

To achieve the above-mentioned goals, this study has been divided into the following tasks:

1. Identification the putative barley *CBF/DREB* transcription factors involved in drought stress responses by data mining exploring different gene and EST data banks
2. Design gene-specific primers based on the identified gene sequences from the previous step
3. Isolation of the genomic sequences of the identified *CBF/DREB* genes with the gene-specific primers from different barley cultivars
4. Expression analysis of the identified *CBF/DREB* genes both under controlled laboratory conditions and field conditions
5. Methylation assay of the selected barley *CBF/DREB* genes
6. Development of new protocols for non-destructive analysis of barley root systems with support and know-how transfer from Prof. Dr. Philip Benfey, Duke University, Durham NC, USA.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

Ten genotypes of spring barley (*Hordeum vulgare*) were utilized in this study. The drought stress tolerant line *Arta* was obtained from Dr. Maria von Korff (MPI Köln). Five old cultivars, *Apex*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Trumpf* were ordered from Dr. Benjamin Kilian (IPK Gatersleben) whereas the four modern cultivars *Barke*, *Eunova*, *Victoriana* and *Wiebke* were received from Prof. Dr. Jens Léon (INRES, University of Bonn).

2.1.2 Chemicals

Chemicals utilized in this work were ordered from the following companies:

- Amersham Bioscience, Freiburg, Germany
- AppliChem, Darmstadt, Germany
- BIOMOL, Hamburg, Germany
- Clontech, Saint-Germain-en-Laye, France
- Fermentas, St. Leon-Rot, Germany
- FLUKA, Buchs, Switzerland
- Hartmann Analytic GmbH, Stöckheim, Germany
- Hoechst AG, Frankfurt, Germany
- Invitrogen/GibcoBRL, Karlsruhe, Germany
- KMF, Lohmar, Germany
- Merck, Darmstadt, Germany
- Macherey-Nagel, Düren, Germany
- PEQLAB, Erlangen, Germany
- Pharmacia, Freiburg Germany
- Roche, Mannheim, Germany
- Roth, Karlsruhe, Germany
- Sigma-Aldrich, Steinheim, Germany

2.1.3 Kits

The following kits were used:

- NucleoSpin® Extract II (Macherey–Nagel, Düren, Germany)
- CloneJET™ PCR Cloning Kit, (Fermentas, St. Leon-Rot, Germany)
- RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas, St. Leon-Rot, Germany)
- HexaLabel™ DNA Labeling Kit (Fermentas, St. Leon-Rot, Germany)

2.1.4 Media

2.1.4.1 Hoagland ́s medium (Sigma-Aldrich, Steinheim, Germany)

Hoagland ́s stock solutions (1 liter; Hoagland *et al.*, 1950)

Solution A

$Ca(NO_3)_2 \cdot 4 H_2O$	298.246 g
NH_4NO_3	20.569 g

Solution B

KNO_3	152.020 g
$MgSO_4 \cdot 7 H_2O$	126.650 g
$ZnSO_4 \cdot 7 H_2O$	0.286 g
$MnSO_4 \cdot H_2O$	0.195 g
$CuSO_4 \cdot 5H_2O$	0.021 g
H_3BO_3	0.725 g
$Na_2MoO_3 \cdot 2 H_2O$	0.011 g
KCl	3.154 g
$NH_4H_2PO_4$	59.030 g

NaFe-DTPA (iron chelate, Sequestene 330) 37.5g/l (store in dark)

* Solution A and B can be autoclaved (filter-sterilization also works)

* NaFe-DTPA should be filter-sterilized.

A half strength Hoagland ́s medium was used for the experiments. For its preparation, 10 ml Hoagland ́s stock solutions A and B were diluted into 5 liter ddH_2O and mixed well. Then 1 ml NaFe-DTPA (sodium ferric diethylenetriamine pentaacetate) was added to the mixture. If required, the pH was adjusted to 5.8 with 5 M KOH. 500 ml of the well-mixed final solutions were added to each glass bottle filled with 0.25g Gelzan before. The medium was autoclaved for 20 min.

2.1.4.2 Yoshida ́s medium

Yoshida ́s stock solutions (Yoshida *et al.*, 1976)

Element	Reagent (AR gerade)	Preparation g/l0 L	g/1 L	
N	NH_4NO_3	914	91.4	
P	$NaH_2PO_4 \cdot 2\ H_2O$	403	40.3	
K	K_2SO_4	714	71.4	
Ca	$CaCl_2 \cdot 2\ H_2O$	886	88.6	
Mg	$MgSO_4 \cdot 7H_2O$	324	32.4	
	Micronutrients			
Mn	$MnCl_2\ 4\ H_2O$	15	1.5	Dissolve separately; then combine with 500 ml of concentrated H_2SO_4. Make up to 10 liter volume with distilled water.
Mo	$(NH4)_6Mo_7O_{24}\ 4\ H_2O$	0.74	0.07	
B	H_3BO_3	9.34	0.93	
Zn	$ZnSO_4 \cdot 7\ H_2O$	0.35	0.04	
Cu	$CuSO_4\ 5\ H_2O$	0.31	0.03	
Fe	$FeCl_3\ 6\ H_2O$	77	7.7	
	Citric acid (monohydrate)		11.9	

To prepare Yoshida ́s medium, 5 ml of each stock solution was added in 4 liter ddH_2O and mixed well. Then 2.185 g MES was added to the mixture. If required, the pH was adjusted to 5.8 with 5 M NaOH. 500 ml of the well-mixed final solutions were added to each glass bottle filled with 0.25g Gelzan before. The medium was autoclaved for 20 min.

2.1.4.3 MS-Medium (Murashige and Skoog, 1962)

To prepare 1 liter MS-medium, 4.6 g MS-salt were dissolved in about 800 ml ddH_2O and mixed well. One milliliter vitamin solution was added to the solution. If required, pH was adjusted to 5.8 with 0.1 M NaOH. The volume was adjusted to 1 liter with ddH_2O. Before autoclaving, 8 g Select Agar was added. The medium was autoclaved for 20 min.

2.1.4.4 LB-Medium (Bertani, 1951)

LB is a nutritionally rich medium, which is primarily used for bacteria cultivation. One liter LB medium contains 10 g peptone, 5 g sodium chloride and 5 g yeast extract. For solid medium, 12 g Select Agar was added to 1 L liquid LB medium. The medium was autoclaved

for 20 min.

2.1.5 Buffers and solutions

Ampicil1in (stock solution)	100 mg/ml in water; filter sterilization; store at -20 ℃; working solution: 1:1000 dilution
100X Denhardt ́s	2% (w/v) Bovine serum albumin (BSA; fraction V) 2% (w/v) Ficoll 400 2% (w/v) Polyvinylpyrrolidone (PVP) filter sterilization; store in aliquots at -20 ℃
10X DNA loading buffer (10 ml)	25 mg Bromophenol blue 25 mg Xylencyanol 200 µl 50X TAE 3 ml Glycerine 6.8 ml ddH_2O
10X MEN	200 mM 3-(N-morpholino) propanesulfonic acid (MOPS) 50 mM sodium acetate 10 mM EDTA store in the dark and at 4 ℃
50X TAE	242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA, pH8.0 add dd H_2O to 1 liter
10X TBE	108 g Tris base 55g boric acid 40 ml EDTA, pH 8.0 add dd H_2O to 1 liter
1X TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0
20X SSC	3 M NaCl 0.3 M sodium citrate adjust the pH to 7.0 with 1M HCl

Buffer	Amount	Component
10X PCR buffer	670 mM	Tris-HCl pH 8.8
	166 mM	$(NH_4)_2SO_4$
	4.5% (v/v)	Trtion® X-100
	2 mg/ml	Gelatin
	20 mM	$MgCl_2$
2X RNA loading dye	95%	formamide
	18 mM	EDTA, pH 8.0
	0.25% (w/v)	SDS
	0.25% (w/v)	Bromophenol Blue
	0.25% (w/v)	Xylene Cyanol FF
		store in aliquots at -20 °C
RNA electrophoresis buffer	820 ml	dd H_2O
	100 ml	10X MEN
	80 ml	37% (v/v) deionized Formaldehyd
RNA hybridization buffer (50 ml)	6.25 ml	dd H_2O
	5 ml	0.1 M PIPES, pH 6.8
	25 ml	formamide
	12.5 ml	20X SSC
	0.5 ml	100X Denhardt´s
	0.5 ml	10% SDS
	250 µl	denatured HS DNA (10 mg/ml)
RNA extraction buffer	38% (v/v)	buffer-saturated phenol
	0.8 M	guanidine thiocyanate
	0.4 M	ammonium thiocyanate
	0.1 M	sodium acetate, pH 5.0
	5% (v/v)	glycerol
DNA extraction buffer	4% (w/v)	CTAB
	100 mM	Tris-HCl, pH 8.0
	20 mM	EDTA, pH 8.0
	1.4 M	NaCl
	0.2% (w/v)	β-mercaptoethanol, pH 8.0
DNA denaturation buffer	1.5 M	NaCl
	0.5 M	NaOH

DNA neutralization buffer	1.5 M Tris-HCl, pH 7.4 1.5 M NaCl
DNA hybridization buffer (50 ml)	31.4 ml dd H_2O 7.5 ml 4M NaCl 5 ml 0.1 M PIPES, pH 6.8 100 µl 0.5 M EDTA, pH 8.0 5 ml 100X Denhardt ́s 500 µl 10% SDS 500 µl denatured HS DNA (10 mg/ml)
Southern-, Northern blot washing buffer	0.1% (w/v) SDS 2X SSC
Vitamin solution	2 mg/ml glycine 0.5 mg/ml Niacin (nicotine acid) 0.5 mg/ml pyridoxine-HCl 0.1 mg/ml thiamine-HCl filter sterilization; store at 4 ℃
Plasmid miniprep solution I	50 mM Glucose 25 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 store at 4 ℃
Plasmid miniprep solution II	0.2 N NaOH 1% (w/v) SDS
Plasmid miniprep solution III	60 ml 5 M potassium acetate 11.5 ml glacial acetic acid 28.5 ml dd H_2O

2.1.6 Primers

All the primers were designed with ``Primer3 plus ´´ (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and ``ApE-A Plasmid Editor ´´(http://biologylabs.utah.edu/jorgensen/wayned/ape/). They were synthesized from Sigma-Genosys (Steinheim, Germany). For preparation of the stock solution all the primers were dissolved into sterile water to a final concentration of 100 µM and stored at -20 ℃. The primers are listed in Table 1. in 5 ́to 3 ́orientation.

Table 1. List of the degenerated primers

Primer Name	Primer Sequence 5´-3´
Isolation of CBFs/DREBs Gene	
HvCBF1 GSP_fwd	GCAACAGTGAGGGCTTCTCG
HvCBF1 GSP_rev	CAGTAGCTCCACAAGCTGACC
HvCBF2 GSP_fwd	AGGGGCAAGACTACATGACG
HvCBF2 GSP_rev	CAAGCTCGCGTAGTAGGACC
HvCBF3 GSP_fwd	TCATCTCACACTCCTCAGTCCTC
HvCBF3 GSP_rev	TCATGTAGTACGAGCCCAGGTC
HvCBF4 GSP_fwd	ACTCCAGTCAAGCGTCTCCAC
HvCBF4 GSP_rev	TTCTCTGGCCTCGCTCTTTC
HvCBF6 GSP_fwd	GGAGATGAGCGGCGAGTC
HvCBF6 GSP_rev	GCCAGGCTCGCGTAGTAAAG
HvCBF11 GSP_fwd	GCTCGTACATGACGGTGTCG
HvCBF11 GSP_rev	CTGATCTCGCAGTCGTAGTCC
HvDREB1 GSP_fwd	GTAGCAAGCGGGAAGGAGAC
HvDREB1 GSP_rev	TAAACCCGTCATCGCCCTCTT
HvDRF1 GSP_fwd	ATGACGGTAGATCGGAAGGGTG
HvDRF1 GSP_rev	CCCTCAAAGAACTCGCTCATCTC
HvDRF1_M_fwd	AAGTTGGCTGGACCTATCTA
HvDRF1_M_rev	CGAATTTCAGCAACCCACTTCC
HvDRF1_End_fwd	CAACCCTGCCTTCCATGATGC
HvDRF1_End_rev	CACTTCCAAAGATCGTGATGC
HvDRF2 GSP_fwd	CAGAGATCGTCCTCCACTTTC
HvDRF2 GSP_rev	TCACTCCAGTATAAACACAGCTCG
Analysis of CBFs/DREBs Gene Expression (RT-PCRs)*	
Actin_fwd	CCCAGCATTGTAGGAAGGC
Actin_rev	CCTCGGTGCGACACGGAGC
HvCBF3_RT_fwd	CGAACGACGCTGCCATGCTC
HvCBF3_RT_rev	GGACCCAGACGACGGAGATA
HvDRF1.1_RT_fwd	TGGAGCAGAGGAAAGTACCC
HvDRF1.1_RT_rev	CATCTCCCTTGGGGTTTTG
HvDRF1.3_RT_fwd	AGCCTGGAAGGAAAAAGCGACCTC
HvDRF1.3_RT_rev	ATCCTGCACAGGGAAGTTGG
OligodT	GTTTTTTTTTTTTTTTTTTTTTTTT
Hsp17_fwd	ATGTCGATCGTGAGGAGGAG
Hsp17_rev	TGCCACTTGTCGTTCTTGTC
Isolation of CBFs/DREBs Gene Promoters (Genome Walking)	
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
HvCBF1_UTR_rev	GCTGGCTGCTGGAGTGTAAG
HvCBF1_GW_rev	ACGTCGAGAAGCCCTCACTG
HvCBF1_Promoter_PSP1	GTTCAGACGCGGACAAGCAC
HvCBF1_Promoter_PSP2	CGGAGTGACCCAACAAGACTCG
HvCBF3_UTR_rev	ACAGTCTGAGCTTGGTGCTTGAG
HvCBF3_GW_rev	GGCAGAGTGCTGGGTGATTG
HvCBF3_Promoter_PSP1	ACTGCTGAATGAGGTCGTCTTG
HvCBF3_Promoter_PSP2	GGTGATACCATCTTCCAAATGC
Other Primers	
pJET1.2_fwd	CGACTCACTATA GGGAGAGCGGC
pJET1.2_rev	AAGAACATCGATTTTCCATGGCAG

* Primers for the isolation of *HvCBF1*, *HvCBF2*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes were also used for the corresponding RT-PCR analysis.

2.1.7 Enzymes, agarose and DNA-marker

Restriction enzymes and their corresponding buffers were ordered from Fermentas (St. Leon-Rot, Germany), New England BioLabs (Frankfurt am Main, Germany), Roche/Boehringer (Mannheim, Germany), Sigma (Munich, Germany) and Invitrogen/GibcoBRL (Karlsruhe, Germany). Agarose used for gel electrophoresis were ordered from Biozym (Hessisch Oldendorf, Germany) and Lonza (Cologne, Germany). The 1 kb DNA ladder was ordered from Invitrogen/GibcoBRL (Karlsruhe, Germany).

2.1.8 Vectors and bacteria

2.1.8.1 pJet/blunt cloning vector

The pJET1.2/blunt is a linearized cloing vector, which was ordered from Fermentas (St. Leon-Rot, Germany). This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate. The pJET1.2/blunt cloning vector multiple cloning site contains two BglII recognition sequences that flank the insertion site. In addition, the vector contains a T7 promoter for *in vitro* and *in vivo* transcription as well as sequencing of the insert.

2.1.8.2 *Escherichia coli* DH10B (Lorow and Jessee, 1990)

Genotype: F$^-$ mcrA Δ (*mrr-hsd*RMS-*mcr*BC) Φ80d*lac* ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ (*ara*,*leu*) 7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ$^-$

This cell was used as host strains for cloning.

2.1.9 Machines and instruments

- Thermal cycler: T3000-Thermocycler (Biometra, Göttingen, Germany)
  PTC 2000 peltier thermal cycler (Bio-Rad, Hercules, USA)
- Power supply: Power PAC 300 electrophoresis power supply (Bio-Rad, Hercules, USA)
- Gel electrophoresis chamber: Minigel (Biometra, Gottingen, Germany)
  EasyCast™ (Owl, Portsmouth, USA)
- UV transilluminator: ECX F15.C (PEQLAB, Erlangen, Germany)
- Imaging system: Typhoon Scanner 9200 Variable Mode Imager (GE Healthcare formerly Amersham Biosciences, Buckinghamshire, UK)
- Incubator shaker: New Brunswick G25 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA)

- Spectrophotometer: Shimadzu BioSpec-nao Microliter Spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany)
- HOBO U30 weather station (Onset Computer Corporation, Bourne, MA, USA)
- HOBO Soil Moisture Smart Sensor: S-SMB-M003 (Onset Computer Corporation, Bourne, MA, USA)
- HOBO Temperature/RH Smart Sensor: S-THB-M002 (Onset Computer Corporation, Bourne, MA, USA)
- HOBO Soil Temperature Smart Sensor: S-TMB-M006 (Onset Computer Corporation, Bourne, MA, USA)

2.1.10 Softwares and online tools

- ApE-A Plasmid Editor Version 2.0.40 (http://biologylabs.utah.edu/jorgensen/wayned/ape/)
- DNA Baser Sequence Assembler (http://www.dnabaser.com/)
- GIA Roots (beta release) (http://giaroots.biology.gatech.edu/)
- ImageQuant Version 5.2 software
- Mega Molecular Evolutionary Genetics Analysis Version 5.05 (http://www.megasoftware.net/)
- Microsoft Office Package 2007
- Vector NTI Advance™ 10
- XnView Version 1.99/1.70 (http://www.xnview.de/)
- Multialgn (http://multalin.toulouse.inra.fr/multalin/)
- ClusterW2-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/)
- HarvEST barley EST data bank (http://harvest.ucr.edu/)
- NCBI GeneBank (http://www.ncbi.nlm.nih.gov/)
- PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html)
- Wikigenes (http://www.wikigenes.org/e/gene/e/853523.html)
- GeneDoc Version 2.7 (http://www.nrbsc.org/downloads/)

2.2 Methods

2.2.1 Growth conditions and stress treatments

2.2.1.1 Drought stress under field conditions

Nine genotypes of spring barley (*Hordeum vulgare*), *Apex*, *Barke*, *Eunova*, *Heils Franken*, *Isaria*, *Pflugs Intensiv*, *Trumpf*, *Victoriana* and *Wiebke* were utilized in this study. The plants were grown in 22 x 22 cm plastic pots containing 11.5 L of Terrasoil® (a mixture of top soil, silica sand, milled lava and peat dust, Terrasoil®, Cordel & Sohn, Salm, Germany) in a plastic greenhouse tunnel in Bonn Poppelsdorf (+50 ° 43' 34.54", +7 ° 5' 15.54"), which enables to have natural growth behaviour under water controlled conditions. The pots were arranged in a spilt plot design with four replications. The seeds were sown on 3rd April, 2010 and 4th April, 2011. Water was supplied with a computer mediated drip irrigation system three times per day (6:15 am, 12:15 pm and 6:15 pm) to keep the VWC (volumetric water content) at 40% in each pot. The drought stress treatment started 30 days after sowing (DAS). Control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the drought stress treated plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. This drought stress protocol was based on preliminary test at INRES with minor modifications. The following parameters were collected by HOBO U30 weather station every 15 min:

- volumetric water content with HOBO Soil Moisture Smart Sensors S-SMB-M003
- soil temperature with HOBO Soil Temperature Smart Sensors S-TMB-M006
- air temperature and radiation with HOBO Temperature/RH Smart Sensors S-THB-M002

2.2.1.2 Drought stress under laboratory conditions

Two genotypes of spring barley (*Hordeum vulgare*), *Barke* and *Arta* were utilized in this experiment. Four-week-old plants were grown in a growth chamber under approximately 120-150 µE m^{-2} s^{-1} light intensity at 22 °C with a day/night cycle of 8/16h. Control plants were watered regularly, whereas water was withheld for two days or five days by drought treated plants, following by rewatering for one week. Leaf, stem and root tissues were harvested after two and five days of the treatment.

2.2.1.3 *in vitro* growth conditions for barley plants

To investigate barley root system architecture, barley seedlings were in a transparent solid growth medium in a growth chamber with the following conditions: 16 hours photoperiod with relative humidity of 50-60%; light intensity of 150 µE m^{-2} s^{-1}; 22 ℃ at daytime and 18 ℃ at night.

2.2.1.4 Growth conditions for bacteria

According to the purpose of the experiments, all *E.coli* strains were cultivated at 37 ℃ in liquid LB medium at 200 rpm or on solid LB agar plate. The cultures were supplemented with appropriate selection markers if required.

2.2.2 Investigation of barley root system architecture

This study was performed in Prof. Dr. Philip Benfey ́s laboratory in the Duke University, Durham NC, USA.

2.2.2.1 Barley seed surface sterilization

In the literature, no protocols for barley seed surface sterilization were reported. Therefore, the efficiency of different seed surface sterilization protocols was tested for barley. These protocols are derived from a common protocol for seeds sterilization used in Prof. Dr. Philip Benfey ́s laboratory (Iyer-Pascuzzi *et al.*, 2010). 1) Barley seeds were sterilized with 35% H_2O_2 for 10 min and rinsed three times with sterile ddH_2O. The sterilized seeds were placed in a Petri-dish containing Hoagland ́s or Yoshida ́s media for one or two days for the pre-germination at 22 ℃ in dark. 2) Barley seeds were sterilized with 35% H_2O_2 for 15 min and rinsed three times with sterile ddH_2O. 3) Barley seeds were dehulled then sterilized with 35% H_2O_2 for 10 min and rinsed three times with sterile ddH_2O. 4) Barley seeds were dehulled then sterilized with 35% H_2O_2 for 15 min and rinsed three times with sterile ddH_2O. 5) Barley seeds were dehulled and sterilized with 35% H_2O_2 for 15 min following by 70% ethanol for five min and rinsing three times with sterile ddH_2O. 6) Barley seeds were dehulled and sterilized with 35% H_2O_2 following by water for 6 hours and rinsing three times with sterile ddH_2O. 7) Barley seeds were sterilized with 10% H_2O_2 for 10 min and rinsed three times with sterile ddH_2O. 8) Barley seeds were sterilized chlorine gas for 6 hours rinsed three times with sterile ddH_2O.

2.2.2.2 Imaging platform

Barley seedlings were imaged using a PhotoCapture 360 turntable and software from Ortery Technologies connected to a Cannon PowerShot G7 digital camera and Dell Latitude 620 laptop computer. By imaging, cylinders or bottles were placed on the automated turntable.

The turntable rotates through a predetermined angle, stops and the camera acquires an image. Images from 40 angels per plant were acquired. The imaging process takes approximately 10 min for each plant. The original images were then converted to two-dimensional (2D) or three-dimensional (3D) images using GiaRoot software developed by the researchers from Duke University and Georgia Institute of Technology (Iyer-Pascuzzi *et al.*, 2010). The principle of the image conversion is based on the pixel counting and comparison. Prior to the image conversion, the diameter of the used container were fixed on the original image. The real measured value of the diameter from the used container was denoted. The software calculates the corresponding pixels basing on the denoted value then automatically converts the chosen image to a binary format using an adaptive thresholding method. Parts of plant such as aerial structure and seed as well as the background of the images were cropped, to reduce the time of image conversion.

2.2.3 Extraction of genomic DNA from barley (Murray and Thompson, 1980)

Genomic DNA was extracted from barley leaf tissues using a protocol from Murray and Thompson (1980) with minor modifications. 200 mg of fresh plant material was ground in liquid nitrogen and the powder was suspended in 1 ml prewarmed extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 4% CTAB (w/v), 0.2% β-mercaptoethanol (v/v)] at 60 ℃ in a circulating water bath for 30 min. After cooling to room temperature, one volume of chloroform:isoamyl alcohol (24:1) was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min. The upper phase was carefully transferred into a fresh 2 ml tube. Three fourth volume of ice-cold isopropanol was added to precipitate the DNA. The extracted DNA was fished out using micropipette into a new tube or pelleted by centrifugation at 13,200 rpm for 5 min. The pellet was washed with ice-cold 80% ethanol. Tubes were inverted on sterile tissue paper for two min to dry the DNA pellets. DNA pellets were then dissolved into 50 µl TE buffer containing 40 µg/µl RNase A. The DNA sample was incubated at 37 ℃ in a water bath for 15 min to get rid of RNA contamination.

2.2.4 Purification of genomic DNA and DNA fragments

2.2.4.1 Purification and precipitation of genomic DNA

To remove proteins and other impurities from a DNA sample, the sample was diluted with sterile ddH_2O up to 200 µl. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 14,000 rpm for 5 min at 4 ℃. The upper phase was carefully transferred into a fresh 1.5 ml tube. 0.1 volume of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol were added, mixed well and incubated at -20 ℃ for 20 min.

The mixture was centrifuged at 14,000 rpm at 4 ℃ for 5 min. The pellets were washed with 70% (v/v) ethanol, air-dried and dissolved into 20 µl sterile TE-buffer.

2.2.4.2 Purification of DNA fragments after digestion with restriction enzymes

Restriction enzymes and ions were separated from the digested DNA sample by adding one volume of phenol then by vortexing to yield a homogenous solution. The mixture was centrifuged at room temperature to separate the aqueous and organic phases. The upper phase was transferred into a fresh 1.5 ml tube. An equal volume of chloroform was added, mixed well and spun down briefly at room temperature. To precipitate the DNA fragments, two volumes of ice-cold 95% (v/v) ethanol, 1/10 volume (v/v) of 3M NaOAc (pH 4.5) and 20 µg of glycogen were added to each tube, mixed well and centrifuged at 14,000 rpm for 15 min at 4 ℃. The pellets were washed with 70% (v/v) ethanol, air-dried and dissolved into 20 µl sterile TE-buffer.

2.2.5 Isolation of *CBF/DREB* genes in barley

2.2.5.1 Identification of *CBF/DREB* genes

To initiate this project, available *Arabidopsis* and wheat *CBF/DREB* protein sequences were used for data mining the NCBI and barley HarvEST data bank (http://harvest.ucr.edu/) in search of barley homologues. cDNA fragments homogeneous to the *Arabidopsis CBF1-3* (also termed *DREB1B*, *-1C* and *-1A*) and *DREB2* (*DREB2A*, *-2B* and *-2C*) genes were obtained using the AP2/EREBP consensus sequences as a query to search the barley EST database.

2.2.5.2 Cloning of DNA Fragments

2.2.5.2.1 Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987)

A standard PCR reaction in a volume of 20 µl was prepared as follows:

2 µl	10 X standard reaction buffer ($MgCl_2$ 15 mM)
0.4 µl	10 mM dNTP mix
0.4 µl	Forward-primer (10 pmol/ µl)
0.4 µl	Reverse-primer (10 pmol/ µl)
0.2 µl	Taq DNA polymerase (5 unit/ µl)
1 µl	DNA template
15.6 µl	ddH_2O

Reaction was homogenized and the PCRs were performed in a thermal cycler. The optimal number of PCR cycles and the annealing temperature was empirically determined for each PCR. A standard PCR program was as follows:

Step	Temperature (℃)	Time (sec)
1	95	180
2	95	30
3	45-65	30
4	72	30/500 bases
5	go to step 2; 35 X	
6	72	600
7	10	forever

2.2.5.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is the most effective way to separate nucleic acids of varying size. The electrophoretic mobility of nucleic acids through an agarose gel is dependent on their molecular size and the pore sizes present in the agarose gel. Pore size can be decreased by increasing the percentage of agarose in the gel. The most utilized agarose gel is from 0.7% [(w/w); resolution: 5-10 kb DNA fragments] to 2% [(w/w); resolution: 0.2-1 kb DNA fragments]. DNA or RNA samples were well-mixed with loading dye and loaded on the gel, which is usually conducted with either TAE or TBE buffer. After staining in ethidium bromide/TAE (TBE) buffer (1 mg/ml) for 10 min, DNA or RNA was visualized under UV light.

2.2.5.2.3 Extraction of DNA fragments from agarose gels

After electrophoresis of the DNA fragments, the fragment of interest was isolated from the agarose gel using NucleoSpin® Extract II Kit. For each 100 mg of agarose gel, 200 µl NTI buffer was added and incubated for 5-10 min at 50 ℃. The mixture was vortexed briefly every 2-3 min until the gel slice is completely dissolved. Up to 700 µl sample can be loaded into a NucleoSpin® column. Sample was centrifuged at room temperature for 1 min at 11,000 g. The remaining sample was loaded, if necessary, and the centrifugation step was repeated. To wash the sample, 700 µl NT3 buffer was added to the column and centrifuged at room temperature for 1 min at 11,000 g. Flow-through was discarded and the previous centrifugation step was repeated to minimize carry-over of chaotropic salts and contaminants, which lower the ratio A_{260}/A_{230}. To elute the DNA, 15-30 µl buffer NE was added into the column and incubated at room temperature for 1 min. The sample was centrifuged at room temperature for 1 min at 11,000 g.

2.2.5.2.4 Ligation

Amplicons of interest were subcloned into the pJET1.2/blunt vector before sequencing, according to the protocol provided with the Kit (CloneJET™ PCR Cloning Kit, Fermentas, St. Leon-Rot, Germany). For this, the 3´-dA overhangs generated by Taq DNA polymerase in the PCR products were removed by a DNA blunting enzyme, a proprietary thermostable DNA polymerase with proofreading activity provided in the kit. The setup of the reaction is as follows:

Component	**Volume**
2 X Reaction buffer	5 µl
PCR product	1-2 µl
Water, nuclease-free	up to 8.5 µl
DNA blunting enzyme	1 µl
Total volume	9 µl

The mixture was vortexed briefly and incubated at 70 ℃ for 5 min. Then the mixture was chilled briefly on ice. To set up the ligation, 0.5 µl pJET1.2/blunt cloning vector (50 ng/µl) and 0.5 µl T4 DNA ligase were added to the mixture and vortexed briefly. The ligation mixture was incubated at room temperature for 5 min. Then the ligation mixture is ready for bacterial transformation.

2.2.5.2.5 Transformation

2.2.5.2.5.1 Preparation of chemically competent *E.coli* cells

A single colony of the *E.coli* stain DH10 was picked from a LB agar plate and cultivated for 16 hours at 37 ℃ then diluted 500-1000 fold into 100 ml LB broth in a 1-liter flask. This bacterial culture was incubated at 37 ℃ with vigorous agitation till OD600 = 0.5. The bacterial cells were transferred into sterile, disposable, ice-cold polypropylene tubes, chilled on ice for 10 min and centrifuged at 5,000 rpm at 4 ℃ for 5 min. The pellet was resuspended by swirling or gentle vortexing in 1 ml ice-cold 0.1 M $CaCl_2$ solution and centrifuged again as above. The pellet was suspended in 9 ml ice-cold 0.1 M $CaCl_2$ solution and centrifuged again as above. Finally, the pellet was suspended in 1 ml 0.1 M $CaCl_2$ and 15% (v/v) glycerol solution. The competent cells were dispensed into aliquots and stored at -80 ℃.

2.2.5.2.5.2 Transformation of chemically competent *E.coli* cells

2.5 µl of the ligation reaction mixture was utilized to transform 50 µl of calcium-competent

E.coli cells, mixed well and incubated on ice for 30 min. The tubes were transferred to rack placed in a circulating water bath preheated to 42 ℃ for 45 seconds. The tubes were chilled briefly on ice. Cells were diluted into 800 µl of LB medium and incubated at 37 ℃ with vigorous agitation for 1 hour. After incubation, 80 µl of cell suspension were spread uniformly on selective agar-plates containing 0.1 mM ampicillin. The plate was inversely incubated at 37 ℃ for 12-16 hours.

2.2.5.2.6 Plasmid mini-prep

The small-scale preparation of plasmid DNA was obtained by the alkaline lysis method (Birnboim and Doly, 1979; Sambrook and Russell, 1989). After identification of a single transformed bacterial colony by colony-PCRs, this single colony was transferred into 2 ml LB medium containing 0.1 mM ampicillin in a loosely capped 15 ml tube. The culture was incubated overnight at 37 ℃ with vigorous shaking. 1.5 ml of the culture was poured into a fresh sterile microfuge tube and centrifuged at 12,000 g for 30 seconds at 4 ℃. The bacterial pellet was resuspended in 100 µl ice-cold Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) by vigorous vortexing and incubated on ice for 5 min. The suspension was then mixed with 200 µl freshly prepared Solution II (0.2 N NaOH; 1% SDS) by inverting the tube rapidly five times. The mixture was incubated on ice for 5 min. 150 µl ice-cold Solution III (60 ml 5M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml H_2O) was added to the mixture and mixed gently by inverting the tube for five times. The mixture was stored on ice for 5 min then centrifuged at 12,000g for 5 min at 4 ℃. The supernatant was transferred into a fresh 1.5 ml tube. One equal volume of phenol: chloroform: isoamyl alcohol was added and mixed by vortexing. After centrifugation at 12,000g for 2 min at 4 ℃, the supernatant was transferred into a fresh 1.5 ml tube. To precipitate the plasmid DNA, 1/10 volume of 3M sodium acetate, pH 5.2 and one volume of ice-cold isopropanol were added and mixed by inverting the tubes for 5 times. After centrifuging at 12,000g for 5 min at 4 ℃, the DNA pellet was washed with 70% ethanol, air-dried and dissolved into 20 µl sterile TE-buffer containing 40 µg/µl RNase A. The dissolved DNA was incubated at 37 ℃ in a water bath for 15 min to remove the RNA contamination.

2.2.6 Isolation of the *CBF/DREB* promoters from barley

2.2.6.1 Digestion of genomic DNA

The Genome Walker DNA library was constructed from a total of five digestion reactions, including four blunt-end digestions *Dra*I, *EcoR*V, *Pvu*II and *Stu*I as well as one *Pvu*II digestion of human genomic DNA as a positive control. A standard digestion reaction in a

volume of 100 µl was prepared as follows:

25 µl High quality genomic DNA (0.1 µg/µl)
4 µl Restriction enzyme (10 units/µl)
10 µl 10 X restriction enzyme buffer
61 µl dd H_2O

The reaction mixture was mixed gently by inverting the tubes for several times and incubated at 37 ℃ for 16-18 hours. Then the DNA fragments were purified as described in 2.2.4.2.

2.2.6.2 Ligation of genomic DNA to genome walker adaptors

Five ligation reactions were set up with the products from the five digestions described in 2.2.6.1. For each reaction, 4 µl of the digested and purified DNA was transferred to a fresh 0.5 ml tube. To each, 1.9 µl of 25 µM genome walker adaptors, 1.6 µl of 10 X ligation buffer and 0.5 µl of T4 DNA ligase (6 units/µl) were added, mixed well and incubated at 16 ℃ overnight. To stop the reactions, the mixture was incubated at 70 ℃ for 5 min. Then 72 µl of TE buffer was added and mixed vigorously.

2.2.6.3 PCR-based DNA walking in the genome walker library

A nested-PCR approach was performed, to get specific PCR products. For primary PCR, 1 µl of each library was utilized, whereas 1 µl of a 50 X dilution of the primary PCR product was used for secondary PCR. For each reaction 50 µl of the mixture was prepared. The two-step of cycle parameters for the primary PCR was as follows:

Step	Temperature (℃)	Time (sec)
1	95	180
2	95	30
3	67	30
4	72	180
5	go to step 2; 6X	
6	95	25
7	63	30
8	67	180
9	go to step 5; 32X	
10	67	450

The two-step of cycle parameters for the secondary PCR is quite similar to the primary PCR with minor modification on annealing temperature and amplification cycles. After gel electrophoresis, the PCR products of interest were excised from the gel and prepared for sequencing (see 2.2.5).

2.2.7 Methylation sensitive Southern blotting (Bird and Southern, 1978; Sambrook and Russell, 1989)

2.2.7.1 Digestion and fractionation

To detect the methylation pattern in the target promoter sequences, barley genomic DNA was digested with the methylation sensitive restriction enzyme *Hpa*II and its isoschizomer *Msp*I. Because of the large genome size, barley genomic DNA was predigested with *Dra*I, which increases the resolution of the subsequent DNA Southern blot. Digestions were carried out at 37 ℃ for 16 hours. A standard digestion reaction in a volume of 40 µl was prepared as follows:

15 µl	High quality genomic DNA (2 µg/µl)
2 µl	*Dra*I (10 units/µl)
2 µl	*Hpa* II/*Msp*I (10 units/µl)
4 µl	10 X restriction enzyme buffer
11 µl	dd H_2O

2.2.7.2 Separation of restriction fragments by agarose gel electrophoresis

At the end of the digestion, the appropriate amount of DNA loading buffer was added and mixed well. The DNA fragments were separated by electrophoresis through a 1% agarose gel cast in 0.5 X TBE without ethidium bromide. The gel was running in 0.5 X TBE buffer at 20 volt for 17 hours. After electrophoresis, the gel was submerged in 0.5 X TBE buffers containing ethidium bromide (0.5 µg/ml) and stained for 15 min. A transparent ruler was placed alongside the gel while gel was imaged under UV light to allow the estimation of the size of the DNA band of interest.

2.2.7.3 Capillary transfer the DNA from agarose gels to nylon membrane

After electrophoresis and imaging, the gel was soaked in several volumes of 0.2 N HCl for 10 min with gentle agitation, which improves transfer of DNA fragments of interest larger than approximately 15 kb. DNA fragments were denatured by soaking the gel for 2 X 25 min in several volumes of denaturation buffer and neutralized by soaking the gel for 2 X 15 min in several volumes of neutralization buffer with gentle agitation. The DNA fragments were blotted onto a positive charged Hybond-N^+ nylon membrane by capillary transfer. The blot was set up from bottom to up as follows:

- A large dish was filled with 20 X SSC with glass plate on top of it to rest the gel. A piece of Whatman 3MM paper, which was larger and longer than the gel, was wrapped

around the glass plate so that both edges of the 3MM paper were in contact with the bottom of the dish. This Whatman 3 MM paper acts as a wick for the capillary transfer.

- The gel was inverted placed on the wet 3MM paper. Air bubbles were smoothed out with a glass rod.
- Hybond-N^+ nylon membrane was cut to the exact size of the gel, wetted with ddH_2O, placed on the top of the gel and smoothed out with a glass rod.
- Two pieces of 3MM paper were cut to the exact size of the gel, wetted in 2 X SSC and placed on the top of the wet nylon membrane.
- A stack of paper towels were cut just smaller than the 3MM paper and placed on the top of the 3MM paper. The gel was surrounded with Parafilm to prevent direct contact between wick and paper towels. A glass plate was put on top of the stack and weighed it down with an additional weight.
- The DNA fragments were transferred onto the nylon membrane overnight.
- After blotting, the DNA was fixed to the membrane with a UV-Crosslinker.

2.2.7.4 Hybridization

The membrane was submerged in the prehybridization buffer in a plastic box and incubated in a water bath at 65 ℃ for at least 2 hours. The radiolabelled DNA probes were prepared following the manufacturer ́s introduction of the HexaLabel™ DNA Labeling Kit (Fermentas; St. Leon-Rot, Germany) with minor modifications. 100 ng DNA templates were mixed well with 10 µl of 5 X reaction buffer containing decanucleotides in a final volume of 40 µl. The mixture was incubated at 95 ℃ for 5 min and chilled down briefly on ice. Subsequently, 3 µl of Mix C, 2 µl of [α-^{32}P]-dCTP and 1 µl of Klenow fragment (5U) were added, mixed well and incubated at 37 ℃ for 5 min. Finally, 4 µl of dNTPs were added followed by incubation at 37 ℃ for 5 min. To stop the reaction, 1 µl of 0.5 M EDTA, pH 8.0 was added. To remove the unincorporated label, the unincorporated dNTPs were removed by a spin column. The labelled DNA fragments were denatured at 95 ℃ for 5 min and cooled down briefly on ice, before it was added to the fresh hybridization buffer. The membrane was hybridized at 65 ℃ in a water bath with gently shaking overnight. The membrane was subsequently washed 2-3 times in the washing buffer containing 2 X SSC and 0.1% (w/v) SDS. The membrane was placed on a PhosphorImager screen and exposed for 6-14 days. The signal was visualized by scanning the PhosphorImager screen on a Typhoon scanner 9200 (GE Healthcare, formerly Amersham Biosciences, Buckinghamshire, UK).

2.2.8 Semi-quantitative RT-PCR

2.2.8.1 Extraction of total RNAs from barley

Total RNA was extracted using a protocol from Valenzula-Avendaño *et al.* (2005) with minor modifications. About 50 mg of each sample tissue were ground to a fine powder using liquid nitrogen and transferred into a 2-ml Eppendorf tubes. 1.5 ml extraction buffer [0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 5.0), 5% glycerol (v/v), 0.1% phenol red] was added. Samples were incubated at room temperature for 10 min. Then samples were centrifuged at 10,000 g at room temperature for 5 min to separate the cell debris. Supernatants were transferred to fresh tubes. To each tube, 2/3 volume of the chloroform-isoamylalcohol (24:1) mixture was added. After invertion for several times, samples were centrifuged at 10,000 g for 5 min at 4 ℃. The upper aqueous layer was transferred into a fresh 2-ml tube. To each tube, 1/3 volume ice cold isopropanol and 1/3 volume 0.8 M sodium citrate/1M sodium chloride were added and mixed thoroughly. Samples were incubated at room temperature for 10 min and subsequently centrifuged at 12,000 g for 5 min at 4 ℃. Pellets were washed with 80% ice cold ethanol. After air drying, pellets were diluted in DEPC-treated water.

2.2.8.2 Removal of genomic DNA from RNA preparations

To remove the DNA contaminant in the RNA samples, 4 µg RNA was treated with 1 µl of DNase I (10 U/µl, Fermentas, St. Leon-Rot, Germany) in 10 µl reaction mixture containing 1 X DNase I buffer (100 mM Tris-HCl ,pH 7.5; 25 mM $MgCl_2$; 1 mM $CaCl_2$) and incubated at 37 ℃ for 30 min. The reaction was terminated by adding 1 µl of 50 mM EDTA to the reactions mixture and incubated at 65 ℃ for 5min.

2.2.8.3 First strand cDNA synthesis

10 µl DNase I treated RNA was mixed gently with 50 pmol Oligo$(dT)_{18}$ and 1 µl of 10 mM dNTPs and incubated at 65 ℃ for 5 min. 4 µl of 5 X First strand buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl; 20 mM $MgCl_2$; 50 mM DTT), 0.5 µl of RiboLock RNase inhibitor and 1 µl of RevertAid reverse transcriptase (100 U/µl) were added, mixed well and centrifuged briefly. The total reactions mixture was incubated at 42 ℃ for 50 min and the reaction was terminated by heating at 70 ℃ for 10 min. Then the synthesized cDNAs are for PCRs available.

2.2.9 Determination of relative water content (RWC)

Relative water content (Slatyer and Barrs, 1965) represents a useful indicator of water balance in a plant. In this study, leaf RWC was determined according the method described by Stocker

(1929). In brief, three whole leaves of each variety were excised and weighed immediately. Then the whole leaves were completely immersed in water for 24 h and reweighed. After subsequent oven drying the leaves at 80 ℃ for 24 h, the final weight was determined and the RWC was calculated according to the formula:

$$RWC = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated weight} - \text{dry weight}} \text{ X } 100\%$$

2.2.10 Phylogenetic analysis

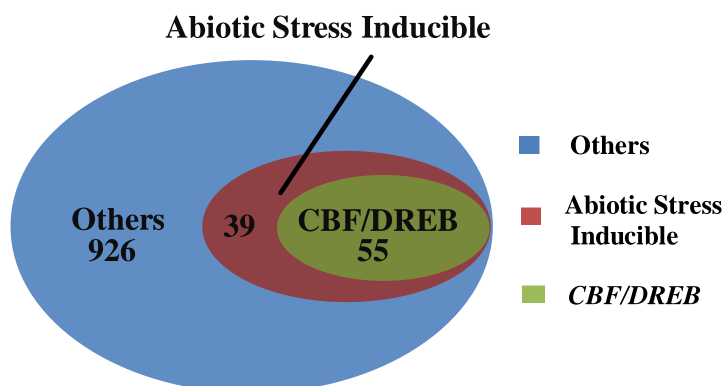
Monocotyledon CBF homologues were identified using *Arabidopsis* and wheat *CBF/DREB* protein sequences as queries against the NCBI GeneBank (http://www.ncbi.nlm.nih.gov/) and barley HarvEST data bank (http://harvest.ucr.edu/). FASTA files of the protein sequences used in this analysis are presented in Appendix. Protein sequences were aligned using ClustalW from the MEGA 5.0 software version 5.1 (http://www.megasoftware.net/) and refined manually using GeneDoc Version 2.7 (http://www.nrbsc.org/downloads/). Phylogenetic analyses on refined alignments were conducted by MEGA 5.0 software and phylogenetic trees were generated using neighbor joining and poisson model on 5050 bootstrap replications.

3. RESULTS

3.1 Identification of barley *CBF/DREB* genes

In this study, a core set of 10 spring barley (*Hordeum vulgare* L.) cultivars (see 2.1.1) were studied to investigate the influence of methylation of the transcription factor *CBF/DREB* genes on their dehydration tolerance. Data mining and analyses of the published *CBF/DREB* sequences in NCBI GeneBank (http://www.ncbi.nlm.nih.gov/) and HarvEST EST data bank (http://harvest.ucr.edu/) suggest that the *CBF/DREB* gene family varies significantly among the different cereal species. To maximize the chance of discovering *CBF/DREB* genes involved in drought stress in barley, 1020 cDNA libraries (853 AP2 domain containing cDNA libraries and 167 *HvCBF/DREB* cDNA libraries) were screened in the HarvEST data bank. Among the 1020 cDNA libraries there are 94 putative abiotic stress inducible cDNA libraries, in which there are 55 *HvCBF/DREB* cDNA libraries corresponding 13 contigs (Fig. 1). The contig numbers are as follows: 17070 (*HvDRF1.3*); 188855 (*HvDREB1*); 21072 and 21648 (*HvDRF1.1*); 22083, 8313, 17946, 23852, 11089 and 30843 (*HvCBF10A*); 46923 (*HvCBF8*); 30770 (*HvCBF11*) and 15347 (*HvCBF3*). In addition, *Arabidopsis* and wheat *CBF/DREB* protein sequences were used as queries against the NCBI to identify barley *CBF/DREB* homologues. Further17 barley *CBF/DREB* genes were identified. They are *HvCBF1* (AY785836), *HvCBF2A* (GU461588), *HvCBF2B* (DQ097684), *HvCBF3* (EU331959), *HvCBF4A* (AY785849), *HvCBF4B* (AY785853), *HvCBF4D* (AY785852), *HvCBF5* (AY785858), *HvCBF7* (AY785863), *HvCBF8B* (AY785876), *HvCBF8C* (AY785875), *HvCBF9* (EU332038), *HvCBF10B* (AY785888), *HvCBF12* (DQ095157), *HvCBF13* (DQ095158), *HvCBF14* (DQ095159) and *HvDRF2* (AF521302). Altogether, a combination

Fig. 1. Screening of barley *CBF/DREB* cDNA libraries involved in abiotic stress



Total: 1020 cDNA Libraries

Fig. 1. Screening of barley *CBF/DREB* cDNA libraries involved in abiotic stress. A total of 1020 cDNA libraries was screened in HarvEST data bank. 94 cDNA libraries contained putative abiotic stress inducible transcripts (labelled in red) and 55 of them would be specific for *CBF/DREB* transcripts (labelled in green).

of cDNA library screening and homolog blasting allowed to identify 30 barley *CBF/DREB* gene sequences in this study. Based on the preliminary studies 10 drought stress inducible *CBF/DREB* genes (listed in Table 2) were determined for this study.

Table 2. Nomenclature and characteristics of barley *CBF/DREB* genes

Gene name	Accession Number	Total amino acid	Stress type	References
HvCBF1	AY785836	217	LT/drought	(Skinner et al., 2005)
HvCBF2	AY785840	221	LT/drought	(Xue, 2003)
HvCBF3	EU331959	249	LT/drought	(Choi et al., 2002)
HvCBF4	AY785848	225	LT/drought	(Skinner et al., 2005)
HvCBF6	AY785859	244	LT/salt/drought	(Skinner et al., 2005)
HvCBF11	AY785889	218	LT/salt	HarvEST-data bank (http://harvest.ucr.edu/)
HvDREB1	HQ647359	278	drought	(Xu et al., 2009)
HvDRF1.1	AAO38209	419	drought	(Xue and Loveridge, 2004)
HvDRF1.3	AAO38211	372	drought	(Xue and Loveridge, 2004)
HvDRF2	AF521302	316	drought	(Nayak et al., 2009)

3.2 Isolation of *CBF/DREB* genes from the barley core set

3.2.1 Isolation of *CBF1* genes from the barley core set

To isolate barley *CBF1* genes from the core set, the in 3.1 identified full-length barley *CBF1* cDNA sequences from the NCBI GeneBank (Accession-Nr.: AY785836) were utilized to design gene specific primers corresponding 5´ and 3´-ends of *HvCBF1* cDNA (Table 1). These gene specific primer pairs were used to amplify the corresponding genes from the genomic DNA of eight different barley genotypes. The *HvCBF1* amplicons were 654 bp in length with an ORF of 218 amino acids. Sequence alignment of the barley *CBF1* cDNA with its genomic DNA indicated that barley *CBF1* gene possesses no intron (Fig. 2A). The multiple sequence alignment of the isolated *HvCBF1* sequences revealed that the *HvCBF1* gene of the cultivars *Eunova*, *Heils Fanken*, *Isaria* and *Victoriana* were 100% identical with the reference gene sequences from the NCBI GeneBank. In *Wiebke*, the presence of a single nucleotide variant of T/C at position 207 of the reference gene led to a non-synonymous mutation from cysteine to arginine amino acid residue. Two SNPs were observed in ORF of the cultivars *Barke* and *Pflugs Intensiv*. In the cultivar *Barke*, one SNP was observed at the

postion 250 from A to G leading to a non-synonymous mutation from threonine to alanine. A further transition from C to T at position 355 led to a non-synonymous mutation from

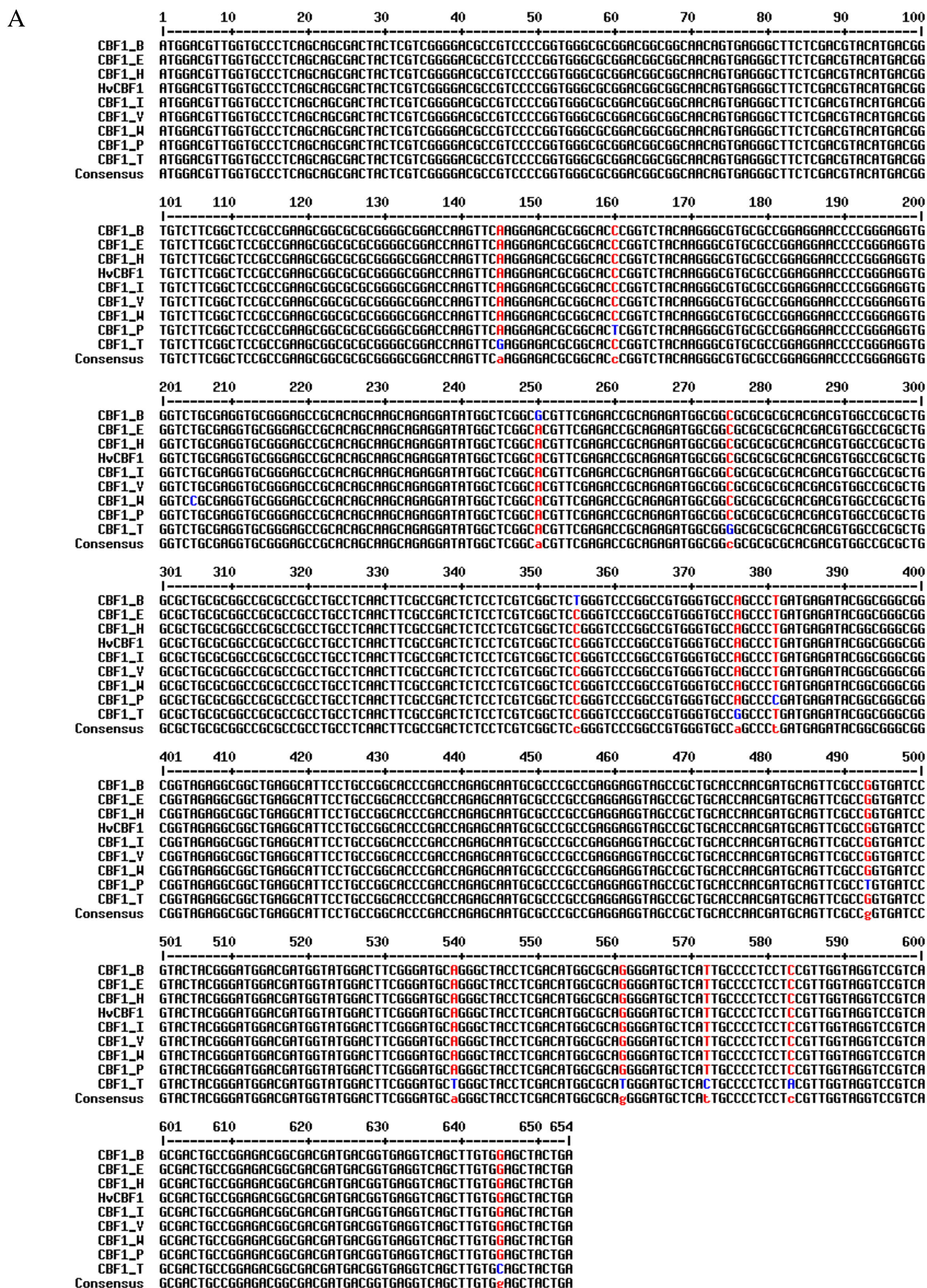


Fig. 2.

B

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          ************************************************:****.************** ***********
CBF1_B    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_E    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_H    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_I    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_P    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHSVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_T    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFEETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_V    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
CBF1_W    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVREVREPHSKQRI   80
HvCBF1    MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRRNPGRWVCEVREPHSKQRI   80
          1.......10........20........30........40........50........60........70........80
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Conservation

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          ***:*******.*************************** *****.**********************************
CBF1_B    WLGAFETAEMAARAHDVAALALRGRAACLNFADSPRRLWVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_E    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_H    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_I    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_P    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_T    WLGTFETAEMAGRAHDVAALALRGRAACLNFADSPRRLRVPAVGAGPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_V    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
CBF1_W    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
HvCBF1    WLGTFETAEMAARAHDVAALALRGRAACLNFADSPRRLRVPAVGASPDEIRRAAVEAAEAFLPAPDQSNAPAEEVAAAPT  160
          .........90.......100.......110.......120.......130.......140.......150.......160
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Conservation

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          **** ************** ******:*** ***.******************* **
CBF1_B    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_E    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_H    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_I    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_P    MQFACDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_T    MQFAGDPYYGMDDGMDFGMLGYLDMAHGMLTAPPTLVGPSATAGDGDDDGEVSLCSY   217
CBF1_V    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
CBF1_W    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
HvCBF1    MQFAGDPYYGMDDGMDFGMQGYLDMAQGMLIAPPPLVGPSATAGDGDDDGEVSLWSY   217
          .........170.......180.......190.......200.......210.......
```

Conservation

C

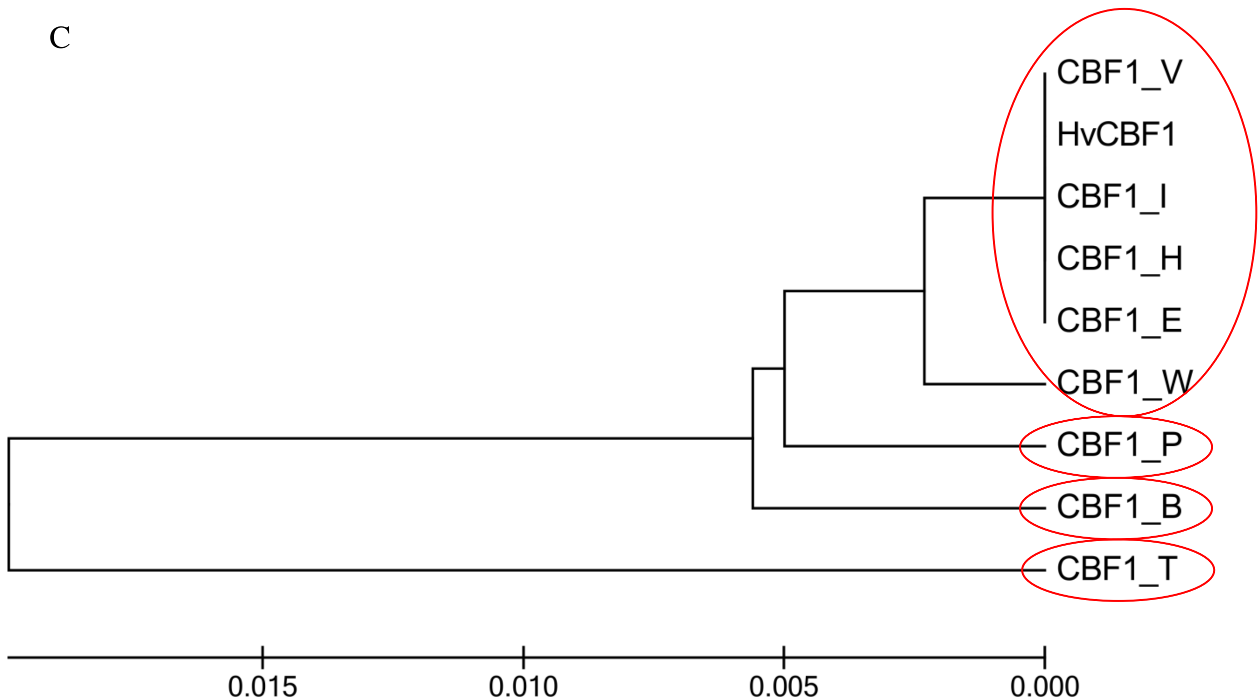

Fig. 2. continued

Fig. 2. DNA sequence analysis of barley *CBF1* genes. (A) Comparison of *CBF1* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF1* genes in eight barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among eight isolated barley *CBF1* genes. A dendrogram was resulted from UPGMA cluster analysis of eight barley cultivars based on the deduced protein sequences of the isolated eight barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF1*: reference gene from NCBI.

arginine to tryptophan. The cultivar *Pflugs Intensiv* had a C/T variant at position 160 and a G/T variant at position 493, which caused non-synonymous mutations from proline to serine and from glycine to cysteine, respectively. In the cultivar *Trumpf*, eight SNPs were observed and most of them occurred at the 3 ́end. Two A/G variants at positions 145 and 376 led to non-synonymous mutations from lysine to glutamic acid and serine to glycine. Two further nucleotide transversions from C to G as well as G to C were observed at positions 275 and 645 leading to non-synonymous mutations from alanine to glycine and tryptophan to cysteine, respectively. The presence of additional A/T, G/T, T/C, C/A variants at positions 539, 561, 572 and 583 led to further non-synonymous mutations from glutamine to leucine, glutamine to histidine, isoleucine to threonine, proline to threonine, respectively (Fig. 2B).

To estimate the genetic diversity between the identified *CBF1* genes in eight different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *CBF1* genes in the eight different barley cultivars are shown in Table 3. To reveal the genetic distance among these *CBF1* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 2C, the isolated barley *CBF1* genes from the eight cultivars were clustered into four groups. The first group contained barley *CBF1* genes of the cultivars *Victoriana*, *Isaria*, *Heils Franken*, *Eunova* and *Wiebke*. This group could be subdivided into two subgroups. The *CBF1* gene of the first four cultivars composed the subgroup I. The subgroup II consisted of the *CBF1* gene from the cultivar *Wiebke*. From the second to the fourth group, each of them contained only one member. It should be noted that a great genetic distance is shown between *Isaria* and *Trumpf*, even though *Isaria* is one of the ancestors of the cultivar *Trumpf*.

Table 3. Estimates of genetic divergence between *CBF1* sequences of barley cultivars

Percentage of genetic divergence		1	2	3	4	5	6	7	8	9
	1.CBF1_B									
	2.CBF1_E	0.3								
	3.CBF1_H	0.3	0.0							
	4.CBF1_I	0.3	0.0	0.0						
	5.CBF1_P	0.8	0.5	0.5	0.5					
	6.CBF1_T	1.5	1.2	1.2	1.2	1.7				
	7.CBF1_V	0.3	0.0	0.0	0.0	0.5	1.2			
	8.CBF1_W	0.5	0.2	0.2	0.2	0.6	1.4	0.2		
	9.HvCBF1	0.3	0.0	0.0	0.0	0.5	1.2	0.0	0.2	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved eight isolated *CBF1* nucleotide sequences and the reference *HvCBF1* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 654 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF1*: reference gene from NCBI.

3.2.2 Isolation of *CBF2* genes from the barley core set

The strategy for the isolation of *CBF2* genes in different barley cultivars was identical as described in 3.2.1. The *CBF2* amplicons were 561 bp in length with an ORF of 187 amino acids. The sequences obtained in nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the barley *CBF2* gene with their cDNAs indicated that no intron sequences were present in the obtained *CBF2* homologues (Fig. 3A). Three SNPs were present only in the refence *HvCBF2* gene from NCBI GeneBank at positions 190, 450 and 467. A variant of A/G and T/C at positions 190 and 450 led to synonymous mutations, whereas a nucleotide transition from G to A at position 467 led to a non-synonymous mutation from glycine to glutamic acid. Further SNPs were observed among the isolated barley *CBF2* amplicons. A C/T variant was observed in the cultivars *Barke* and *Heils Franken*, which led to a synonymous mutation. In the cultivar *Barke*, a further nucleotide transition from T to C at position 223 led to a non-synonymous mutation from serine to proline. In the cultivars *Arta*, *Pflugs intensiv* and *Isaria*, an A/G variant at position 369 and a further G/T variant at position 372 also led to synonymous mutations. A further nucleotide transition from C to T was observed in the cultivar *Isaria* at position 324 leading to a non-synonymous mutation from phenylalanine to serine. In the cultivar *Wiebke*, two single nucleotide variants from C to A and T to C at positions 419 and 550 led to non-synonymous mutations from leucine and tyrosine to histidine, respectively. An additional C/A nucleotide transversion at position 340 did not change the corresponding amino acid residue. In the cultivar *Eunova*, a G/A variant at position 198 and a C/T variant at position 249 also led

to synonymous mutations. Another G/A variant at position 358 led to a non-synonymous mutation from alanine to threonine. As shown in the isolated *HvCBF1* amplicons, the most nucleotide variants occurred in the isolated *HvCBF2* sequences of the cultivar *Trumpf*. Nucleotide variants from C to A and C to T at positions 303 and 327 did not change the corresponding amino acid residues. Two C/G transversions at positions 252 and 485 led to non-synonymous mutations from asparagine to lysine and threonine to serine. Another transversion from A to T was present at position 356 leading to a non-synonymous mutation from histidine to leucine (Fig. 3B).

To estimate genetic diversity between the identified *CBF2* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *CBF2* genes in nine cultivars are shown in Table 4. To reveal the genetic distance among these *CBF2* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 3C, the isolated *CBF2* genes from nine different barley cultivars were clustered into four groups. The first group contained *CBF2* genes of the cultivars *Arta*, *Pflugs Intensiv*, *Isaria*, *Barke*, *Heils Franken* and *Victoriana*. This group could be subdivided into three subgroups. The barley *CBF2* genes of the first three cultivars composed the subgroup I. The *CBF2* gene of the cultivar *Barke* belonged to the subgroup II. In the subgroup III, the *CBF2* gene of the cultivars *Heils Franken* and *Victoriana* was present. From the third to the fifth group, each of them contained only one member. The reference gene *HvCBF2* formed the second cluster. The genetic distance of the isolated barley *CBF2* genes is quite similar to that shown in *HvCBF1* genes.

Table 4. Estimates of genetic divergence between *CBF2* sequences of barley cultivars

Percentage of genetic divergence	1	2	3	4	5	6	7	8	9	10
1. CBF2_A										
2. CBF2_B	0.7									
3. CBF2_E	1.1	0.7								
4. CBF2_H	0.5	0.2	0.5							
5. CBF2_I	0.2	0.9	1.2	0.7						
6. CBF2_P	0.0	0.7	1.1	0.5	0.2					
7. CBF2_T	0.9	1.6	2.0	1.4	1.1	0.9				
8. CBF2_V	0.4	0.4	0.7	0.2	0.5	0.4	1.2			
9. CBF2_W	0.9	0.9	1.2	0.7	1.1	0.9	1.8	0.5		
10. HvCBF2	0.9	0.9	1.2	0.7	1.1	0.9	1.8	0.5	1.1	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *CBF2* nucleotide sequences and the reference *HvCBF2* gene from the NCBI. All positions containing gaps and missing

data were eliminated. There were a total of 561 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF2*: reference gene from NCBI.

A

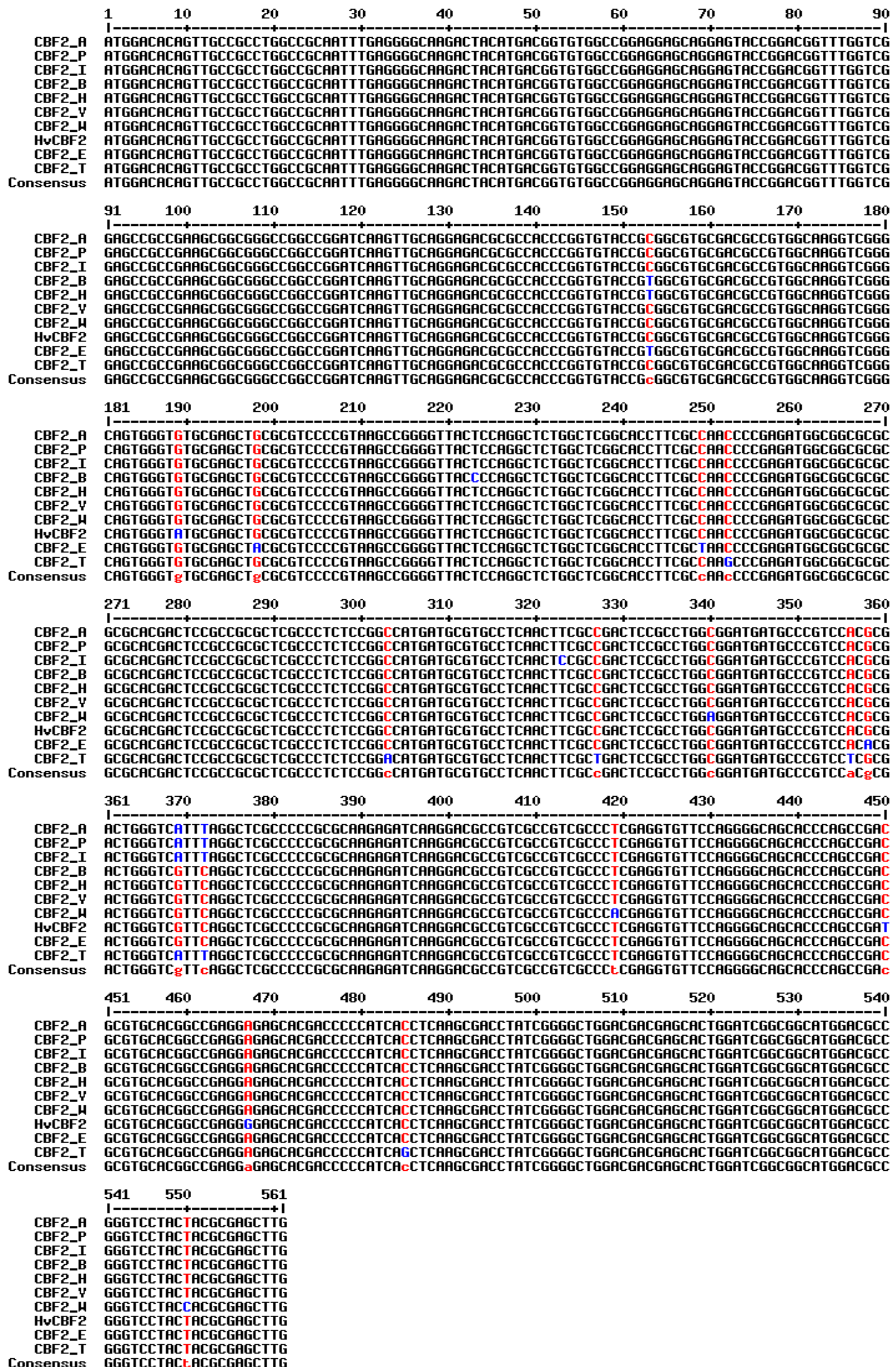

Fig. 3.

B

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         ******************************************************************************.*****
CBF2_I   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_P   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_V   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
HvCBF2   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_W   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_H   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_A   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_B   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYPRLWLG   80
CBF2_E   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
CBF2_T   MDTVAAWPQFEGQDYMTVWPEEQEYRTVWSEPPKRRAGRIKLQETRHPVYRGVRRRGKVGQWVCELRVPVSRGYSRLWLG   80
         1.......10........20........30........40........50........60........70........80
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Conservation

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         ***:*********************** ********** :******************* **************** ****
CBF2_I   TFANPEMAARAHDSAALALSGHDACLNSADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_P   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_V   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
HvCBF2   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEGSTTP   160
CBF2_W   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVAHEVFQGQHPADACTAEESTTP   160
CBF2_H   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_A   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_B   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_E   TFANPEMAARAHDSAALALSGHDACLNFADSAWRMMPVHTTGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
CBF2_T   TFAKPEMAARAHDSAALALSGHDACLNFADSAWRMMPVLATGSFRLAPAQEIKDAVAVALEVFQGQHPADACTAEESTTP   160
         ........90.......100.......110.......120.......130.......140.......150.......160
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Conservation

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         *:*********************:***
CBF2_I   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_P   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_V   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
HvCBF2   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_W   ITSSDLSGLDDEHWIGGMDAGSYHASL   187
CBF2_H   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_A   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_B   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_E   ITSSDLSGLDDEHWIGGMDAGSYYASL   187
CBF2_T   ISSSDLSGLDDEHWIGGMDAGSYYASL   187
         .......170.......180.......
```

Conservation

C

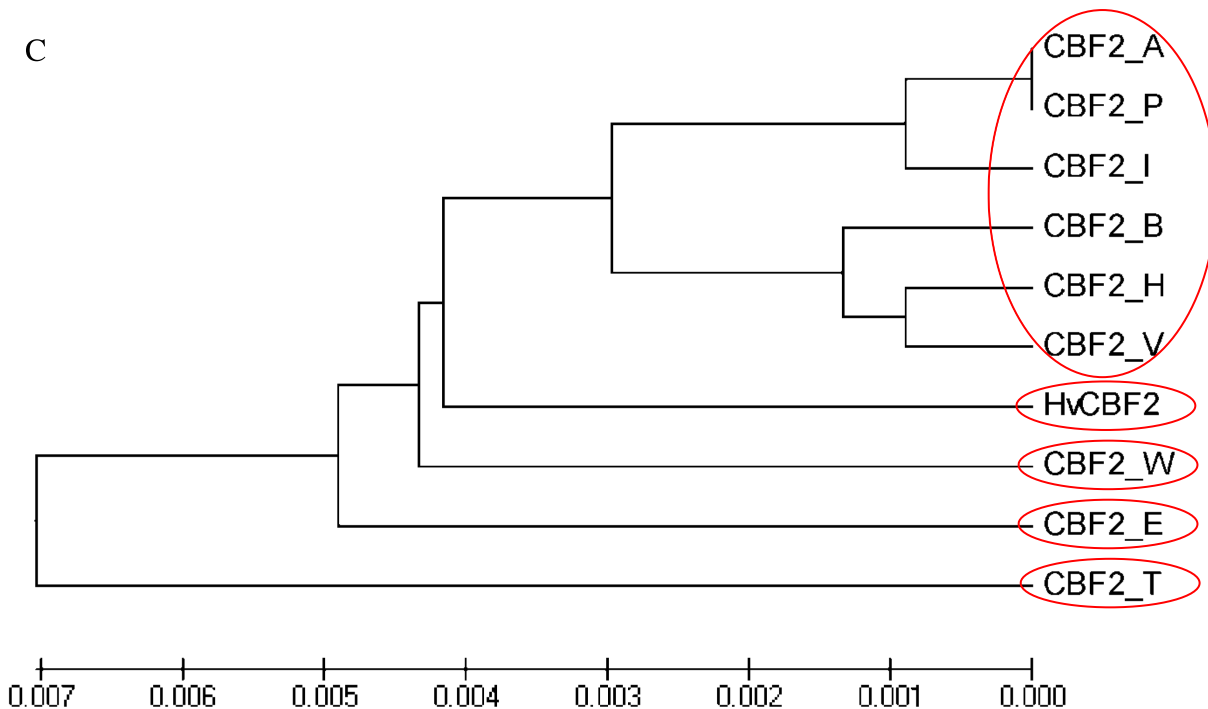

Fig. 3. continued

Fig. 3. DNA sequence analysis of barley *CBF2* genes. (A) Comparison of *CBF2* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF2* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among nine isolated barley *CBF2* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF2*: reference gene from NCBI.

3.2.3 Isolation of *CBF3* genes from the barley core set

The barley *CBF3* genes, the barley *CBF3* homologues were isolated from nine different genotypes using degenerate primers (Table 1). These amplicons were 730 bp in length with a 99 bp long 5 ´UTR and an ORF of 210 amino acid residues. The sequences obtained in nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the barley *CBF3* gene with their cDNAs indicated that no intron sequences were present in the obtained *CBF3* homologues (Fig. 4A). A/T and C/T nucleotide variants were present at positions 10 and 73 of the 5 ´UTR region. The barley genotypes *Arta*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Trumpf* shared die nucleotide mutations at positions 150, 192, 220, 228 and 229. Only a C/T variant at position 220 led to a non-synonymous mutation from arginine to cysteine. The identical mutation was observed in the variety *Eunova* at position 394. Four additional non-synonymous mutations were also identified. Each of a G/A nucleotide transition at positions 109 and 110 in the variety *Arta* led to changes in the amino acid residues from glycine to asparagine. The cultivars *Victoriana* and *Wiebke* shared a G/A nucleotide transition at position 169 leading to non-synonymous mutation from alanine to threonine (Fig. 4B). Another alanine/threonine mutation was identified in the cultivars *Barke*, *Victoriana* and *Wiebke* caused by a G/A nucleotide variant at position 637. In contrast, the presence of a single nucleotide variant C/T at position 423 in the genotypes *Trumpf*, *Victoriana* and *Wiebke* led to a synonymous mutation. Another two common synonymous mutations were observed at position 678 in the varieties *Heils Franken*, *Pflugs Intensiv* and *Isaria* as well as at position 129 in the cultivars *Vitcoriana* and *Wiebke*. Five additional synonymous mutations were identified in the nine isolated barley *CBF3* homologues. Two nucleotide transversions were present at positions 558 and 712 in the

A

```
           1       10        20        30        40        50        60        70        80        90        100
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     TCATCTCACTCTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_H     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_P     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_I     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_T     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_B     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCTAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_E     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_V     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
CBF3_W     TCATCTCACACTCCTCAGTCCTCAGTAAGCTCAAGCACCAAGCTCAGACTGTTCAAGAAGGAAGCCGCCTGCCAATCACCCAGCACTCTGCCGGTAGCCA
HvCBF3                                                                                                        A
Consensus  tcatctcac.ctcctcagtcctcagtaagctcaagcaccaagctcagactgttcaagaaggaagccgcctgc.aatcacccagcactctgccggtagccA

           101     110       120       130       140       150       160       170       180       190       200
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     TGGACATGAACCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTATCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCGAAGCGCCC
CBF3_H     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTATCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCGAAGCGCCC
CBF3_P     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTATCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCGAAGCGCCC
CBF3_I     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTATCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCGAAGCGCCC
CBF3_T     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTATCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCGAAGCGCCC
CBF3_B     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTGTCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCCAAGCGCCC
CBF3_E     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTGTCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCCAAGCGCCC
CBF3_V     TGGACATGGGCCTTGAGGTCTCGAGCTCGTCCCCCTCCTCCTCGTCGGTGTCGTCCTCGCCCGAGCACACGGCGAGGCGGGCGTCGCCGGCCAAGCGCCC
CBF3_W     TGGACATGGGCCTTGAGGTCTCGAGCTCGTCCCCCTCCTCCTCGTCGGTGTCGTCCTCGCCCGAGCACACGGCGAGGCGGGCGTCGCCGGCCAAGCGCCC
HvCBF3     TGGACATGGGCCTTGAGGTCTCGAGCTCCTCCCCCTCCTCCTCGTCGGTGTCGTCCTCGCCCGAGCACGCGGCGAGGCGGGCGTCGCCGGCCAAGCGCCC
Consensus  TGGACATGGGCCTTGAGGTCTCGAGCTCcTCCCCCTCCTCCTCGTCGGTgTCGTCCTCGCCCGAGCACgCGGCGAGGCGGGCGTCGCCGGCcAAGCGCCC

           201     210       220       230       240       250       260       270       280       290       300
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     CGCTGGGCGCACCAAGTTCTGCGAGACAAGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_H     CGCTGGGCGCACCAAGTTCTGCGAGACAAGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_P     CGCTGGGCGCACCAAGTTCTGCGAGACAAGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_I     CGCTGGGCGCACCAAGTTCTGCGAGACAAGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_T     CGCTGGGCGCACCAAGTTCTGCGAGACAAGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_B     CGCTGGGCGCACCAAGTTCCGGGAGACGCGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_E     CGCTGGGCGCACCAAGTTCCGGGAGACGCGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_V     CGCTGGGCGCACCAAGTTCCGGGAGACGCGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
CBF3_W     CGCTGGGCGCACCAAGTTCCGGGAGACGCGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
HvCBF3     CGCTGGGCGCACCAAGTTCCGGGAGACGCGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC
Consensus  CGCTGGGCGCACCAAGTTCcGgGAGACgcGGCACCCGGTGTACCGCGGCGTGCGGCGCCGGGGCAACACCGAACGGTGGGTCTGCGAGGTGCGCGTCCCC

           301     310       320       330       340       350       360       370       380       390       400
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_H     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_P     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_I     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAGCGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_T     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_B     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_E     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_V     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
CBF3_W     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
HvCBF3     GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG
Consensus  GGCAAGCGCGGTGCTCGGCTCTGGCTCGGGACGTACGCCACGGCTGAGGTCGCCGCGCGCGCGAACGACGCTGCCATGCTCGCCCTGGGCGGCCGCTCCG

           401     410       420       430       440       450       460       470       480       490       500
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_H     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_P     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_I     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_T     CCGCGTGCCTCAACTTCGCCGATTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_B     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_E     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_V     CCGCGTGCCTCAACTTCGCCGATTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
CBF3_W     CCGCGTGCCTCAACTTCGCCGATTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
HvCBF3     CCGCGTGCCTCAACTTCGCCGACTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC
Consensus  CCGCGTGCCTCAACTTCGCCGAcTCCGCGTGGCTGCTCGCCGTGCCGTCCGCCCTGTCCGATCTCGCAGACGTCCGGCGCGCGGCTGTCGAGGCCGTCGC

           501     510       520       530       540       550       560       570       580       590       600
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_H     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_P     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_I     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_T     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGATGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_B     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCAGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_E     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
CBF3_V     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCATCACTATCTCCGTCGTCTGGGTCC
CBF3_W     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
HvCBF3     GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC
Consensus  GGATTTCCAGCGACGGGAGGCTGCCGATGGCTCCCTCGCCATCGCTGTCGCTAAGGAGGCCTCCTCTGGCGCTCCTTCACTATCTCCGTCGTCTGGGTCC

           601     610       620       630       640       650       660       670       680       690       700
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
CBF3_A     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
CBF3_H     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGATATGTTCAGGCTTGACTTGTTCC
CBF3_P     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGATATGTTCAGGCTTGACTTGTTCC
CBF3_I     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGATATGTTCAGGCTTGACTTGTTCC
CBF3_T     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
CBF3_B     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCACCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
CBF3_E     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
CBF3_V     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCACCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
CBF3_W     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCACCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
HvCBF3     GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCGCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAAATGTTCAGGCTTGACTTGTTCC
Consensus  GACAGTGCCGGTTCGACGGGGACGTCGGAACCTTCCgCCAATGGAGTGTTCGAGGGGCCCGTTGTAATGGACAGTGAaATGTTCAGGCTTGACTTGTTCC

           701     710       720       730
           |--------+---------+---------|
CBF3_A     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_H     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_P     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_I     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_T     CGGAAATGGACATGGGCTCGTACTACATGA
CBF3_B     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_E     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_V     CGGAAATGGACCTGGGCTCGTACTACATGA
CBF3_W     CGGAAATGGACCTGGGCTCGTACTACATGA
HvCBF3     CGGAAATGGACCTGGGCTCGTACTACATGA
Consensus  CGGAAATGGACCTGGGCTCGTACTACATGA
```

Fig. 4.

B

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         ***.*******************:**************** ***************************************
CBF3_A   MDMNLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFCETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_B   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFRETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_E   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFRETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_H   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFCETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_I   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFCETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_P   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFCETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_T   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFCETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_V   MDMGLEVSSSSPSSSSVSSSPEHTARRASPAKRPAGRTKFRETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
CBF3_W   MDMGLEVSSSSPSSSSVSSSPEHTARRASPAKRPAGRTKFRETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
HvCBF3   MDMGLEVSSSSPSSSSVSSSPEHAARRASPAKRPAGRTKFRETRHPVYRGVRRRGNTERWVCEVRVPGKRGARLWLGTYA   80
         1.......10........20........30........40........50........60........70........80
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Conservation

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         ********.********* *****************************************************:*******
CBF3_A   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_B   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_E   TAEVAARANDAAMLALGGCSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_H   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_I   TAEVAARASDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_P   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_T   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKDASSGAPS   160
CBF3_V   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
CBF3_W   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
HvCBF3   TAEVAARANDAAMLALGGRSAACLNFADSAWLLAVPSALSDLADVRRAAVEAVADFQRREAADGSLAIAVAKEASSGAPS   160
         .........90.......100.......110.......120.......130.......140.......150.......160
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Conservation

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         *******************:************:***********:*****
CBF3_A   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
CBF3_B   LSPSSGSDSAGSTGTSEPSTNGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
CBF3_E   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
CBF3_H   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSDMFRLDLFPEMDLGSYYM   210
CBF3_I   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSDMFRLDLFPEMDLGSYYM   210
CBF3_P   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSDMFRLDLFPEMDLGSYYM   210
CBF3_T   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSEMFRLDLFPEMDMGSYYM   210
CBF3_V   LSPSSGSDSAGSTGTSEPSTNGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
CBF3_W   LSPSSGSDSAGSTGTSEPSTNGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
HvCBF3   LSPSSGSDSAGSTGTSEPSANGVFEGPVVMDSEMFRLDLFPEMDLGSYYM   210
         .......170.......180.......190.......200.......210
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Conservation

C

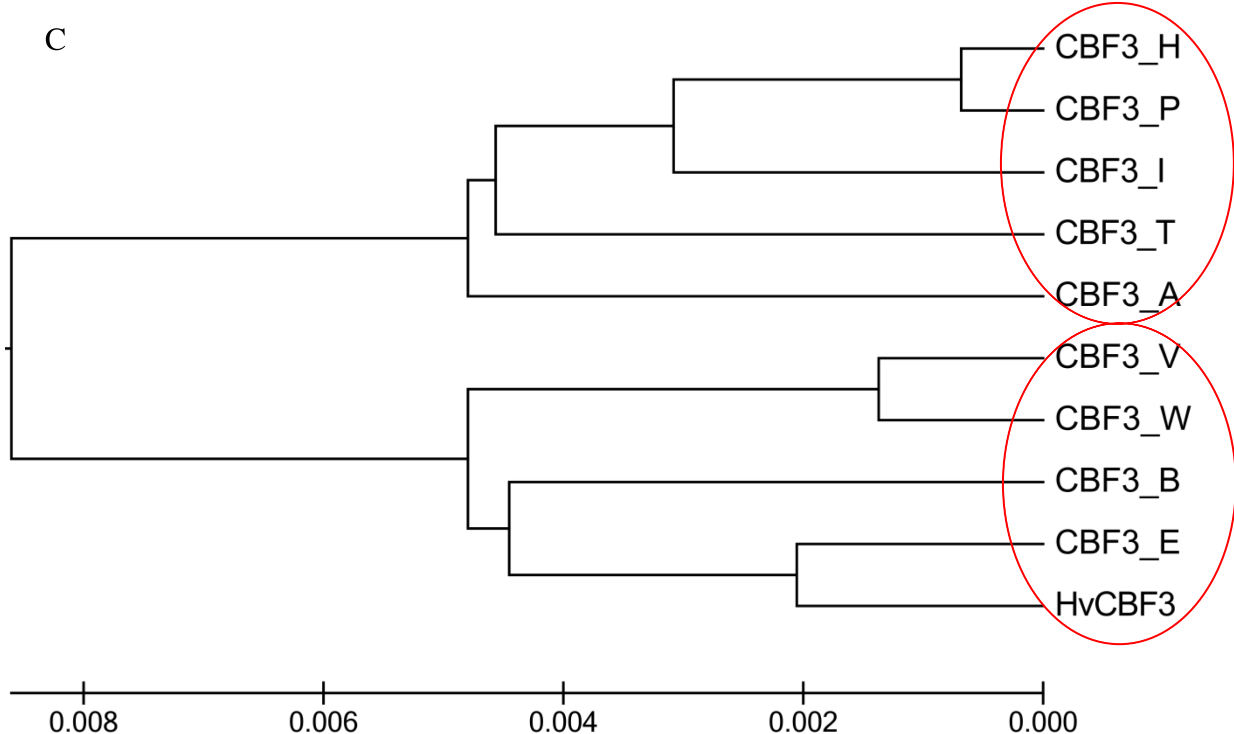

Fig. 4. continued

Fig. 4. DNA sequence analysis of barley *CBF3* genes. (A) Comparison of *CBF3* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF3* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among nine isolated barley *CBF3* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF3*: reference gene from NCBI.

variety *Trumpf* while another nucleotide transversion was identified at position 365 in the variety *Isaria*. The cultivars *Barke* and *Victoriana* shared two T/A nucleotide variants at position 567 and 576, respectively.

To estimate genetic diversity between the identified *CBF3* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *CBF3* genes in nine cultivars are shown in Table 5. To reveal the genetic distance among these *CBF3* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 4C, the isolated *CBF3* genes from nine cultivars are clustered into two groups. The first group contained *CBF3* genes of the cultivars *Heils Franken*, *Pflugs Intensiv*, *Isaria*, *Trumpf* and *Arta*. This group could be subdivided into three subgroups. The barley *CBF3* genes of first three cultivars composed the subgroup I. The subgroup II and subgroup III consisted of *CBF3* genes of the cultivars *Arta* and *Trumpf*, respectively. Similar to the group I, the group II was also subdivided into three groups. The subgroup I consisted of the *CBF3* genes of the cultivars *Victoriana* and *Wiebke* while the *CBF3* gene of the cultivar *Eunova* and the reference gene from the NCBI GeneBank composed the subgroup III. The subgroup II contained the *CBF3* gene of the cultivar *Barke*. As the barley core set was determined for the CROP.SENSe.net project (http://www.cropsense.uni-bonn.de/), eight old and eight modern barley varieties were chosen. The cultivars *Barke*, *Eunova*, *Victoriana* and *Wiebke* belong to the modern varieties while the cultivars *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Wiebke* belong to the old varieties. As shown in Fig. 4C, the phylogenetic distance among the isolated *CBF3* genes revealed that there is a genetic demarcation between the modern and old cultivars in the barley core set.

Table 5. Estimates of genetic divergence between *CBF3* sequences of barley cultivars

Percentage of genetic divergence

	1	2	3	4	5	6	7	8	9	10
1. CBF3_A										
2. CBF3_B	2.1									
3. CBF3_E	1.6	1.0								
4. CBF3_H	0.7	1.6	1.2							
5. CBF3_I	1.2	2.2	1.8	0.5						
6. CBF3_P	0.8	1.8	1.4	0.1	0.7					
7. CBF3_T	1.1	2.1	1.6	0.7	1.2	0.8				
8. CBF3_V	2.1	1.1	1.0	1.6	2.2	1.8	1.8			
9. CBF3_W	2.1	1.1	1.0	1.6	2.2	1.8	1.8	0.3		
10. HvCBF3	1.2	0.8	0.4	1.1	1.6	1.2	1.5	0.8	0.8	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *CBF3* nucleotide sequences and the reference *HvCBF3* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 730 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF3*: reference gene from NCBI.

3.2.4 Isolation of *CBF4* genes from the barley core set

The *CBF4* homologues were isolated using the gene-specific primers to amplify the corresponding genes with the genomic DNA from nine different barley genotypes. The amplicons were 659 bp in length with a 42 bp long 5 ´UTR and an ORF of 205 amino acid residues. The sequences obtained in nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *CBF4* genes with their cDNAs indicated that no intron sequences were present in the obtained *CBF4* homologues (Fig. 5A). A total of eight SNPs was identified in the nine isolated *CBF4* homologues. Three of them did not result in any changes in the corresponding amino acid residues while the rest led to non-synonymous mutations (Fig. 5B). Synonymous mutations were caused by a G/C nucleotide transversion and two C/T nucleotide transitions. A G/C nucleotide variant was identified at position 138 in the genotypes *Victoriana* and *Wiebke*. Two C/T transitions were identified at position 345 in the varieties *Victoriana* and *Wiebke* as well as at position 288 in the cultivar *Eunova*, respectively. In case of non-synonymous mutations, three G/C nucleotide transversions, a G/T nucleotide transversion and a common C/T nucleotide transition were identified. These three nucleotide transversions were observed at positions 567, 590 and 615 in the cultivar *Barke*. The G/C variant at positions 567 and 615 led to the same non-synonymous mutation from glutamine to histidine, while the G/C variant at position 590 resulted in non-synonymous mutation from glycine to alanine. In addition, the G/C variant at position 590 was also observed in the variety *Victoriana*. A common G/T

nucleotide transversion was identified at position 517 in the varieties *Arta*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Trumpf* leading to changes in amino acid codon from alanine to serine. A common C/T nucleotide transition was present in the variety *Barke* and *Victoriana* at position 634 leading to a non-synonymous mutation from proline to serine.

To estimate genetic diversity between the identified *CBF4* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *CBF4* genes in nine cultivars are shown in Table 6. To reveal the genetic distance among these *CBF4* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 5C, the isolated *CBF4* genes in nine cultivars were clustered into three groups. The first group contained *CBF4* genes of the cultivars *Pflugs Intensiv*, *Trumpf*, *Isaria*, *Heils Franken*, *Arta* and *Eunova*. This group could be subdivided into two subgroups. The barley *CBF4* genes of the first five cultivars composed the subgroup I, while the *CBF4* gene of the cultivar *Eunova* belonged to the subgroup II. The *CBF4* genes of the cultivars *Victoriana* and *Wiebke* composed the second group. The third group consisted of only one member, namely the *CBF4* gene of the cultivar *Barke*. The distribution of the isolated barely *CBF4* genes is quite similar to that shown in the isolated barley *CBF3* genes. However, the phylogenetic distance among the isolated *CBF4* genes is much greater than that of the isolated *CBF3* genes. The *CBF4* gene of the cultivar *Eunova* could be considered as a transition between the old and new cultivars in the barley core set.

Table 6. Estimates of genetic divergence between *CBF4* sequences of barley cultivars

Percentage of genetic divergence

	1	2	3	4	5	6	7	8	9	10
1. CBF4_A										
2. CBF4_B	0.8									
3. CBF4_E	0.3	0.8								
4. CBF4_H	0.0	0.8	0.3							
5. CBF4_I	0.0	0.8	0.3	0.0						
6. CBF4_P	0.0	0.8	0.3	0.0	0.0					
7. CBF4_T	0.0	0.8	0.3	0.0	0.0	0.0				
8. CBF4_V	0.8	0.6	0.8	0.8	0.8	0.8	0.8			
9. CBF4_W	0.5	0.9	0.5	0.5	0.5	0.5	0.5	0.3		
10. HvCBF4	0.2	0.6	0.2	0.2	0.2	0.2	0.2	0.6	0.3	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *CBF4* nucleotide sequences and the reference *HvCBF4* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 659 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF4*: reference gene from NCBI.

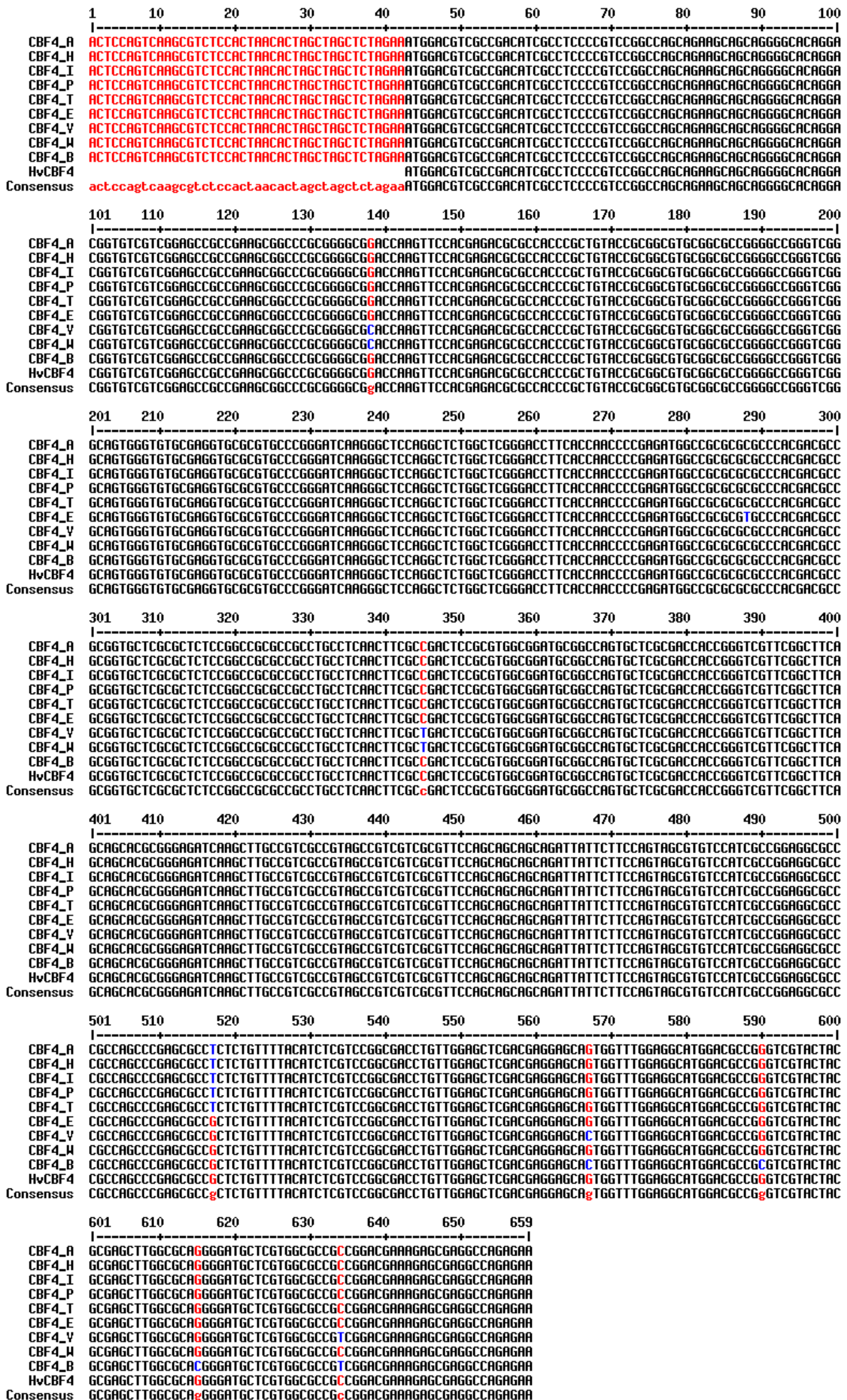

Fig. 5.

B

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           ********************************************************************************
CBF4_A     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_B     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_E     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_H     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_I     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_P     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_T     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_V     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
CBF4_W     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
HvCBF4     MDVADIASPSGQQKQQGHRTVSSEPPKRPAGRTKFHETRHPLYRGVRRRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMA   80
           1.......10........20........30........40........50........60........70........80
Conservation

           ******************************************************************************:*
CBF4_A     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSASL   160
CBF4_B     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSAAL   160
CBF4_E     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSAAL   160
CBF4_H     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSASL   160
CBF4_I     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSASL   160
CBF4_P     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSASL   160
CBF4_T     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSASL   160
CBF4_V     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSAAL   160
CBF4_W     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSAAL   160
HvCBF4     ARAHDAAVLALSGRAACLNFADSAWRMRPVLATTGSFGFSSTREIKLAVAVAVVAFQQQQIILPVACPSPEAPASPSAAL   160
           ........90.......100.......110.......120.......130.......140.......150.......160
Conservation

           **************:*******.*******:******.*******
CBF4_A     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_B     FYISSGDLLELDEEHWFGGMDAASYYASLAHGMLVAPSDERARPE   205
CBF4_E     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_H     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_I     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_P     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_T     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
CBF4_V     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPSDERARPE   205
CBF4_W     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
HvCBF4     FYISSGDLLELDEEQWFGGMDAGSYYASLAQGMLVAPPDERARPE   205
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Conservation
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C

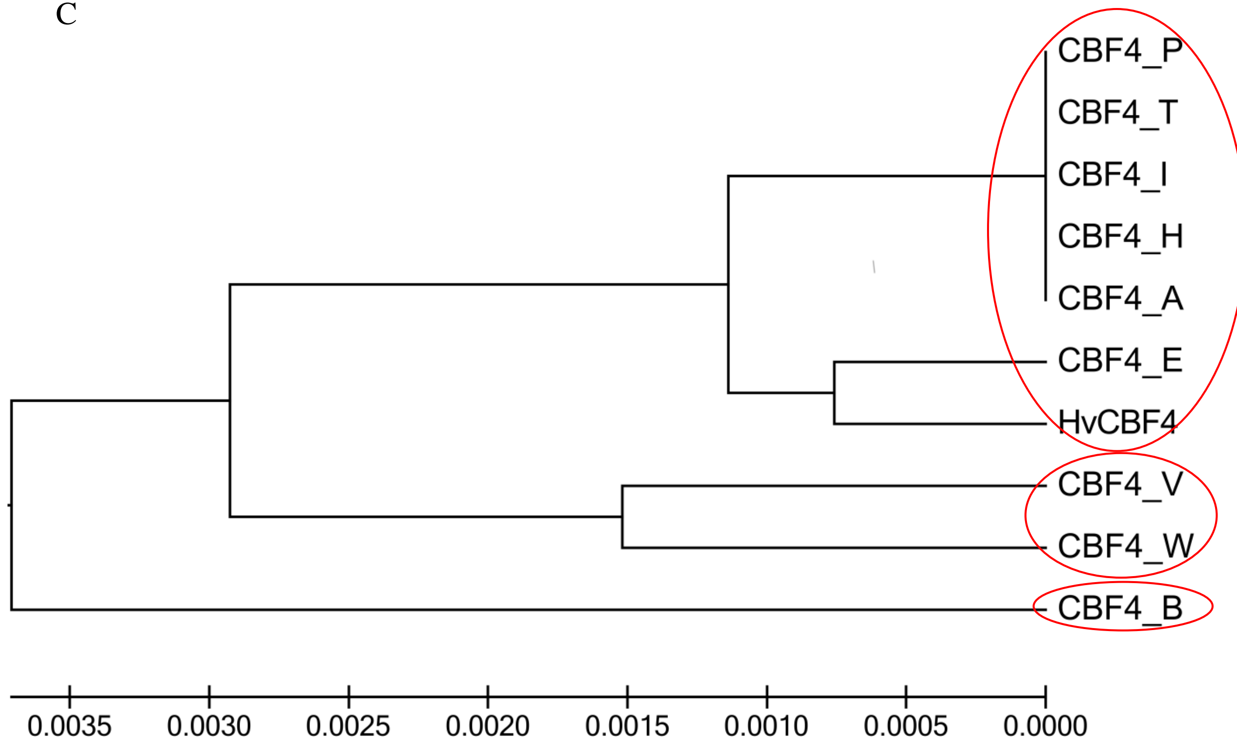

Fig. 5. continued

Fig. 5. DNA sequence analysis of barley *CBF4* genes. (A) Comparison of *CBF4* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF4* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among nine isolated barley *CBF4* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF4*: reference gene from NCBI.

3.2.5 Isolation of *CBF6* genes from the barley core set

The *CBF6* homologues were isolated using gene-specific primers. The corresponding genes were amplified with the genomic DNA from nine different barley genotypes. The amplicons were 632 bp in length with an ORF of 210 amino acid residues. The sequences obtained from nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *CBF6* genes with their cDNAs indicated that no intron sequences were present in the obtained *CBF6* homologues (Fig. 6A). A total of 16 SNPs was identified in the nine isolated barley *CBF6* homologues. Ten of them led to synonymous mutations. A T/C nucleotide transition was present only in the reference *CBF6* gene from the NCBI GeneBank. Three nucleotide variants were identified in the cultivars *Arta*, *Heils Franken*, *Isaria* and *Pflugs Intensiv* at positions 501, 543 and 606. A common nucleotide transversion from G to C was observed at position 183 in the cultivars *Barke*, *Trumpf*, *Victoriana* and *Wiebke*. Three C/T nucleotide variants were identified at positions 172, 213 and 411 in the cultivars *Heils Franken*, *Victoriana* and *Trumpf*, respectively. A C/G and a T/G nucleotide variant were identified at positions 366 and 615 in the cultivar *Arta*. In case of non-synonymous mutations, six nucleotide transversions were identified. Two common A/C nucleotide transversions were identified at positions 529 and 563 in the cultivars *Arta*, *Heils Franken*, *Isaria* and *Pflugs Intensiv* leading to non-synonymous mutations from isoleucine to leucine and asparitic acid to cysteine, respectively. Another common A/C transversion was observed at position 56 in the cultivars *Barke*, *Wiebke*, *Victoriana* and *Trumpf* leading to a non-synonymous mutation from glutamic acid to alanine. In the cultivars *Arta*, *Heils Franken*, *Isaria* and *Pflugs Intensiv*, the presence of an additional nucleotide transversion from G to T at position 502 caused a non-synonymous mutation from

glycine to cysteine. The rest two non-synonymous mutations were caused by single nucleotide transversion at positions 143 and 245 in the cultivars *Pflugs Intensiv* and *Victoriana*, respectively. The previous one led to non-synonymous mutation from proline to leucine, while the last one resulted in changes in amino acid residues from glycine to serine (Fig. 6B).

To estimate genetic diversity between the identified *CBF6* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *CBF6* genes from nine cultivars are shown in Table 7. To reveal the genetic distance among these *CBF6* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 6C, the isolated *CBF6* genes from nine cultivars were clustered into two groups. The group I contained *CBF6* genes of the cultivars *Barke*, *Wiebke*, *Trumpf*, *Victoriana* and *Eunova*. This group could be subdivided into four subgroups. CBF6 genes of the first two cultivars composed the subgroup I. From subgroup II to subgroup IV, each one contained only one member. The *CBF6* genes of the cultivars *Trumpf* and *Victoriana* composed the subgroup II and III, respectively. The *CBF6* gene of the cultivars *Eunova* and the reference *CBF6* gene from the NCBI GeneBank belonged to the subgroup IV. Moreover, the *CBF6* genes of the cultivars *Arta*, *Heils Franken*, *Isaria* and *Pflugs Intensiv* composed the group II. Similar to the group I, the group II could also be subdivided into three subgroups. The *CBF6* genes of the cultivars *Arta* and *Heils Franken* belonged to the subgroup I and subgroup II, respectively. The last two isolated *CBF6* genes composed the subgroup III. As shown in the isolated *CBF3* and *CBF4* genes (Fig. 4C, 5C), a demarcation between the old and modern cultivars could also be observed among the isolated *CBF6* genes. The *CBF6* gene of the cultivar *Eunova* played a role of transition between the old and modern barley cultivars in the core set. Instead, the *CBF6* gene of the cultivar *Eunova* demonstrated closer phylogenetic distance to the new barley cultivars.

Table 7. Estimates of genetic divergence between *CBF6* sequences of barley cultivars

Percentage of genetic divergence

	1	2	3	4	5	6	7	8	9	10
1. CBF6_A										
2. CBF6_B	1.6									
3. CBF6_E	1.3	0.3								
4. CBF6_H	0.5	1.4	1.1							
5. CBF6_I	0.3	1.3	0.9	0.2						
6. CBF6_P	0.5	1.4	1.1	0.3	0.2					
7. CBF6_T	1.7	0.2	0.5	1.6	1.4	1.6				
8. CBF6_V	1.9	0.3	0.6	1.7	1.6	1.7	0.5			
9. CBF6_W	1.6	0.0	0.3	1.4	1.3	1.4	0.2	0.3		
10. HvCBF6	1.4	0.5	0.2	1.3	1.1	1.3	0.6	0.8	0.5	

Table 7. The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *CBF6* nucleotide sequences and the reference *HvCBF6* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 632 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF6*: reference gene from NCBI.

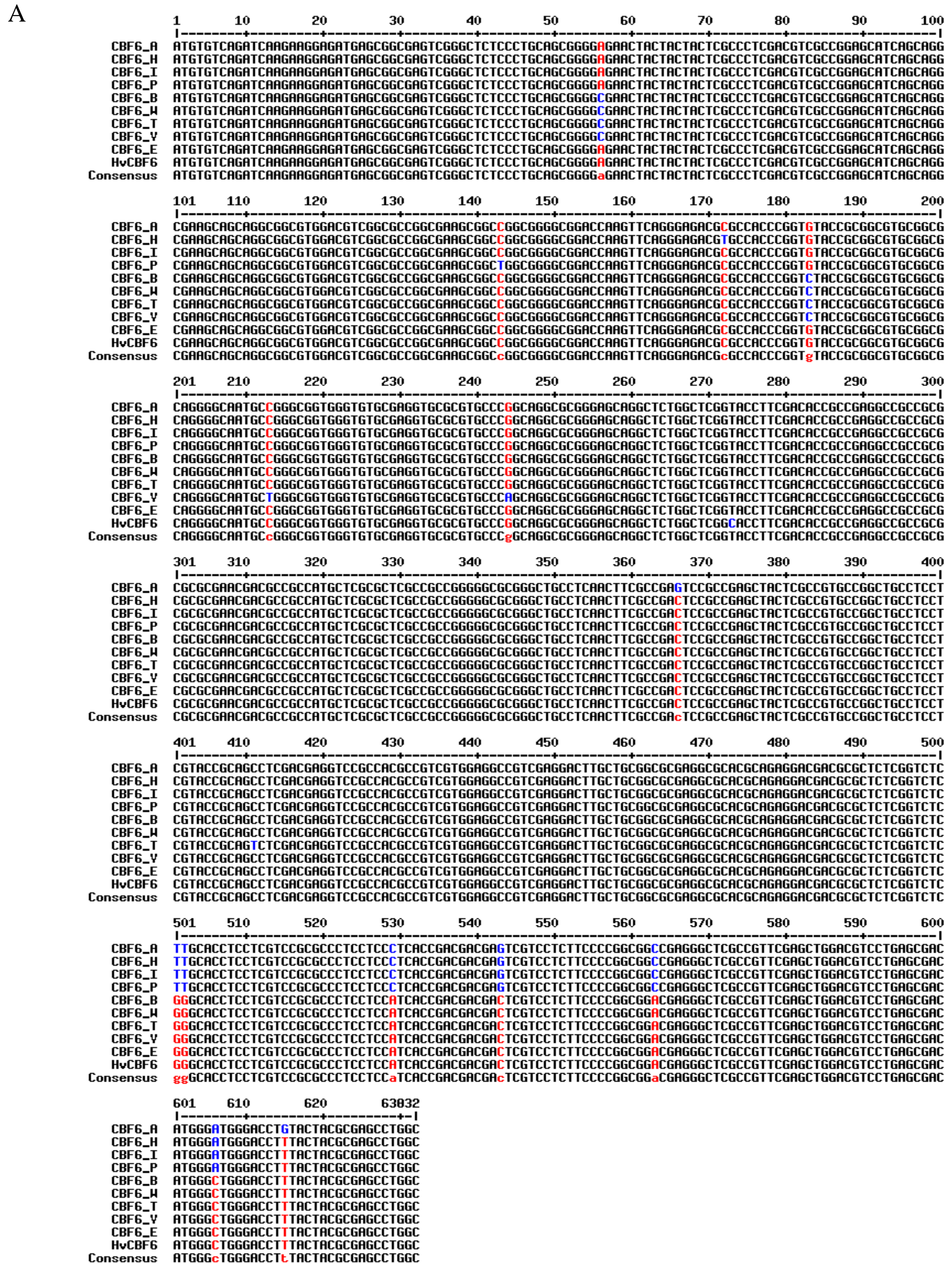

Fig. 6.

B

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                 ****************** ****************************** *********************************
CBF6_A    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_B    MCQIKKEMSGESGSPCSGANYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_E    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_H    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_I    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_P    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRLAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_T    MCQIKKEMSGESGSPCSGANYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_V    MCQIKKEMSGESGSPCSGANYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
CBF6_W    MCQIKKEMSGESGSPCSGANYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
HvCBF6    MCQIKKEMSGESGSPCSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETRHPVYRGVRRRGNAGRWVCEVRV   80
          1.......10........20........30........40........50........60........70........80
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Conservation

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          *.***************************************:*************************************
CBF6_A    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFAESAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_B    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_E    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_H    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_I    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_P    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_T    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_V    PSRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
CBF6_W    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
HvCBF6    PGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCLNFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAE   160
          ........90.......100.......110.......120.......130.......140.......150.......160
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Conservation

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          ******* ********:***:****** **********************
CBF6_A    DDALSVSCTSSSAPSSLTDDESSSSPAAEGSPFELDVLSDMGWDLYYASL   210
CBF6_B    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
CBF6_E    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
CBF6_H    DDALSVSCTSSSAPSSLTDDESSSSPAAEGSPFELDVLSDMGWDLYYASL   210
CBF6_I    DDALSVSCTSSSAPSSLTDDESSSSPAAEGSPFELDVLSDMGWDLYYASL   210
CBF6_P    DDALSVSCTSSSAPSSLTDDESSSSPAAEGSPFELDVLSDMGWDLYYASL   210
CBF6_T    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
CBF6_V    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
CBF6_W    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
HvCBF6    DDALSVSGTSSSAPSSITDDDSSSSPADEGSPFELDVLSDMGWDLYYASL   210
          .......170.......180.......190.......200.......210
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Conservation

C

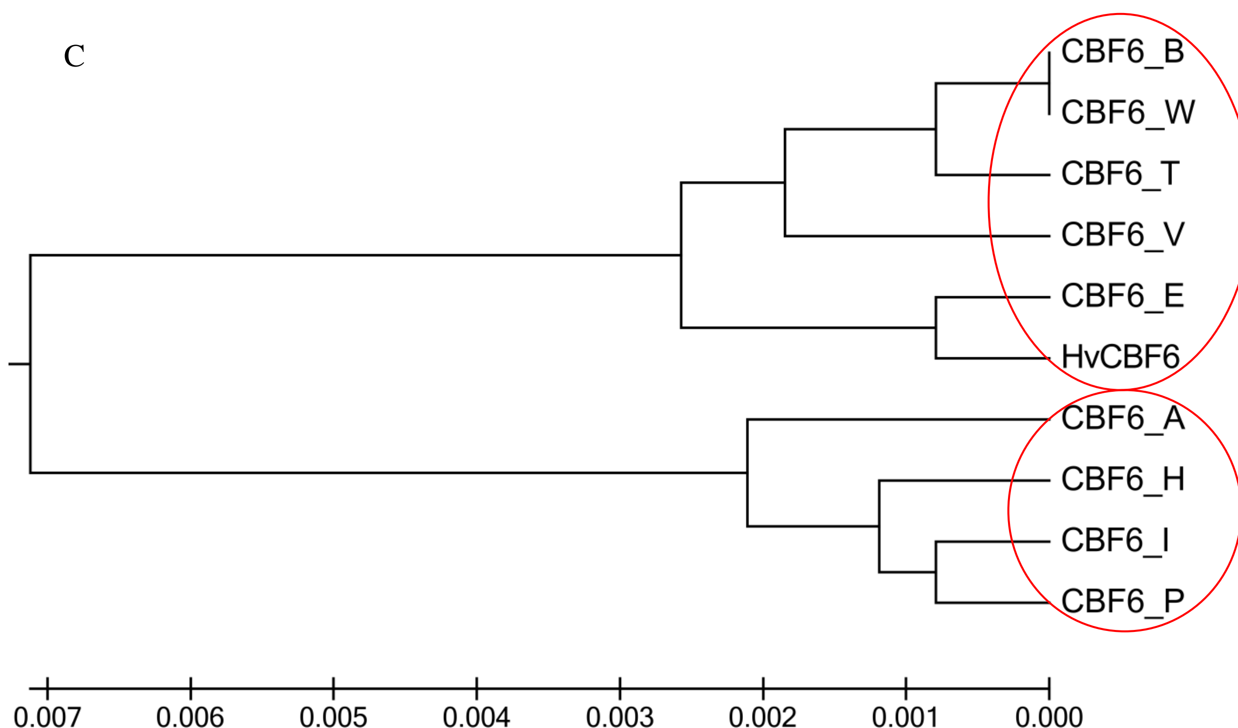

Fig. 6. continued

Fig. 6. DNA sequence analysis of barley *CBF6* genes. (A) Comparison of *CBF6* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF6* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among nine isolated barley *CBF6* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF6*: reference gene from NCBI.

3.2.6 Isolation of *CBF11* genes from the barley core set

The *CBF11* homologues were isolated using gene-specific primers to amplify the corresponding gene with the genomic DNA from nine different barley genotypes. The amplicons were 641 bp in length with an ORF of 213 amino acid residues. The sequences obtained from nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *CBF11* genes with their cDNAs indicated that no intron sequences were present in the obtained *CBF11* homologues (Fig. 7A). A total of eight SNPs was identified in the nine isolated barley *CBF11* homologues. Four nucleotide variants led to synonymous mutations. Two G/A transitions were identified at positions 105 and 351 in the cultivars *Trumpf* and *Barke*, respectively. An additional nucleotide transition from C to T was present at position 207 in the cultivar *Barke*. The presence of a T/G nucleotide variant at position 555 in the cultivar *Isaria* led also to a synonymous mutation. In case of non-synonymous mutations, four additional nucleotide variants were identified, including three T/C nucleotide transitions and one A/T nucleotide transversion. The presence of a T/C nucleotide variant at position 194 in the cultivar *Isaria* led to a non-synonymous mutation from valine to alanine, while another T/C nucleotide variant at position 317 in the cultivar *Barke* resulted in a change in the amino acid residue from leucine to proline. The third T/C nucleotide transition was identified at position 514 in the variety *Victoriana* leading to a non-synonymous mutation from tyrosine to histidine. A heterogeneous nucleotide transition from A to G was identified at position 559 in the cultivar *Heils Franken* leading to a non-synonymous mutation from methionine to valine (Fig. 7B).

To estimate genetic diversity between the identified *CBF11* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was

A

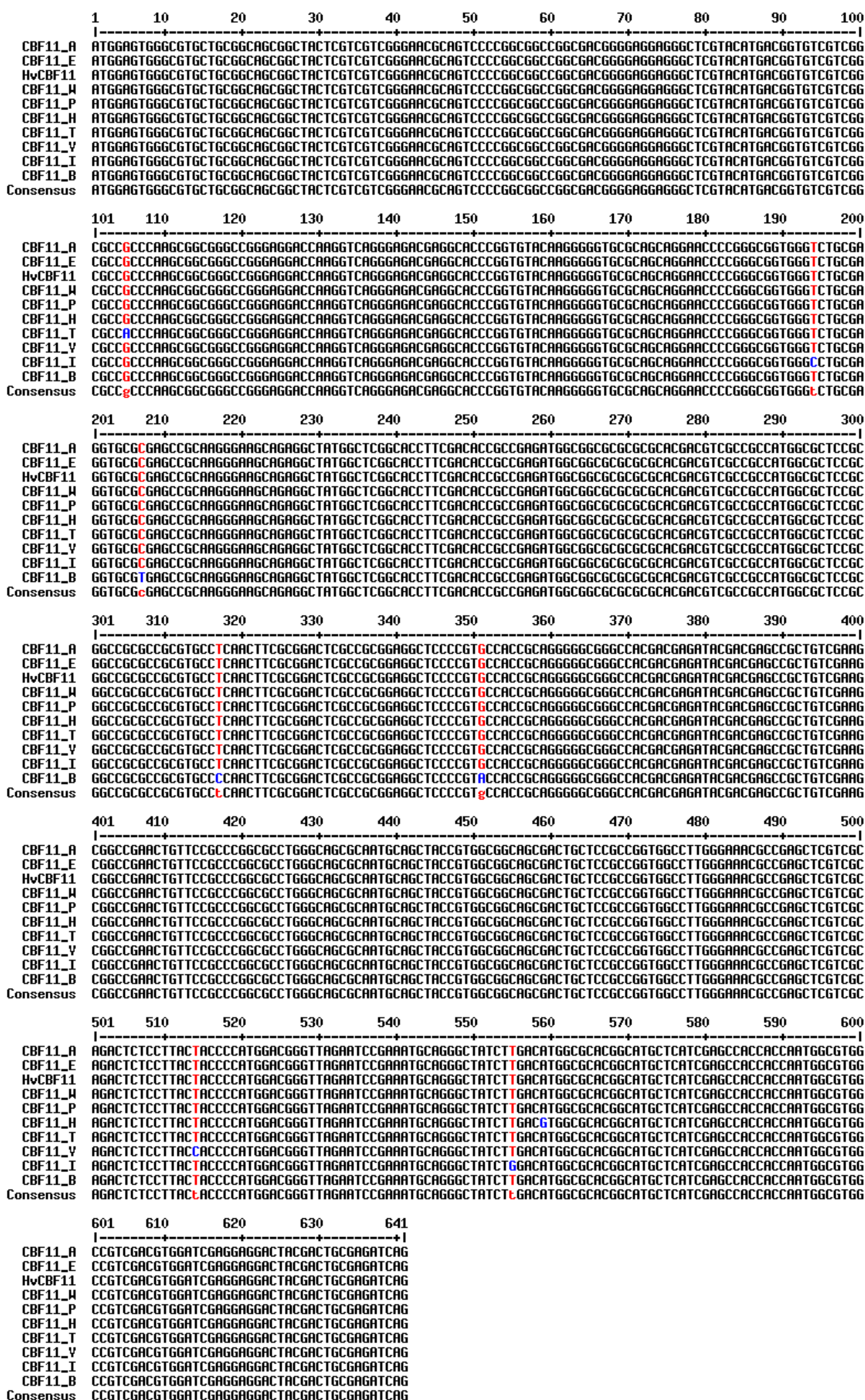

Fig. 7.

B

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           ****************************************************************.***************
CBF11_A    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRFPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_B    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_E    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_H    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_I    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWACEVREPQGKQRLWLG   80
CBF11_P    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_T    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_V    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
CBF11_W    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
HvCBF11    MEWACCGSGYSSSGTQSPAAGDGEEGSYMTVSSAPPKRRAGRTKVRETRHPVYKGVRSRNPGRWVCEVREPQGKQRLWLG   80
           1.......10........20........30........40........50........60........70........80
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Conservation

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           ************************* ******************************************************
CBF11_A    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_B    TFDTAEMAARAHDVAAMALRGRAACPNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_E    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_H    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_I    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_P    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_T    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_V    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
CBF11_W    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
HvCBF11    TFDTAEMAARAHDVAAMALRGRAACLNFADSPRRLPVPPQGAGHDEIRRAAVEAAELFRPAPGQRNAATVAAATAPPVAL  160
           ........90.......100.......110.......120.......130.......140.......150.......160
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Conservation

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           ***********:**************:**************************
CBF11_A    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_B    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_E    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_H    GNAELVADSPYYPMDGLESEMQGYLDVAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_I    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_P    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_T    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_V    GNAELVADSPYHPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
CBF11_W    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
HvCBF11    GNAELVADSPYYPMDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEI  213
           .......170.......180.......190.......200.......210...
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Conservation

C

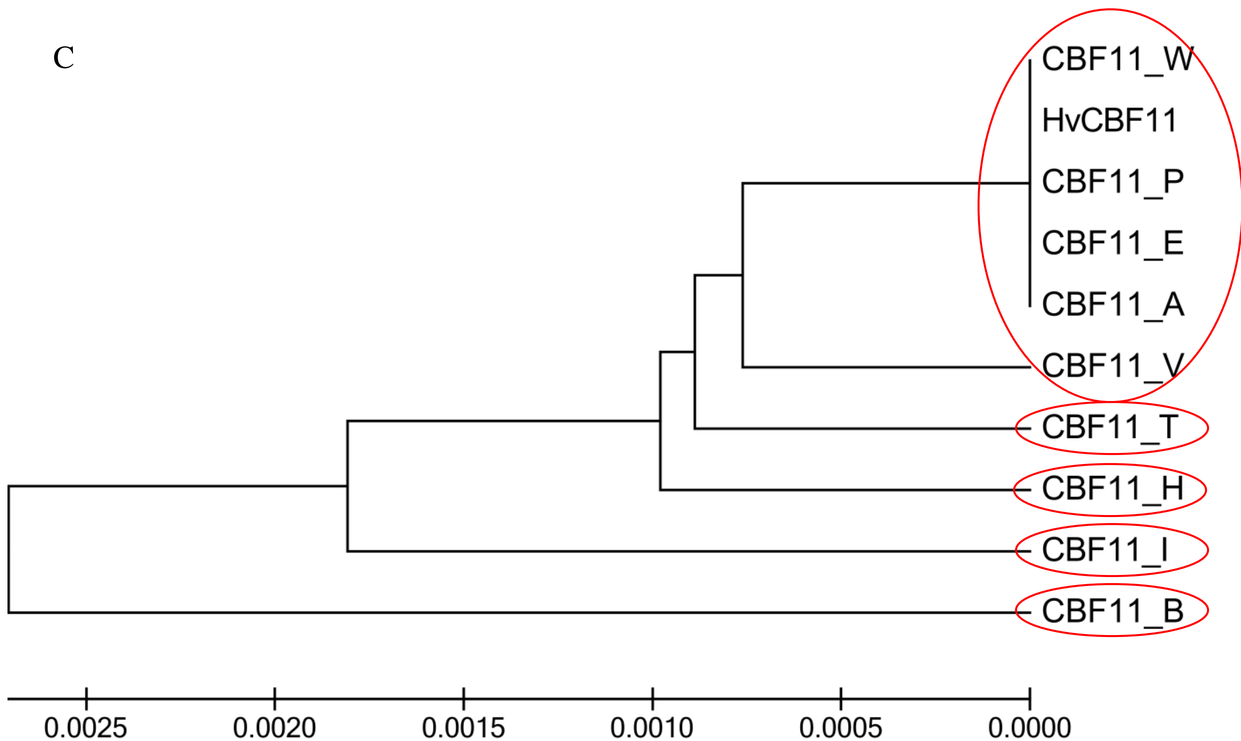


Fig. 7. continued

Fig. 7. DNA sequence analysis of barley *CBF11* genes. (A) Comparison of *CBF11* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *CBF11* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain bracketed with signature motifs (labelled with red square frames) is labelled with a black square frame. (C) Genetic distance among nine isolated barley *CBF11* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF11*: reference gene from NCBI.

computed. The genetic diversity and differentiation among the isolated *CBF11* genes in nine cultivars are shown in Table 8. To reveal the genetic distance among these *CBF11* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 7C, the isolated barley *CBF11* genes in nine cultivars were clustered into five groups. The first group contained *CBF11* genes of the cultivars *Wiebke*, *Pflugs Intensiv*, *Isaria*, *Arta* and *Victoriana*. This group could be subdivided into two subgroups. The *CBF11* genes of the first four cultivars composed the subgroup I. The *CBF11* genes of the cultivar *Victoriana* belonged to the subgroup II. From the second to the fifth group, each one contained only one member. It should be noted that the classification of the isolated barley *CBF11* genes is quite similar to the isolated barley *CBF1* genes (Fig. 2C).

Table 8. Estimates of genetic divergence between *CBF11* sequences of barley cultivars

Percentage of genetic divergence	1	2	3	4	5	6	7	8	9	10
1. CBF11_A										
2. CBF11_B	0.5									
3. CBF11_E	0.0	0.5								
4. CBF11_H	0.2	0.6	0.2							
5. CBF11_I	0.3	0.8	0.3	0.5						
6. CBF11_P	0.0	0.5	0.0	0.2	0.3					
7. CBF11_T	0.2	0.6	0.2	0.3	0.5	0.2				
8. CBF11_V	0.2	0.6	0.2	0.3	0.5	0.2	0.3			
9. CBF11_W	0.0	0.5	0.0	0.2	0.3	0.0	0.2	0.2		
10. HvCBF11	0.0	0.5	0.0	0.2	0.3	0.0	0.2	0.2	0.0	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *CBF11* nucleotide sequences and the reference *HvCBF11* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 641 positions in the final dataset. Evolutionary analyses

were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvCBF11*: reference gene from NCBI.

3.2.7 Isolation of *DREB1* genes from the barley core set

The *DREB1* homologues were isolated using gene-specific primers to amplify the corresponding gene with the genomic DNA from nine different barley genotypes. The amplicons were 1541 bp in length with an ORF of 279 amino acid residues. The sequences obtained from nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *DREB1* genes with their cDNAs indicated that a 704 bp long intron with GT-AG borders was present in the obtained *DREB1* homologues (Fig. 8A). A total of 16 SNPs was identified in the nine isolated barley *DREB1* homologues. Five of them were present in the intron region. Two T/C nucleotide variants were identified at positions 622 and 700 in the cultivars *Victoriana* and *Eunova*, respectively. In the cultivar *Eunova*, an additional C/T nucleotide variant was present at position 77. A C/A and an A/C nucleotide variant were observed at positions 95 and 591 in the cultivars *Pflugs Intensiv* and *Victoriana*, respectively. A total of 11 SNPs were identified in the two exons of the nine isolated barley *DREB1* homologues. Seven of them led to synonymous mutations. Three G/A nucleotide transitions were present at positions 1241, 1364 and 1431 in the cultivars *Arta*, *Trumpf* and *Wiebke*, respectively. The G/A variant at position 1431 was also identified in the cultivar *Trumpf*. Two A/G nucleotide transitions were present at positions 999 and 1122 in the cultivars *Barke* and *Heils Franken*, while another two T/C nucleotide transitions were identified at position 941 and 1034 in the cultivars *Wiebke* and *Barke*, respectively. In case of non-synonymous mutations, two nucleotide transversions and two nucleotide transitions were identified. In the cultivar *Trumpf*, a nucleotide transversion from G to T and another nucleotide transversion from A to T at positions 51 and 1210 led to non-synonymous mutations from arginine to methionine and glutamic acid to valine, respectively. A T/C and an A/G nucleotide transitions at positions 1279 and 1366 in the cultivars *Arta* and Victorina led to further non-synonymous mutations from phenylalanine to serine and glutamic acid to alanine (Fig. 8B).

To estimate genetic diversity between the identified *DREB1* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *DREB1* genes in nine cultivars are shown in Table 9. To reveal the genetic distance among these *DREB1* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 8C, the isolated *DREB1* genes in nine cultivars were

A

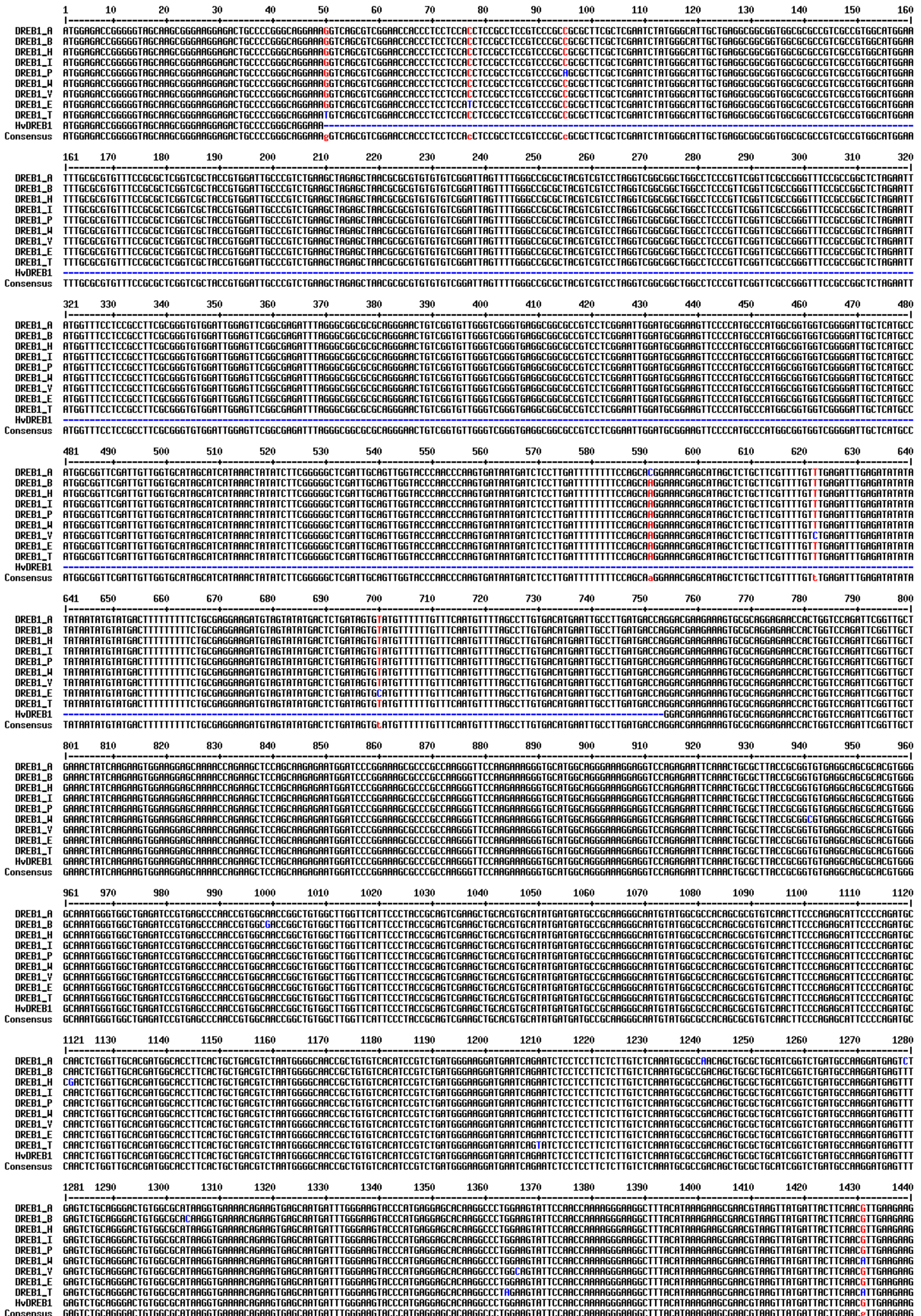
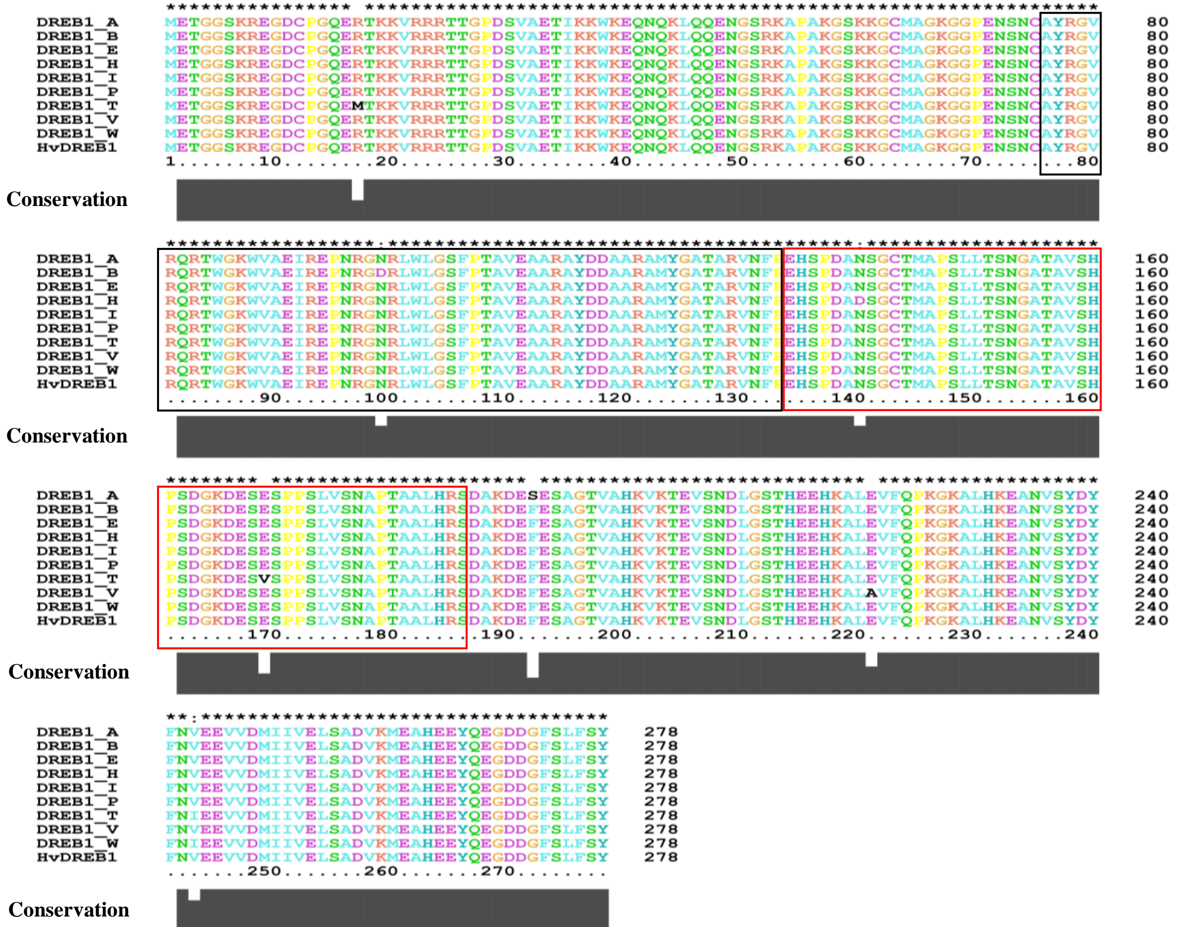


Fig. 8.

B



C

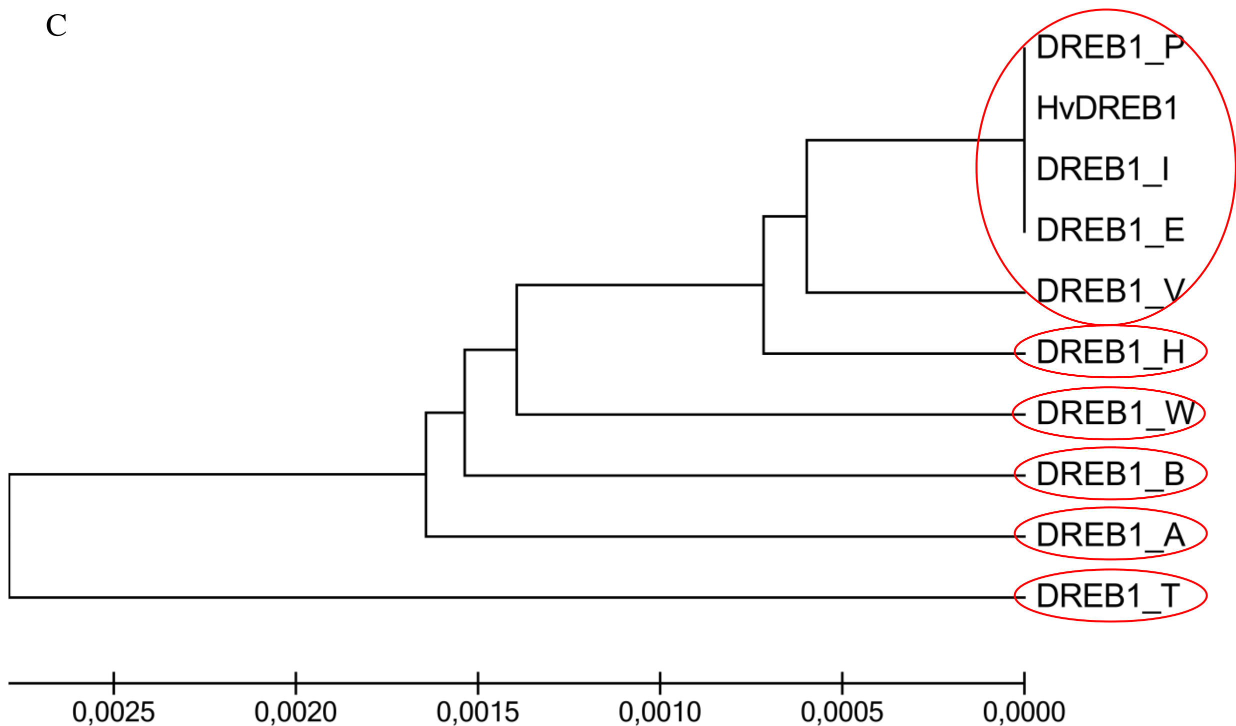


Fig. 8. continued

Fig. 8. DNA sequence analysis of barley *DREB1* genes. (A) Comparison of *DREB1* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *DREB1* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain is labelled with a black square frame. Ser- and Thr-rich 53-amino acid region adjacent to the AP2 domain is labelled with a red square frame. (C) Genetic distance among nine isolated barley *DREB1* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvDREB1*: reference gene from NCBI.

clustered into six groups. The group I contained *DREB1* genes of the cultivars *Pflugs Intensiv*, *Isaria*, *Eunova* and *Victoriana*. This group could be subdivided into two subgroups. *DREB1* genes of the first three cultivars composed the subgroup I. The subgroup II consisted of the *DREB1* genes of the cultivar *Victoriana*. From the second to the fifth group, each one contained only one member. As shown in the isolated *CBF1* genes (Fig 2C), a great genetic distance between *Isaria* and *Trumpf* could also be observed in the isolated *DREB1* genes.

Table 9. Estimates of genetic divergence between *DREB1* sequences of barley cultivars

Percentage of genetic divergence

	1	2	3	4	5	6	7	8	9	10
1. DREB1_A										
2. DREB1_B	0.5									
3. DREB1_E	0.2	0.2								
4. DREB1_H	0.4	0.4	0.1							
5. DREB1_I	0.2	0.2	0.0	0.1						
6. DREB1_P	0.2	0.2	0.0	0.1	0.0					
7. DREB1_T	0.7	0.7	0.5	0.6	0.5	0.5				
8. DREB1_V	0.4	0.4	0.1	0.2	0.1	0.1	0.6			
9. DREB1_W	0.5	0.5	0.2	0.4	0.2	0.2	0.5	0.4		
10. HvDREB1	0.2	0.2	0.0	0.1	0.0	0.0	0.5	0.1	0.2	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *DREB1* nucleotide sequences and the reference *HvDREB1* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 1541 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvDREB1*: reference gene from NCBI.

3.2.8 Isolation of *DRF1* genes from the barley core set

The *DRF1* homologues were isolated using gene-specific primers to amplify the corresponding gene with the genomic DNA from nine different barely genotypes. The

amplicons were 3077 bp in length. The sequences obtained from nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *DRF1* genes with their cDNAs indicated that three introns with GT-AG borders were present in the obtained *DRF1* homologues. The introns were in a range of 1078, 109 and 633 bp, respectively (Fig. 9A). A total of 12 SNPs was identified in the nine isolated barley *DRF1* homologues. Four of them were identified in the intron regions. Two T/C nucleotide transitions were identified at positions 263 and 905 in the cultivars *Barke* and *Isaria*, respectively. In the cultivar *Isaria*, two additional nucleotide transitions from A to G were present at positions 1165 and 1766. Another eight SNPs were observed in the exon region. Xue and Loveridge (2004) revealed that three forms of *HvDRF1* transcripts were produced through alternative splicing. *HvDRF1.1* and *HvDRF1.3* were two isoforms encoding AP2 proteins, while *HvDRF1.2* as the majority of *HvDRF1* mRNA was misspliced. *HvDRF1.1* gene possessed all four exons, while only exon 1 and 4 were presented in the *HvDRF1.3* gene. Among the eight SNPs in the exon regions, a T/C nucleotide transition was only observed at position 1175 in the exon 2 of the isolated *HvDRF1* homologues from the cultivar *Victoriana* leading to a non-synonymous mutation from cysteine to arginine in the isoform *HvDRF1.1* (Fig. 9B). *HvDRF1.1* and *HvDRF1.3* isoforms shared the rest six exonal SNPs. Only one of them led to synonymous mutation. It was caused by an A/G nucleotide transversion at postion 2301 in the cultivar *Arta*. In case of non-synonymous mutations, one nucleotide transversion and five nucleotide transitions were observed. A common C/G transversion at position 2874 in the cultivars *Arta*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Trumpf* led to a non-synonymous mutation from arginine to glycine. A T/C nucleotide transition was observed at position 2560 in the cultivar *Pflugs intensiv*e lead to other non-synonymous mutations from isoleucine to threonine. Three A/G nucleotide transitions were observed at positions 2236, 2768 and 2830 in the cultivar *Arta* leading to non-synonymous mutations from glutamine to arginine, isoleucine to methionine and alanine to asparatic acid, respectively. The A/G nucleotide transition at position 2768 was also present in the reference *HvDRF1* gene. In addition, an additional T/G variant at position 2554 occurred only in the reference *HvDRF1* gene leading to another non-synonymous mutation from leucine to arginine. It should be noted that a GGC nucleotide insertion was only present at positions 48, 49 and 50 in all nine isolated barley *DRF1* homologues (Fig. 9B, 9C).

To estimate genetic diversity between the identified *DRF1* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *DRF1* genes from nine

A

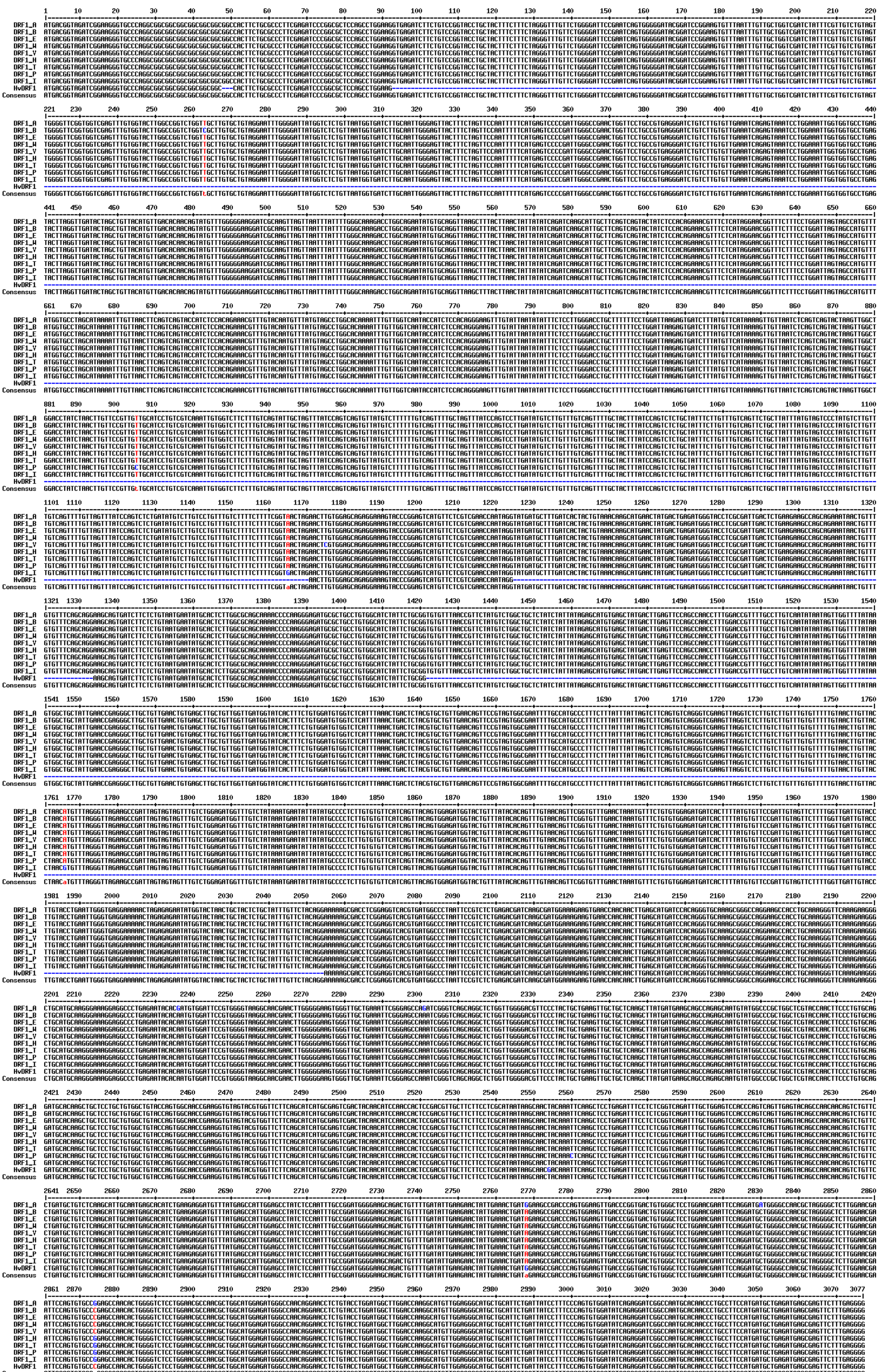


Fig. 9.

B

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           ********* ********************** ************************************************************
DRF1.1_E   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_V   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTRGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_W   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_B   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_P   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_T   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_H   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
DRF1.1_I   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
HvDRF1.1   MTVDRKGAQ-AAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   89
DRF1.1_A   MTVDRKGAQAAAAAAAATSAPFEIPALQPGRTCGAEESTRSHVLVEPIGSSDLLCNEYALLAQQNPKGDALPVASILRKKRPRRSRDGPN   90
           1.......10........20........30........40........50........60........70........80........90
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Conservation

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           ****************************************************:********************:********************
DRF1.1_E   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_V   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_W   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_B   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_P   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_T   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_H   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
DRF1.1_I   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   180
HvDRF1.1   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQA   179
DRF1.1_A   SVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKGCMQGKGGPENTRCGFRGVRQRTWGKWVAEIREPDRVSRLWLGTFPTAEVAAQA   180
           .......100.......110.......120.......130.......140.......150.......160.......170.......180
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Conservation

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           ***************************************************************** * ***********************
DRF1.1_E   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_V   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_W   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_B   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_P   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQTQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_T   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_H   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
DRF1.1_I   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
HvDRF1.1   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQRQIQAPEISSRSDLLESTQSVEYSQQ   269
DRF1.1_A   YDEAARAMYGPLARTNFPVQDAQAAPAVAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQ   270
           .......190.......200.......210.......220.......230.......240.......250.......260.......270
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Conservation

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           ********************************************:******************** ************** ********
DRF1.1_E   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWN   360
DRF1.1_V   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWN   360
DRF1.1_W   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWN   360
DRF1.1_B   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWN   360
DRF1.1_P   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWN   360
DRF1.1_T   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWN   360
DRF1.1_H   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWN   360
DRF1.1_I   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWN   360
HvDRF1.1   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLMEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWN   359
DRF1.1_A   QSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIEELLKLMEADPVEVDPVTVGSWNEFQDDGANARGSWNEFQCAGANTGVSWN   360
           .......280.......290.......300.......310.......320.......330.......340.......350.......360
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Conservation

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           ***********************************************************
DRF1.1_E   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_V   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_W   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_B   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_P   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_T   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_H   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
DRF1.1_I   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
HvDRF1.1   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   418
DRF1.1_A   ANAGMEMGQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG   419
           .......370.......380.......390.......400.......410.........
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Conservation

Fig. 9. continued

C

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         ********* ***********************************************************************
DRF1.3_P MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_T MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_I MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_H MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_E MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_V MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_B MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
HvDRF1.3 MTVDRKGAQ-AAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
DRF1.3_A MTVDRKGAQAAAAAAAATSAPFEIPALQPGRKKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKPPAKGSKKG  80
         1.......10........20........30........40........50........60........70........80
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Conservation

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         ***********:*********************:**********************************************
DRF1.3_P CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_T CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_I CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_H CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_E CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_V CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_B CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
HvDRF1.3 CMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
DRF1.3_A CMQGKGGPENTRCGFRGVRQRTWGKWVAEIREPDRVSRLWLGTFPTAEVAAQAYDEAARAMYGPLARTNFPVQDAQAAPA  160
         ........90.......100.......110.......120.......130.......140.......150.......160
```

Conservation

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         ************************************* * ****************************************
DRF1.3_P VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQTQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_T VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_I VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_H VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_E VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_V VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_B VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
HvDRF1.3 VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQRQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
DRF1.3_A VAVPVATEGVVRGSSASCESTTTSNHSDVASSSHNKQLQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEE  240
         .......170.......180.......190.......200.......210.......220.......230.......240
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Conservation

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         ******************************:****************** ************** ***************
DRF1.3_P DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWNANAGMEM  320
DRF1.3_T DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWNANAGMEM  320
DRF1.3_I DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWNANAGMEM  320
DRF1.3_H DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCAGANTGVSWNANAGMEM  320
DRF1.3_E DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWNANAGMEM  320
DRF1.3_V DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWNANAGMEM  320
DRF1.3_B DVYEPLEPISNLPDGEADCFDIEELLKLIEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWNANAGMEM  320
HvDRF1.3 DVYEPLEPISNLPDGEADCFDIEELLKLMEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWNANAGMEM  320
DRF1.3_A DVYEPLEPISNLPDGEADCFDIEELLKLMEADPVEVDPVTVGSWNEFQDDGANARGSWNEFQCAGANTGVSWNANAGMEM  320
         .......250.......260.......270.......280.......290.......300.......310.......320
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Conservation

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         ****************************************************
DRF1.3_P GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_T GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_I GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_H GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_E GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_V GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_B GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
HvDRF1.3 GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
DRF1.3_A GQQEPLYLDGLDQGMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEG  372
         .......330.......340.......350.......360.......370..
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Conservation

Fig. 9. continued

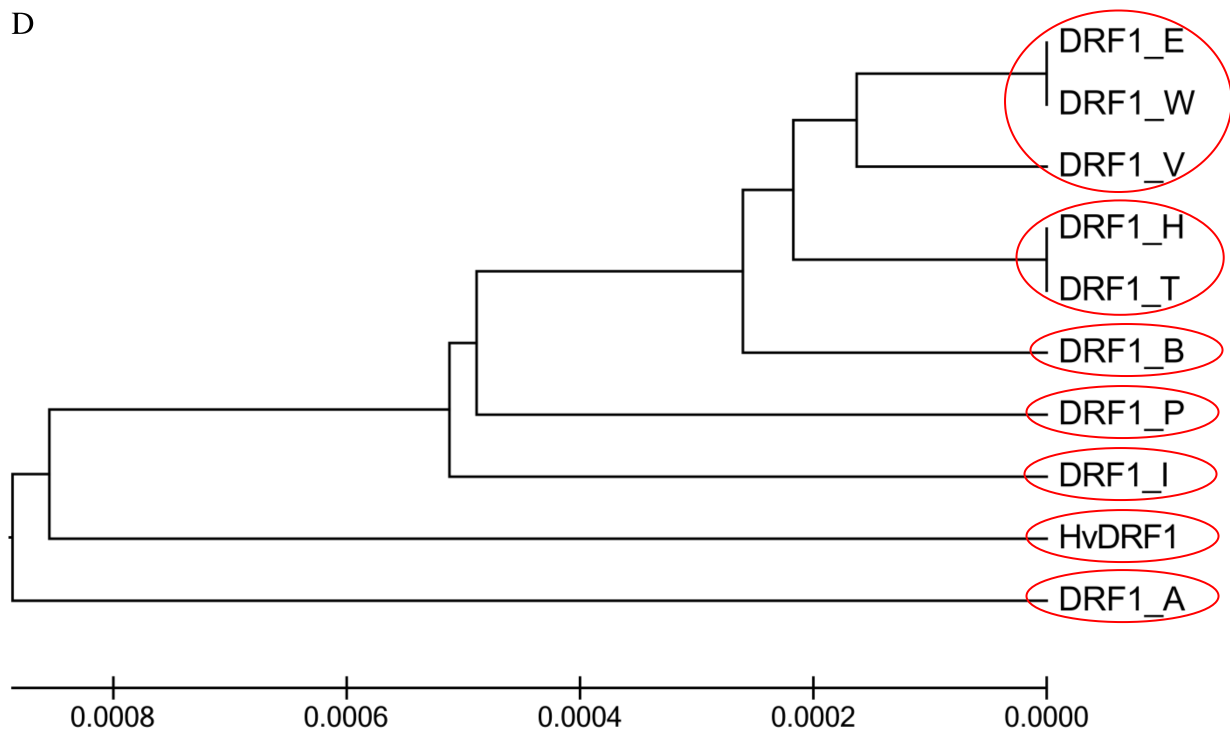


Fig. 9. DNA sequence analysis of barley *DRF1* genes. (A) Comparison of *DRF1* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B), (C) Comparison of deduced protein sequences of the *DRF1.1* and *DRF1.3* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain is labelled with a black square frame. Ser- and Thr-rich 98-amino acid region adjacent to the AP2 domain is labelled with a red square frame. (D) Genetic distance among nine isolated barley *DRF1* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvDRF1*: reference gene from NCBI.

cultivars are shown in Table 10. To reveal the genetic distance among these *DRF1* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 9D, the isolated *DRF1* genes in nine cultivars were clustered into six groups. The group I contained *DRF1* genes of the cultivars *Eunova*, *Wiebke* and *Victoriana*. This group could be subdivided into two subgroups. The first two cultivars composed the subgroup I. The subgroup II consisted of the *DRF1* genes of the cultivar *Victoriana*. The *DRF1* genes of the cultivars *Heils Franken* and *Trumpf* composed the group II. From the third to the seventh group, each one contained only one member. It should be

noted that the reference *HvDRF1* gene from the NCBI GeneBank formed the group VI.

Table 10. Estimates of genetic divergence between *DRF1* sequences of barley cultivars

Percentage of genetic divergence

	1	2	3	4	5	6	7	8	9	10
1. DRF1_A										
2. DRF1_B	0.2									
3. DRF1_E	0.2	0.0								
4. DRF1_H	0.1	0.1	0.0							
5. DRF1_I	0.2	0.1	0.1	0.1						
6. DRF1_P	0.2	0.1	0.1	0.1	0.1					
7. DRF1_T	0.1	0.1	0.0	0.0	0.1	0.1				
8. DRF1_V	0.2	0.1	0.0	0.1	0.1	0.1	0.1			
9. DRF1_W	0.2	0.0	0.0	0.0	0.1	0.1	0.0	0.0		
10. HvDRF1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.1	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *DRF1* nucleotide sequences and the reference *HvDRF1* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 3077 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvDRF1*: reference gene from NCBI.

3.2.9 Isolation of *DRF2* genes from the barley core set

The *DRF2* homologues were isolated using gene-specific primers to amplify the corresponding gene with the genomic DNA from nine different barley genotypes. The amplicons were 1047 bp in length. The sequences obtained from nine different genotypes were multiple aligned and analyzed for the presence of SNPs. The multiple sequence alignment of the isolated barley *DRF2* genes with their cDNAs indicated that a 96 bp long intron with GT-AG borders was present in the obtained *DRF2* homologues (Fig. 10A). A total of 12 SNPs was identified in the nine isolated barley *DRF2* homologues. Five of them led to synonymous mutations. Two A/G nucleotide transitions were present at positions 531 and 633 in the cultivars *Heils Franken* and *Pflugs Intensiv*, respectively. An additional C/T nucleotide transition was identified at position 684 in the cultivar *Pflugs Intensiv*. The fourth synonymous mutation caused by an A/T nucleotide transversion was observed in the cultivar *Eunova* at position 137. It should be noted that a G/A nucleotide transition was only identified at position 579 in the reference *HvDRF2* gene from the NCBI GeneBank. In case of non-synonymous mutations, seven SNPs were identified in the nine isolated *HvDRF2* homologues. However, two neighbour SNPs were identified at position 974 and 975 in the cultivars *Arta*, *Barke*, *Trumpf* and *Pflugs Intensiv*. These GC/TT nucleotide variants led to a non-synonymous mutation from glycine to valine. Another common C/T nucleotide transition was identified at position 902 leading to a non-synonymous mutation from threonine to

methionine. Coincidentally, the change of amino acid residue from methionine to threonine was caused by a T/C nucleotide transition at postion 1010 in the cultivar *Victoriana*. In the cultivar *Arta*, the presence of an A/T nucleotide transversion at position 209 resulted in a non-synonymous mutation from leucine to isoleucine. In the cultivar *Wiebke*, an A/G nucleotide transition was observed at position 311 leading to a non-synonymous mutation from asparagine to serine. The last non-synonymous mutation from proline to threonine was caused by a C/A nucleotide variant at position 853 in the cultivar *Pflugs Intensiv* (Fig.10B).

To estimate genetic diversity between the identified *DRF2* genes in nine different barley cultivars, the proportion of nucleotide differences between each pair of sequences was computed. The genetic diversity and differentiation among the isolated *DRF2* genes in nine cultivars are shown in Table 11. To reveal the genetic distance among these *DRF2* genes, a dendrogram was constructed by MEGA 5.0 software using UPGMA and p-distance model (Tamura *et al.*, 2011). As shown in Fig. 10C, the isolated *DRF2* genes in nine cultivars were clustered into two groups. The group I contained the *DRF2* genes of the cultivars *Isaria*, *Victoriana*, *Wiebke*, *Eunova* and *Heils Franken*, while the *DRF2* genes of the cultivars *Arta*, *Pflugs Intensiv*, *Barke* and *Trumpf* composed the group II. This group could be subdivided into three subgroups. The *DRF2* genes of the cultivars *Arta* and *Pflugs Intensiv* belonged to the subgroup I and subgroup II, respectively. The last two *DRF2* genes from the cultivars *Barke* and *Trumpf* composed the subgroup III.

Table 11. Estimates of genetic divergence between *DRF2* sequences of barley cultivars

Percentage of genetic divergence	1	2	3	4	5	6	7	8	9	10
1. DRF2_A										
2. DRF2_B	0.2									
3. DRF2_E	0.4	0.4								
4. DRF2_H	0.5	0.5	0.3							
5. DRF2_I	0.3	0.3	0.1	0.2						
6. DRF2_P	0.4	0.2	0.6	0.7	0.5					
7. DRF2_T	0.2	0.0	0.4	0.5	0.3	0.2				
8. DRF2_V	0.4	0.4	0.2	0.3	0.1	0.6	0.4			
9. DRF2_W	0.4	0.4	0.2	0.3	0.1	0.6	0.4	0.2		
10. HvDRF2	0.4	0.4	0.2	0.3	0.1	0.6	0.4	0.2	0.2	

The percentage of nucleotide differences between each pair of sequences was computed. Analyses were conducted using the p-distance model. The analysis involved nine isolated *DRF2* nucleotide sequences and the reference *HvDRF2* gene from the NCBI. All positions containing gaps and missing data were eliminated. There were a total of 1047 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; HvDRF2: reference gene from NCBI.

A

```
          1       10        20        30        40        50        60        70        80        90        100       110       120
DRF2_A    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_B    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_T    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_P    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_E    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_I    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_W    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_V    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
DRF2_H    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGTCCGGCTCGTCGGGCCTGGTGTGGGACGAGCGGTTCCTGACCGAGGGCGCCGAGAGAATACGGTACTAACTGCTACTCTGC
HvDRF2    ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGG---------------------------------------------------------------------------------
Consensus ATGGAAGGGCTGCAGAGATCGTCCTCCACTTTCAGAAGGtccggctcgtcgggcctggtgtgggacgagcggttcctgaccgagggcgccgagagaatacggtactaactgctactctgc

          121     130       140       150       160       170       180       190       200       210       220       230       240
DRF2_A    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGATACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_B    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_T    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_P    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_E    TATTTGTTCTACAGGATAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_I    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_W    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_V    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
DRF2_H    TATTTGTTCTACAGGAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
HvDRF2    ---------------AAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA
Consensus tatttgttctacaggAAAAAGCGACCTCGAAGGTCACGTGATGGGCCTAATTCAGTCTCTGAGACGATCAAGCAATGGAAAGAAGTGAAACAACAACTCGAGCATGATCCACAGGGTGCA

          241     250       260       270       280       290       300       310       320       330       340       350       360
DRF2_A    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_B    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_T    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_P    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_E    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_I    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_W    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAGTACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_V    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
DRF2_H    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
HvDRF2    AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG
Consensus AAGCGGGCGAGGAAGCCACCAGCAAAGGGTTCAAAGAAGGGCTGCATGCAAGGGAAAGGAGGCCCTGAGAATACACAATGTGGATTCCGTGGGGTAAGGCAACGAACTTGGGGGAAGTGG

          361     370       380       390       400       410       420       430       440       450       460       470       480
DRF2_A    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_B    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_T    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_P    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_E    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_I    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_W    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_V    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
DRF2_H    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
HvDRF2    GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT
Consensus GTTGCTGAAATTCGGGAGTCAAATCGGGTCAGCAGGCTCTGGTTGGGGACGTTCCCTACTGCTGAAGTTGCTGCTCAAGCTTATGATGAAGCAGCCAAAGCAATGTATGGCCCGCTGGCT

          481     490       500       510       520       530       540       550       560       570       580       590       600
DRF2_A    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_B    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_T    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_P    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_E    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_I    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_W    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_V    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
DRF2_H    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTGCAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAGTCGACTACAACATCCAACCAC
HvDRF2    CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAATCGACTACAACATCCAACCAC
Consensus CGCACCAACTTCCCTGTGCAGGATGCACAAGCTGCTCCTACTGTGGTTGTACAAGTGGCAACCGAAGGTGTTGTACGTGGTTCTTCAGCATCATGCGAgTCGACTACAACATCCAACCAC

          601     610       620       630       640       650       660       670       680       690       700       710       720
DRF2_A    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_B    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_T    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_P    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAGCTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_E    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_I    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_W    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_V    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
DRF2_H    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCTACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
HvDRF2    TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT
Consensus TCCGACGTTGCTTCTTCCTCGCATAATAAGCAACTACAAATTCAAGCTCCTGAGATTTCCTCTCAGTCAGATTTGCTGGAGTCCACCCAGTCAGTTGAGTACATCCAACAACAGTCTGTT

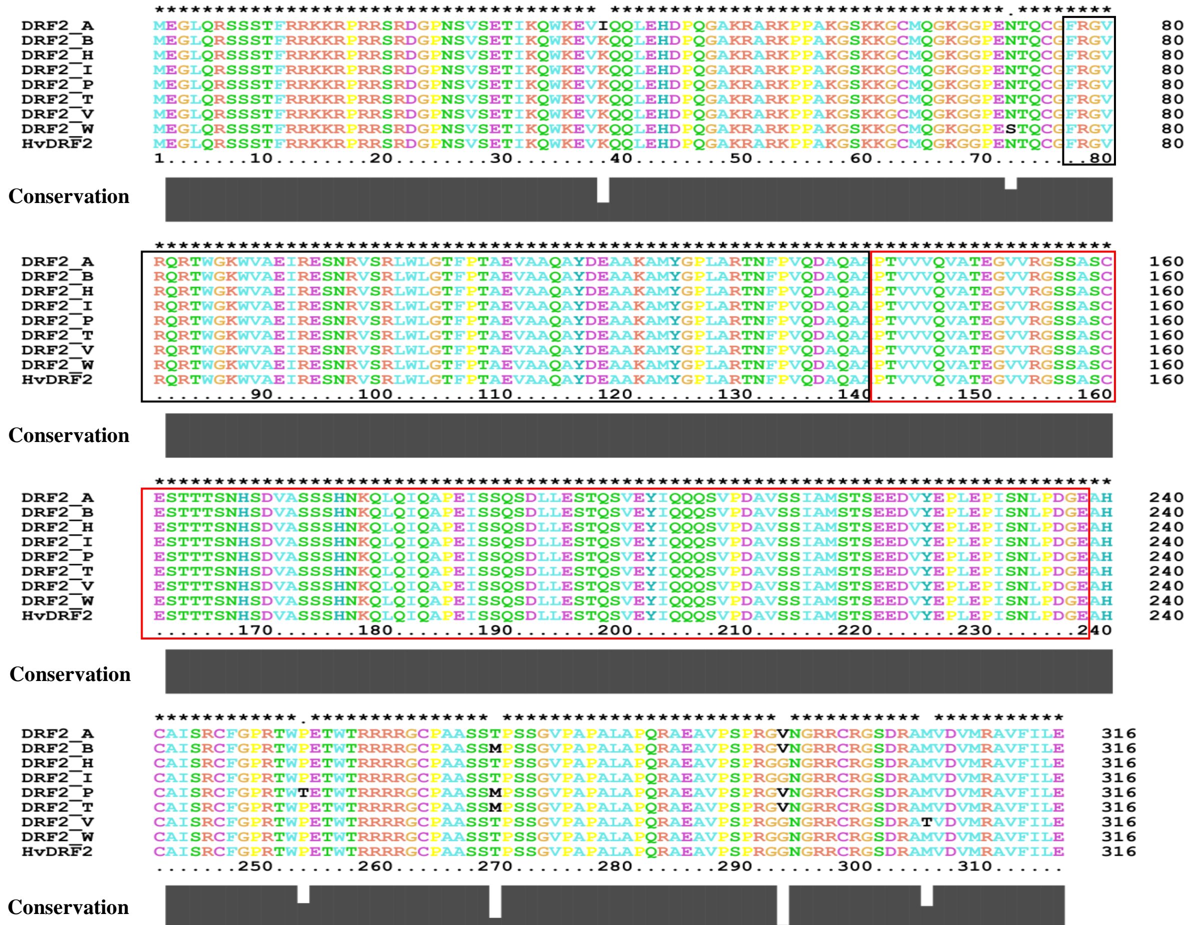
          721     730       740       750       760       770       780       790       800       810       820       830       840
DRF2_A    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_B    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_T    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_P    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_E    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_I    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_W    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_V    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
DRF2_H    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
HvDRF2    CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA
Consensus CCTGATGCTGTCTCAAGCATTGCAATGAGCACATCTGAAGAGGATGTCTATGAGCCATTGGAGCCTATCTCCAATTTGCCAGATGGGGAAGCACACTGCGCGATCAGCAGGTGTTTCGGA

          841     850       860       870       880       890       900       910       920       930       940       950       960
DRF2_A    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_B    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTATGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_T    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTATGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_P    CCAAGGACGTGGACTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTATGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_E    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_I    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_W    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_V    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
DRF2_H    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
HvDRF2    CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTACGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA
Consensus CCAAGGACGTGGCCTGAGACATGGACCCGCCGTCGCCGAGGGTGTCCGGCGGCATCCTCTAcGCCATCTTCGGGGGTTCCGGCTCCGGCTCTGGCCCCGCAGCGCGCCGAGGCCGTGCCA

          961     970       980       990       1000      1010      1020      1030      1040  1047
DRF2_A    AGCCCAAGAGGAGTTAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_B    AGCCCAAGAGGAGTTAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_T    AGCCCAAGAGGAGTTAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_P    AGCCCAAGAGGAGTTAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_E    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_I    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_W    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_V    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAACGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
DRF2_H    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
HvDRF2    AGCCCAAGAGGAGGCAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
Consensus AGCCCAAGAGGAGgcAACGGTCGGCGGTGTAGGGGTTCTGATCGGGCAATGGTGGATGTCATGCGAGCTGTGTTTATACTGGAGTGA
```

Fig. 10.

B



C

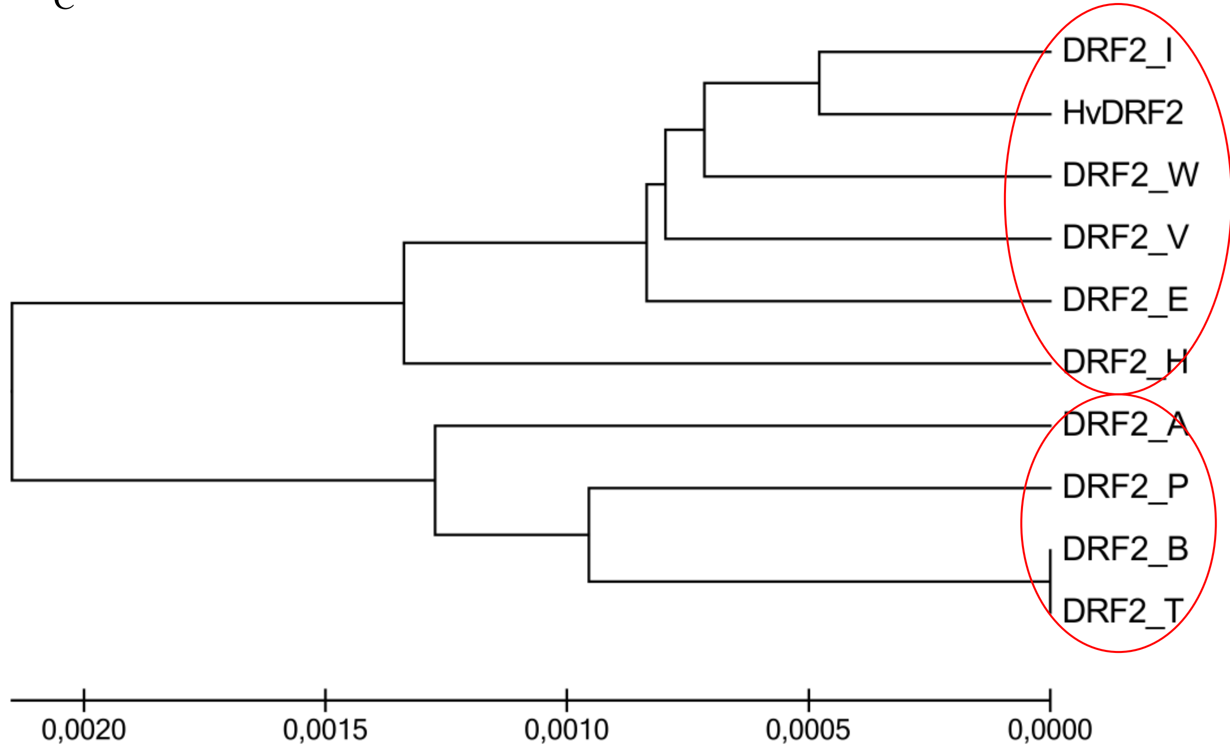


Fig. 10. continued

Fig. 10. DNA sequence analysis of barley *DRF2* genes. (A) Comparison of *DRF2* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour. (B) Comparison of deduced protein sequences of the *DRF2* genes in nine barley cultivars using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. The AP2 domain is labelled with black square frame. Ser- and Thr-rich 98-amino acid region adjacent to the AP2 domain is labelled with a red square frame. (C) Genetic distance among nine isolated barley *DRF2* genes. A dendrogram was resulted from UPGMA cluster analysis of nine barley cultivars based on the deduced protein sequences of the isolated nine barley cultivars. The dendrogram was constructed by MEGA 5.0 software. Branch lengths represent the distance of genetic diversity. A: *Arta*; B: *Barke*; E: *Eunova*; H: *Heils Franken*; I: *Isaria*; P: *Pflugs Intensiv*; T: *Trumpf*; V: *Victoriana*; W: *Wiebke*; *HvDRF2*: reference gene from NCBI.

3.3 Phylogeny of the ten candidate barley *CBF/DREB* genes

To clarify the phylogenetic relationships of the ten isolated barley *CBF/DREB* genes and other AP2/EREBP proteins isolated from *Arabidopsis*, rice, maize, sorghum and wheat, multiple alignment analyses were carried out using amino acid sequences of the AP2/ERF domain. Multiple sequence alignment revealed that AP2/ERF domain of *CBF/DREB* genes has high sequence similarity among both monocot and dicot species. Low sequence similarities could be observed in certain areas of the conservation diagram. However, the variability of the sequences is very low and in most cases there are only two different amino acid residues presented at the same position. In addition, the alignment indicated that residues Gly3, Val4, Arg5, Arg7, Glu16, Arg18, Arg27, Trp29, Leu30, Gly31, Ala39, Ala40, Ala46 and Asn59 are completely conserved among the 29 analyzed *CBF/DREB* genes (Fig. 11A). Altogether, the AP2/ERF domain is highly conserved in *CBF/DREB* genes of monocot and dicot species. Based on these observations, a phylogenetic tree based on the alignment of AP2/ERF domains of these 29 proteins was constructed. Ten candidate barley *CBF/DREB* genes can be grouped into three phylogenetic subgroups, designated HvCBF1, HvCBF4 and HvDREB1 (Fig. 11B). HvCBF1 subgroup is ancestral to HvCBF4- and HvDREB1-subgroups. In addition, the distribution of the *CBF/DREB* genes of the monocot and dicot plants in the reconciled tree indicates the clear demarcation. *CBF/DREB* genes such as *HvDRF1.1*, *HvDRF1.3*, *HvDRF2*, *HvDREB1*, *OsDREB2*, *OsDREB2B*, *OsDREB2C*, *OsDBF1*, *SbDREB2*, *AtDREB2A*, *AtDREB2B*, *AtDREB2C*, *ZmDREB1C*, *ZmDREB2A* and *ZmDBF1* are grouped in cluster I. Cluster II contains four clades including one clade of four *Arabidopsis CBF/DREB1* genes and anther three clade of monocot *CBF/DREB1* genes, which compose the HvCBF1- and HvCBF4-subgroups. Interestingly, this distribution of *CBF/DREB* genes in the reconciled

tree coincides with their responses to drought stress. *CBF/DREB* genes of the cluster I are inducible by drought stress. In contrast, *CBF/DREB* genes of the cluster II are not drought-inducible (*AtCBF1/DREB1B*, *AtCBF2/DREB1C* and *AtCBF3/DREB1A*) or slightly inducible, e.g. *AtCBF4/DREB1D* (Lata and Prasad, 2011).

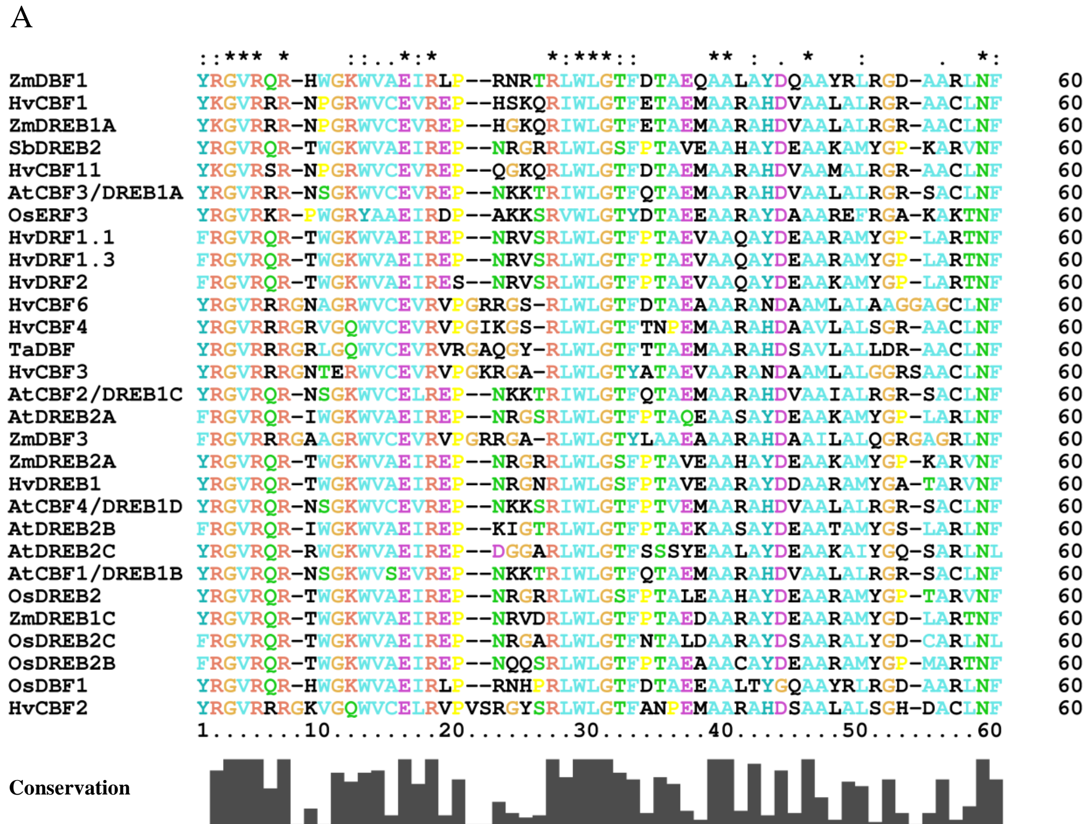


Fig. 11. Investigation of *CBF/DREB* protein sequences in monocot and dicot plants. (A) Comparison of the amino acid sequences of the AP2/ERF domain of the 10 candidate barley *CBF/DREB* genes with those in other monocot and dicot plants using ClustalX version 2.1. Amino acids are designated in single-letter code. An asterisk indicates identical, a colon indicates closely related, and a period indicates distantly related amino acid. The partially conserved amino acid residues are labelled with different colours. In contrast, identical colour represents the amino acid residues with 100% homology. The conservation degree of the amino acid residues is shown in a bar chart at the bottom of the alignment. (B) Phylogenetic relationships of AP2/ERF domains of the ten candidate barley *CBF/DREB* genes and other published AP2/EREBP proteins isolated from *Arabidopsis*, rice, maize, sorghum and wheat in the NCBI GeneBank. The phylogenetic tree was conducted by MEGA 5.0 software using neighbor joining and poisson model on 5050 bootstrap replications. Branch lengths represent the distance. The utilized proteins are the following: *Hordeum vulgare*: All the deduced protein sequences derives from the *de novo* sequenced *CBF/DREB* gene of the cultivar *Barke*; *Arabidopsis thaliana AtCBF1/DREB1B* (BAA33435), *AtCBF2/DREB1C* (BAA33436), *AtCBF3/DREB1A* (BAA33434), *AtCBF4/DREB1D* (AED96156), *AtDREB2A* (BAA36705), *AtDREB2B* (BAA36706), *AtDREB2C* (AEC09816); *Oryza sativa OsDREB1A* (AAN02486), *OsDREB1B* (AAN02488), *OsDREB2* (AAN02487), *OsDREB2A* (AFB77198), *OsDREB2B* (Q5W6R4), *OsDREB2C* (Q84ZA1), *OsERF3* (BAB16083), *OsDBF1* (AAP56252); *Zea mays ZmDREB1A* (ACG40680), *ZmDREB1C* (ACO72991), *ZmDREB2A* (ACG47772), *ZmDBF1*

(AAM80486), *ZmDBF3* (NP_001105651); *Triticum aestivum TaDBF* (AAZ08560); *Sorghum bicolor SbDREB2* (ACA79910).

B.

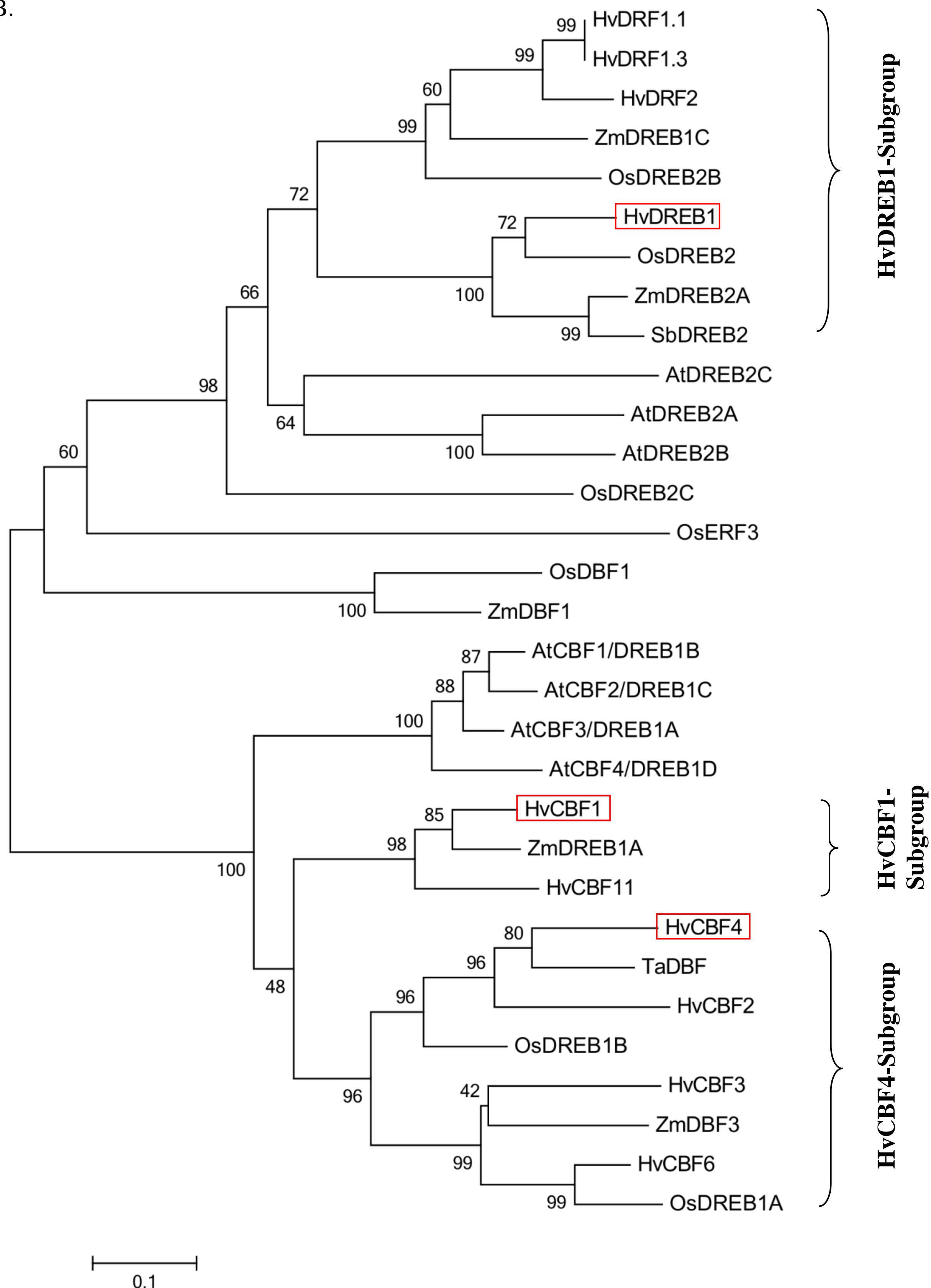


Fig. 11. continued

3.4 Expression analysis of *HvCBF/DREBs* under drought stress conditions

3.4.1 Expression analysis of *HvCBF/DREBs* under drought stress conditions in the plastic greenhouse tunnel

3.4.1.1 Expression profiles of the *HvCBF1* gene

Eight genotypes of spring barley (*Hordeum vulgare*), *Barke*, *Eunova*, *Heils Franken*, *Isaria*, *Pflugs Intensiv*, *Trumpf*, *Victoriana* and *Wiebke* were utilized in this study. The plants were grown in a plastic greenhouse tunnel in Bonn Poppelsdorf (+50 ° 43' 34.54", +7 ° 5' 15.54"). The expression pattern of the *HvCBF1* gene was analyzed in barley under well-watered growth conditions and progressive drought stress conditions in 2010 and 2011. The expression pattern of the *HvCBF1* gene for the cultivar *Apex* was additionally analyzed in 2011 (Fig.12). After exposure to drought, the expression of the *HvCBF1* gene was differentially altered in the eight cultivars under study. By using the samples from 2010, the examination of the expression pattern of the *HvCBF1* gene in leaf tissues led to define two groups of cultivars: early drought responsive (labelled with purple square frame) and late drought responsive cultivars (labelled with red square frame). The early drought responsive cultivars included *Heils Franken*, *Pflugs Intensiv* and *Wiebke*. Noticeable changes in the *HvCBF1* expression were observed in leaf tissues of these plants within seven days after the start of the experiment in contrast to the other cultivars. The *HvCBF1* expression level increased in the cultivars *Pflugs Intensiv*e and *Wiebke*; whereas it reduced in the cultivar *Heils Franken*. Only a slight increase in *HvCBF1* expression was observed in leaf tissues of the cultivars *Wiebke* and *Pflugs Intensiv* after 14 and 21 days exposure to drought stress. However, their expressions did not seem to change (*Wiebke*) or dropped faintly (*Pflugs Intensiv*) after exposure to drought for 28 days. No further changes were observed in the *HvCBF1* expression level in the cultivar *Heils Franken* after exposure to drought for 14 and 21 days. Furthermore, its expression was strongly down-regulated after exposure to drought for 28 days. In contrast to the early drought responsive cultivars, changes in the *HvCBF1* gene expression in the late drought responsive cultivars occurred late after 14 days of drought stress. This group consisted of the cultivars *Barke*, *Eunova*, *Isaria*, *Trumpf* and *Victoriana*. Depending on whether the *HvCBF1* expression was down- or up-regulated, the late drought responsive cultivars could be subdivided into two subgroups. *Barke*, *Eunova* and *Isaria* represented the subgroup I and displayed a strong up-regulation of *HvCBF1* after 14 and 21 days of drought stress treatments. However, with further prolonged drought stress treatments, their expression levels went down to a very low level. After 28 days long drought stress treatments,

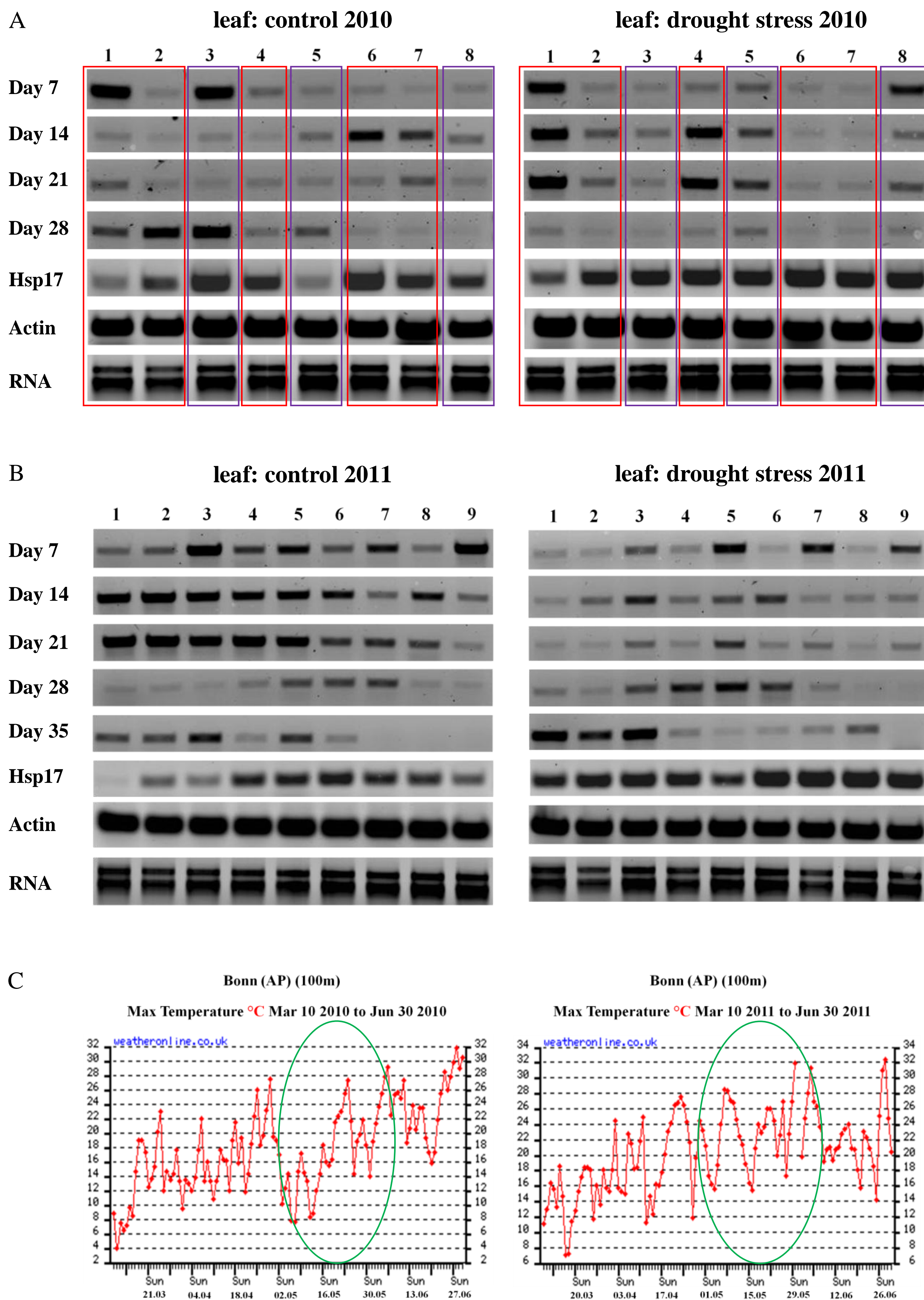


Fig. 12

Fig. 12. Expression analysis of the *HvCBF1* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF1* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in the years 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. (C) Maximal temperature from Mar. 10 to Jun. 30 in 2010 and 2011 in Bonn, Germany. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex.*

the *HvCBF1* expression was hardly detected in the stressed plants. The subgroup II comprised the cultivars *Trumpf* and *Victoriana*. As shown in the subgroup I, the *HvCBF1* expression level was unchanged until plants were exposed to drought for 14 days. However, their expression was down-regulated in response to the progressive drought stress. Similar to the varieties in subgroup I, their expression of *HvCBF1* could hardly be detected after 28 days exposure to drought (Fig. 12A).

The progressive drought stress experiments were repeated in 2011 using the same barley varieties with identical treatments. Unlike in 2010, the expression pattern of *HvCBF1* was found significantly low in most cultivars in the first three weeks after exposure to drought. However, a strong up-regulation of the *HvCBF1* gene could be detected in *Heils Franken*, *Isaria* and *Pflugs Intensiv* after exposure to drought for 28 days. Such big differences in the *HvCBF1* expression levels in 2010 and 2011 were unexpected, but could be due to the fluctuations in environmental conditions. To ascertain this, the variation in ambient temperature recorded in the field during the period of stress application was analyzed. The whole drought stress treatments lasted from the jointing stage (19th week) to the booting stage (23th week). As the drought stress treatments began on May 10, 2010, the maximal temperature in Bonn was 17 ℃. In contrast, this value was 28 ℃ on May 11, 2011 (Fig. 12C). In addition, the average temperature and the radiation efficiency in 2010 were much lower than those in 2011 during the first week of drought stress experiments. The average temperature was 10.8 ℃ during the first week (19th week) of drought stress treatments in 2010, whereas this value was 17.3 ℃ in 2011 (Tab.12). No significant average temperature differences were observed at the second and third week during the drought stress experiment in 2010 and 2011. However, significant difference in maximal temperature and radiation efficiency were observed in the same period between these two years. It should be noted that the maximal temperature on Jun. 1, 2011 was 32 ℃, as the samples were harvested at the third week of the drought stress experiment. In contrast, this value was 14 ℃ in 2010. The high temperature and radiation efficiency indicated rapid dehydration. This high temperature

period during the first three weeks of the drought stress in 2011 paralleled overall the down-regulation of the *HvCBF1* gene. To ascertain this, the expression of the *Hsp17.8* was analyzed, which is heat shock inducible (Guo *et al.*, 2009). As shown in Fig 12B, a strong up-regulation of the *Hsp17.8* gene was observed at the third week of drought stress treatment in 2011.

Table 12. Climate data during the drought stress assay

Week	rel. Humidity [%]	Temperatur [℃]	Radiation Efficiency [W/m²]	Week	rel. Humidity [%]	Temperatur [℃]	Radiation Efficiency [W/m²]
2010/15	57.8	11.3	133.8	2011/14	69.6	13.7	134.2
2010/16	53.9	12.1	169.5	2011/15	61.0	11.6	139.4
2010/17	58.7	17.0	142.6	2011/16	54.7	17.7	184.2
2010/18	69.7	10.2	100.1	2011/17	57.5	17.0	185.2
2010/19	72.6	10.8	98.2	2011/18	48.4	15.5	194.0
2010/20	60.0	16.5	178.3	2011/19	59.0	17.3	180.7
2010/21	61.6	18.3	159.5	2011/20	67.9	17.6	161.6
2010/22	61.1	19.9	186.5	2011/21	51,9	17,5	212,6
2010/23	65.1	21.3	157.8	2011/22	60.8	20.4	207.1
2010/24	57.5	18.1	162.0	2011/23	69.9	18.1	180.4
2010/25	52.0	22.3	213.2	2011/24	70.1	18.5	154.7
2010/26	50.3	27.6	188.3	2011/25	78.9	17.3	119.3

3.4.1.2 Expression profiles of the *HvCBF2* gene

The expression of the *HvCBF2* gene in leaf tissues was analyzed by RT-PCR as described in 3.4.1.2. Similar to the *HvCBF1* gene, the expression pattern of the *HvCBF2* gene in eight different barley cultivars in the year 2010 could be divided in to two groups: early drought responsive and late drought responsive cultivars (Fig. 13A). The late drought responsive group contained the the cultivars *Barke* and *Victoriana* (labelled with purple square frame). In this group, changes in *HvCBF2* expression level were recorded one week later than another six cultivars after exposure to drought. But these two cultivars responded to drought in a different way. In the cultivar *Barke*, a very low *HvCBF2* expression level was detected in the control plants at the second week. Its expression was down-regulated in the stressed plants, which was hardly detected. Moreover, with the prolonged stress treatment, a relatively high *HvCBF2* expression level was observed in the control and stressed plants at the third and fourth week, respectively. No difference in the *HvCBF2* expression was observed in the cultivar *Barke* at the third week, while its expression was down-regulated at the fourth week. Compared to *Barke*, the cultivar *Victoriana* responded positively to drought at the second and third week. Similar to *Barke*, the expression of the *HvCBF2* gene was down regulated. In case of the early drought responsive cultivars, changes in *HvCBF2* expression level were observed after exposure to drought for one week. Depending on whether the *HvCBF2* expression was

down- or up-regulated, the early drought responsive cultivars could be subdivided into two subgroups. The subgroup I consisted the cultivars *Eunova*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Wiebke* (labelled with red square frame). The cultivars *Trumpf* composed the subgroup II. (labelled with green square frame). Compared the the subgroup II, the cultivars in the subgroup I responded positively to drought. In the subgroup I, the cultivars *Eunova* and *Wiebke* displayed the same responses to drought. The expression of the *HvCBF2* gene was up-regulated at the first week of drought stress treatments. However, their expression was down regulated at the week II, III and IV. The cultivars *Heils Franken*, *Isaria* and *Pflugs Intensiv* displayed the similar *HvCBF2* expression pattern as shown in the cultivars *Eunova* and *Wiebke*. However, the expression of the *HvCBF2* gene was hardly detected at the second week after exposure to drought. The cultivar *Trumpf* as a unique member of the subgroup II

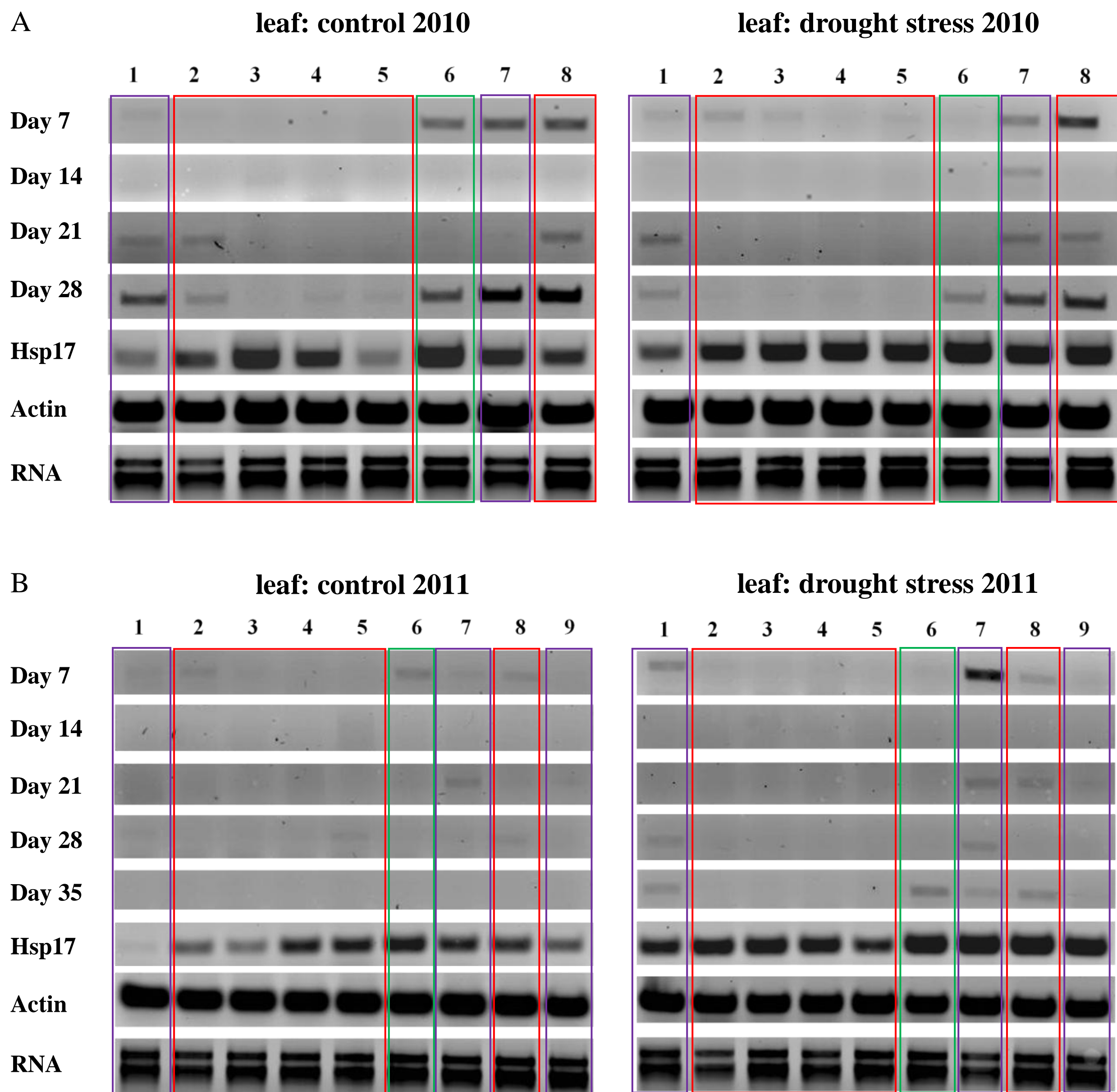


Fig. 13

Fig. 13. Expression analysis of the *HvCBF2* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF2* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex.*

responded negatively to drought during the whole stress experiment.

The progressive drought stress experiments were repeated in 2011 using the same barley cultivars with identical treatments. As shown in Fig. 13B, the *HvCBF2* expression was very weak in both control and stressed plants. However, the arrangement in groups was coincident with that shown in 2010. In addition, the expression pattern of the cultivar *Apex* was similar to the cultivar *Barke* and *Victoriana*. Unlike in the year 2010, the cultivar *Barke* and *Pflugs Intensiv* responded swiftly to drought in 2011. Changes in the *HvCBF2* expression could be positively observed after exposure to drought for one week. Moreover, a strong up-regulation of the *HvCBF2* gene was present in the cultivar Pflugs Inensiv. It should be noted that the expression of the *HvCBF2* gene was hardly detected in the cultivars *Eunova*, *Heils Franken*, *Isaria* and *Pflugs Intensiv* during the whole experiment. Only a very weak *HvCBF2* expression was observed in the control plants of the cultivar *Eunova* at the first week and *Pflugs Intensiv* at the fourth week, respectively.

3.4.1.3 Expression profiles of the *HvCBF3* gene

The expression of the *HvCBF3* gene in leaf tissues was analyzed by RT-PCR. Unlike the *HvCBF1* and *HvCBF2* genes, changes in *HvCBF3* expression were observed in all eight different barley cultivars after exposure to drought for one week (Fig. 14A). Depending on whether the *HvCBF3* expression was down- or up-regulated, the expression pattern of the *HvCBF3* gene in eight different barley cultivars in the year 2010 could be divided in to two groups: positive drought responsive (labelled with green square frame) and negative drought responsive cultivars (labelled with red square frame). The cultivars *Barke*, *Trumpf* and *Heils Franken* composed the negative drought responsive group. This group could be further subdivided into two subgroups. The subgroup I contained the cultivars *Barke* and *Trumpf*, while the cultivar *Heils Franken* composed the subgroup II. In case of the cultivar *Heils Franken*, the expression of the *HvCBF3* gene was down-regulated during the whole drought stress experiment in 2010. Moreover, its maximal expression level was observed in the control plants after exposure to drought for four weeks. In contrast to the subgroup II, the

HvCBF3 expression was up-regulated in the cultivars of subgroup I at the second and third week. In the cultivar *Barke*, its maximal expression level was present in the stressed plants at the second week. In the cultivar *Trumpf*, the maximal expression level of the *HvCBF3* gene was observed in the stressed plants at the third week. Moreover, the *HvCBF3* expression was down-regulated in both cultivars. In case of the positive drought responsive, three subgroups were present. The cultivars *Eunova*, *Isaria* and *Pflugs Intensiv* composed the subgroup I. After exposure to drought, an up-regulation of the *HvCBF3* gene was observed in the first three weeks. However, because of the presence of maximal expression level in the control plants at week IV, a down-regulation of the *HvCBF3* gene was observed in these cultivars. The cultivar *Victoriana* and *Wiebke* composed the subgroup II and III, respectively. The

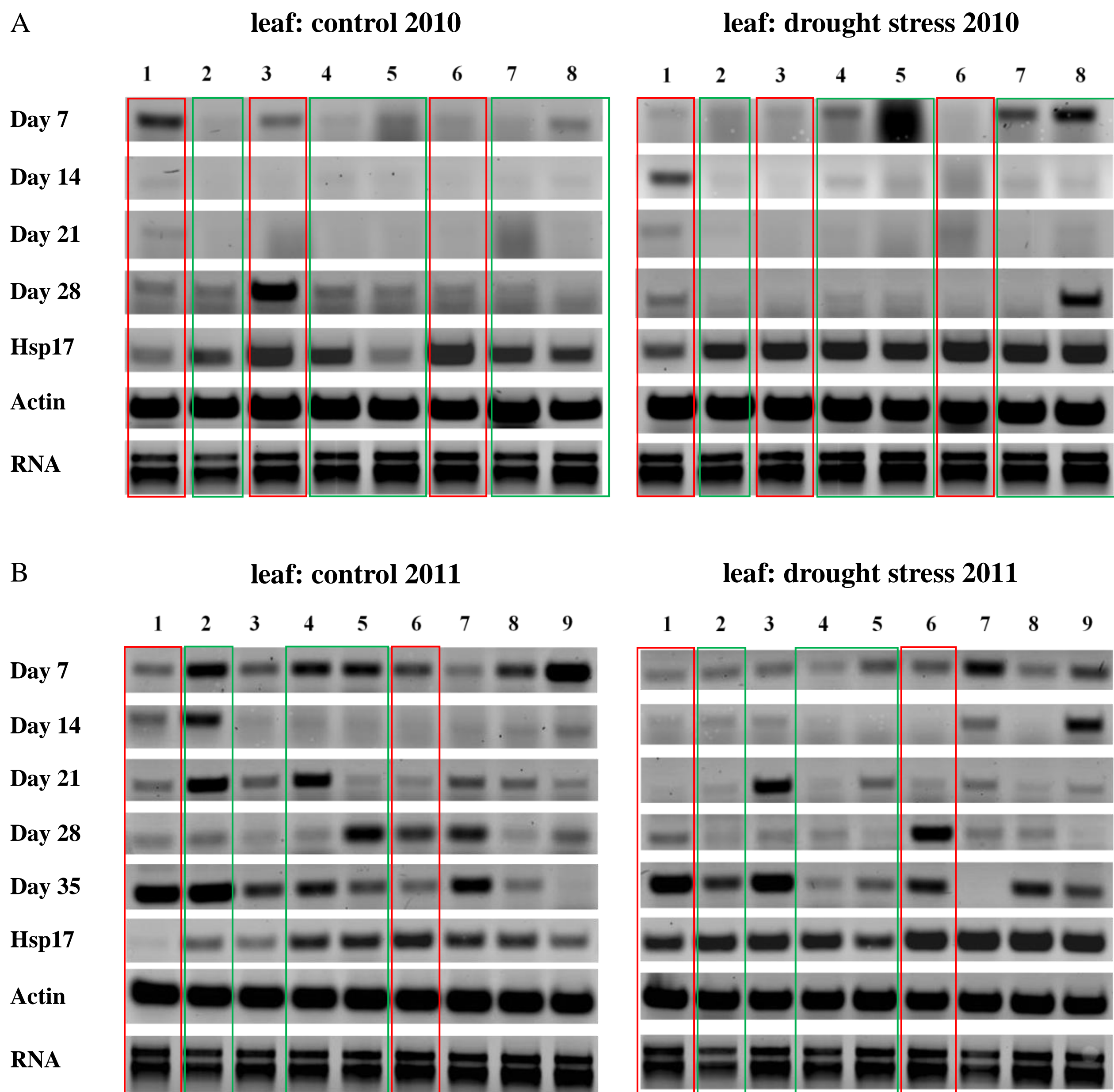


Fig. 14

Fig. 14. Expression analysis of the *HvCBF3* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF3* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

expression pattern of the both cultivars was similar. After exposure to drought, the expression of the *HvCBF3* gene was up-regulated and reached its maximal expression level at the first week. Its expression was reduced to a low level at the second and third week. However, at the fourth week, an up-regulation was observed in the cultivar *Wiebke*, while a down-regulation was present in the cultivar *Victoriana*.

In case of the *HvCBF3* expression pattern in the year 2011, a strong down-regulation was observed similar to the observations in the *HvCBF1* and *HvCBF2* genes. Unlike the *HvCBF2* gene, the arrangement in groups for the *HvCBF3* gene in 2011 was partially coincident with that shown in 2010. The *HvCBF3* expression pattern of the cultivars *Barke* and *Trumpf* was similar in the year 2010 and 2011. They responded negatively to drought. Moreover, an identical *HvCBF3* expression pattern was observed in the cultivars *Eunova*, *Isaria* and *Pflugs Intensiv*, which belonged to the subgroup I of the positive drought responsive group in the year 2010. However, the *HvCBF3* expression of these cultivars was down-regulated during the whole drought stress experiments in 2011, except a slight up-regulation in the cultivar *Pflugs Intensiv* at the third week. As described in 3.4.1.1, rapid dehydration caused by high temperature led to a reduction of the *HvCBF3* transcripts in leaf tissues.

3.4.1.4 Expression profiles of the *HvCBF4* gene

The expression of the *HvCBF4* gene in leaf tissues was analyzed in 2010 and 2011by RT-PCR. Unlike the *HvCBF1*, *HvCBF2* and *HvCBF3* genes, the *HvCBF4* gene responded at first positively to rapid drought caused by high temperature in 2011. During exposure to drought, an up-regulation of the *HvCBF4* gene was observed in nine different barley cultivars. But its expression depended on the time scale. Therefore, its expression pattern could be also divided into early drought responsive and late drought responsive cultivars as shown in *HvCBF1* in 2010. Early drought responsive cultivars contained the cultivars *Barke*, *Eunova*, *Heils Franken*, *Isaria Pflugs Intensiv*, *Victoriana* and *Apex* (labelled in green square frame). Changes in *HvCBF4* expression occurred after exposure to drought for 7 days. A strong up-regulation of the *HvCBF4* gene was observed and reached its maximal expression level. With

prolonged drought treatments, its expression was repressed and reduced to a relatively low level. This phenomenon was confirmed in the above-mentioned three *HvCBF* genes. Compared to these seven cultivars, changes in *HvCBF4* expression occurred 14 days later in the cultivars *Trumpf* and *Wiebke* (labelled in black square frame).

In the year 2010, a negative regulation of the *HvCBF4* gene was observed in leaf tissues of eight different barley cultivars. A strong down-regulation of the *HvCBF4* gene was observed after exposure to drought for 7 days. This large alteration in the *HvCBF4* gene expression in 2010 and 2011 was attributed to the great temperature differences, as described in 3.4.1.1. Moreover, based on their expression patterns, the eight different barley cultivars were divided

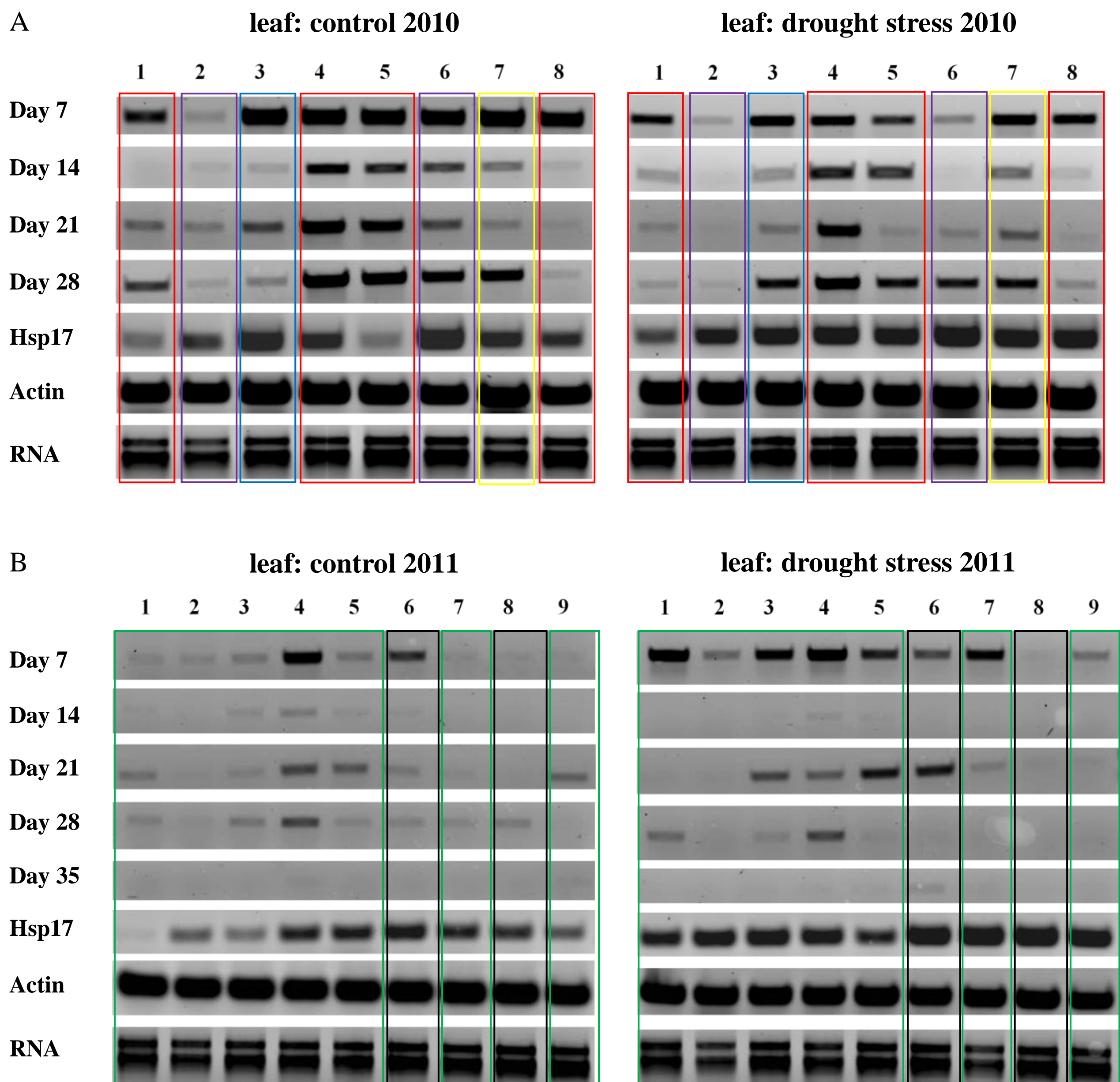


Fig. 15

Fig. 15. Expression analysis of the *HvCBF4* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF4* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

into four groups. The cultivars *Barke*, *Isaria*, *Pflugs Intensiv* and Wieke demonstrated identical *HvCBF4* expression patterns and composed the group I (labelled with red square frame). The group II contained the cultivars *Eunova* and *Trumpf* (labelled with purple square frame). The cultivars *Heils Franken* and *Victoriana* composed the group III and IV, respectively (labelled with blue and yellow square frame).

3.4.1.4 Expression profiles of the *HvCBF6* gene

The expression of the *HvCBF6* gene in leaf tissues was analyzed in 2010 and 2011by RT-PCR. In 2011, the *HvCBF6* genes from nine different cultivars demonstrated similar expression patterns characterized in the *HvCBF3* genes in 2010. Depending on whether the *HvCBF6* expression was down- or up-regulated, the *HvCBF6* genes from nine different barley cultivars were clustered into two groups, positive drought responsive (labelled with green square frames) and negative drought responsive cultivars (labelled with black square frames, Fig. 16B). The genotypes *Barke*, *Victoriana*, *Wiebke*, *Pflugs Inensiv* and *Trumpf* belonged to positive drought responsive cultivars. This group could be subdivided into two subgroups. The first three cultivars composed the subgroup I, while the last two cultivars formed the subgroup II. After exposure to drought for seven days, the *HvCBF6* expression was up-regulated and reached the maximal level in the subgroup I. With prolonged drought treatments, its expression was marginally reduced in the stressed plants from the second to the fourth week. Compared to the subgroup I, the *HvCBF6* expression was down-regulated in the stressed plants during the second and third week. During the fourth week, the expression was recovered. Moreover, an up-regulation of the *HvCBF6* gene was observed in the cultivar *Trumpf*; while it was down-regulated in the cultivar *Pflugs Intensiv*. Compared to the positive drought responsive cultivars, a down-regulation of the *HvCBF6* expression was observed in the cultivars *Eunova*, *Heils Franken*, *Isaria* and *Apex* after exposure to drought for seven days. These four cultivars composed therefore the negative drought responsive group. As shown in the positive drought responsive cultivars, the *HvCBF6* expression was also marginally reduced in the stressed plants of the negative drought responsive cultivars from the second to the fourth week of drought stress treatments.

A time course dependent *HvCBF6* expression was also observed in 2010. The examination of the *HvCBF6* gene expression in leaf tissues allow to define two groups of cultivars: early drought responsive and late drought responsive cultivars. Changes in the *HvCBF6* gene expression were recordd in the cultivars *Victoriana* and *Wiebke* after exposure to drought for 14 days. These two cultivars displayed a declined expression in the *HvCBF6* genes and composed the late drought responsive group (labelled with red square frames, Fig. 16A). In contrast, a down-regulation was observed seven days earlier in the cultivars *Barke*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Trumpf*. Moreover, an increased *HvCBF6* gene

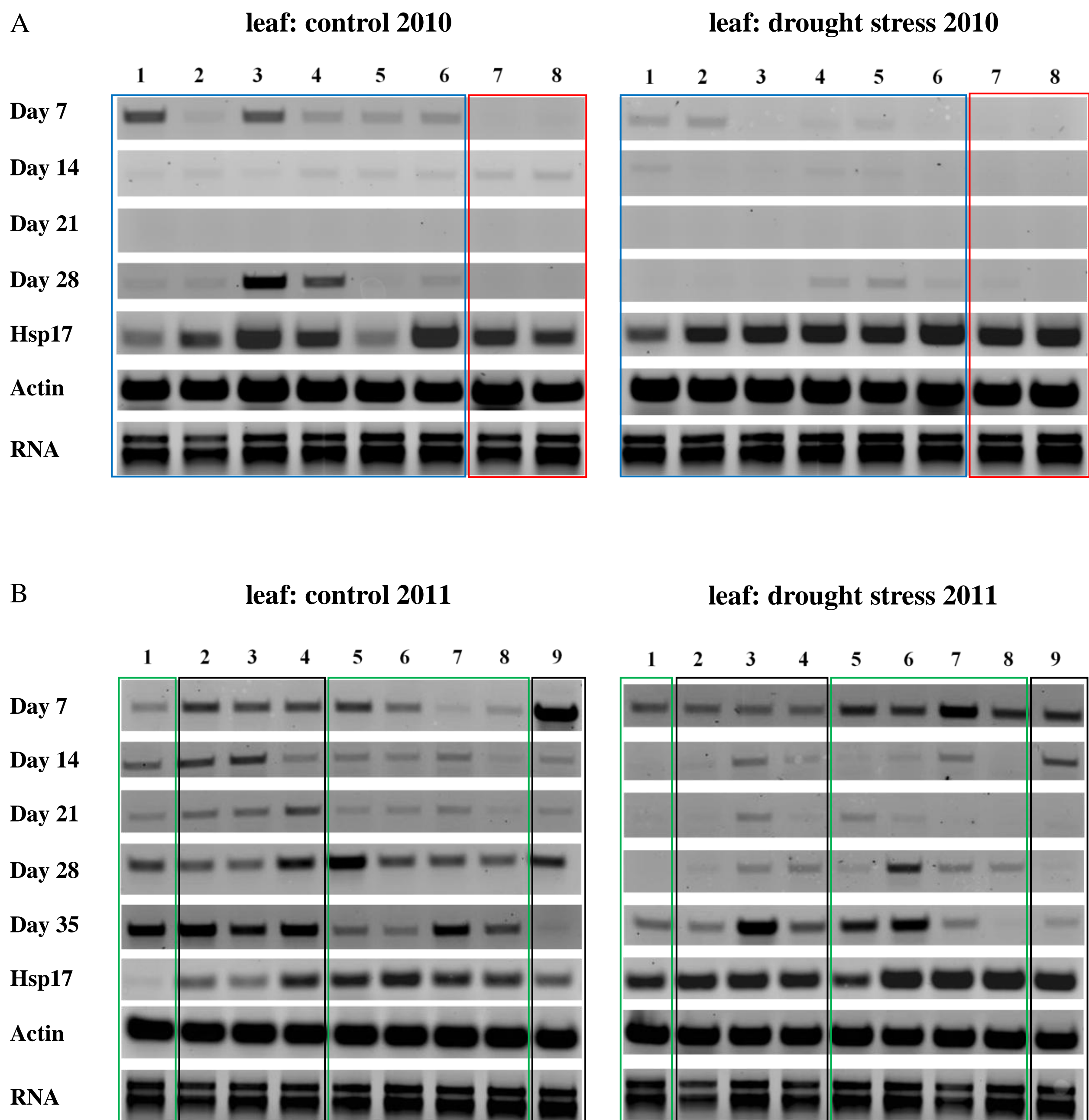


Fig. 16

Fig. 16. Expression analysis of the *HvCBF6* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF6* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

expression was observed in the cultivar *Eunova* during the first week of drought stress experiments. Therefore, these six cultivars composed the early drought responsive barley group (labelled with blue square frames).

3.4.1.5 Expression profiles of the *HvCBF11* gene

The *HvCBF11* expression pattern was obtained by RT-PCR as described in 3.4.1.1. Compared the *HvCBF11* expression pattern in the years 2010 and 2011, a strong down-regulation of the *HvCBF11* gene was observed in 2011 in the first three weeks after exposure to drought, which was also confirmed for *HvCBF1* and *HvCBF3* gene expression. Similar to these two genes, rapid dehydration caused by high temperature also repressed the *HvCBF11* expression. A down-regulation of the *HvCBF11* gene was observed in all nine cultivars after exposure to drought for seven days in 2011 (Fig. 17B). However, the rapid dehydration made a greater impact on the *HvCBF11* gene expression in the cultivars *Barke* and *Eunova* (labelled with black square frames). Its expression was hardly detected in the stressed leaf tissues in the first three weeks after exposure to drought. In addition, the expression of the *HvCBF11* gene did not recover rapidly after the rehydration.

Similar to the *HvCBF1* gene, the *HvCBF11* gene expression in the eight barley cultivars demonstrated timing differences in 2010. The cultivars *Eunova*, *Trumpf* and *Victoriana* responded to drought seven days later than another five cultivars. Therefore, these three cultivars composed the late drought sensitive group, in which changes in *HvCBF11* gene expression were detected after exposure to drought for 14 days. Only a slight up-regulation of the *HvCBF11* gene was observed in these three cultivars (labelled with blue square frames). In contrast, changes in *HvCBF11* gene expression could be already detected in the cultivars *Barke*, *Heils Franken*, *Isaria*, *Pflugs Intensiv* and *Wiebke* after seven days exposure to drought. These five cultivars comprised the early drought responsive group. Depending on whether the *HvCBF11* expression was down- or up-regulated, the early drought responsive cultivars could be subdivided into two subgroups. The cultivars *Barke*, *Heils Franken* and *Isaria* represented the subgroup I and displayed a down-regulation of the *HvCBF11* gene after

exposure to drought for seven days (labelled with yellow square frames). The cultivars *Pflugs Intensiv* and *Wiebke* belonged to the subgroup II. An up-regulation of the *HvCBF11* gene was observed at the identical time point as shown in the subgroup I and reached its maximal expression level (labelled with red square frames).

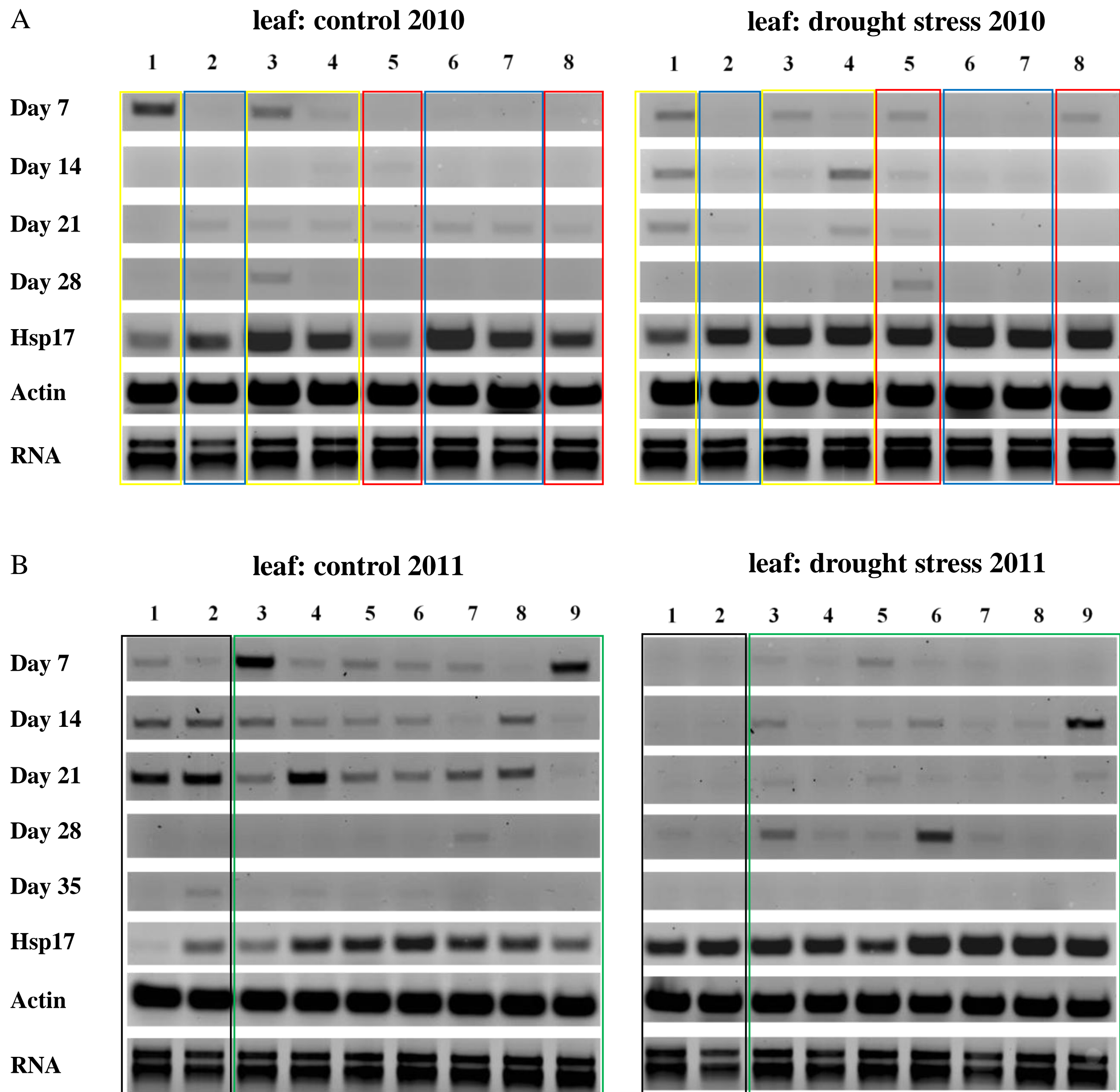


Fig. 17. Expression analysis of the *HvCBF11* gene in different barley cultivars by RT-PCR. (A) The expression of the *HvCBF11* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in 2010 and (B) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

3.4.1.6 Constitutive expression of the *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes

The expression of the *HvDRF1.1*, *HvDRF1.3* and *HvDREB1* genes in leaf tissues was analyzed in 2010 and 2011 by RT-PCR. Unlike the above-analyzed *HvCBF* genes, a strong expression of the *HvDRF1.1*, *HvDRF1.3* and *HvDREB1* genes was observed in both control and stressed plants during the whole drought stress treatments in 2010. No differences in the expression level were present between the control and stressed plants in these three genes (Fig. 18A, C and E). These results were reproducible on independent RNA preparations in 2011 (Fig. 18B, D and F). Therefore, the *HvDRF1.1*, *HvDRF1.3* and *HvDREB1* genes were constitutively expressed upon to drought stress.

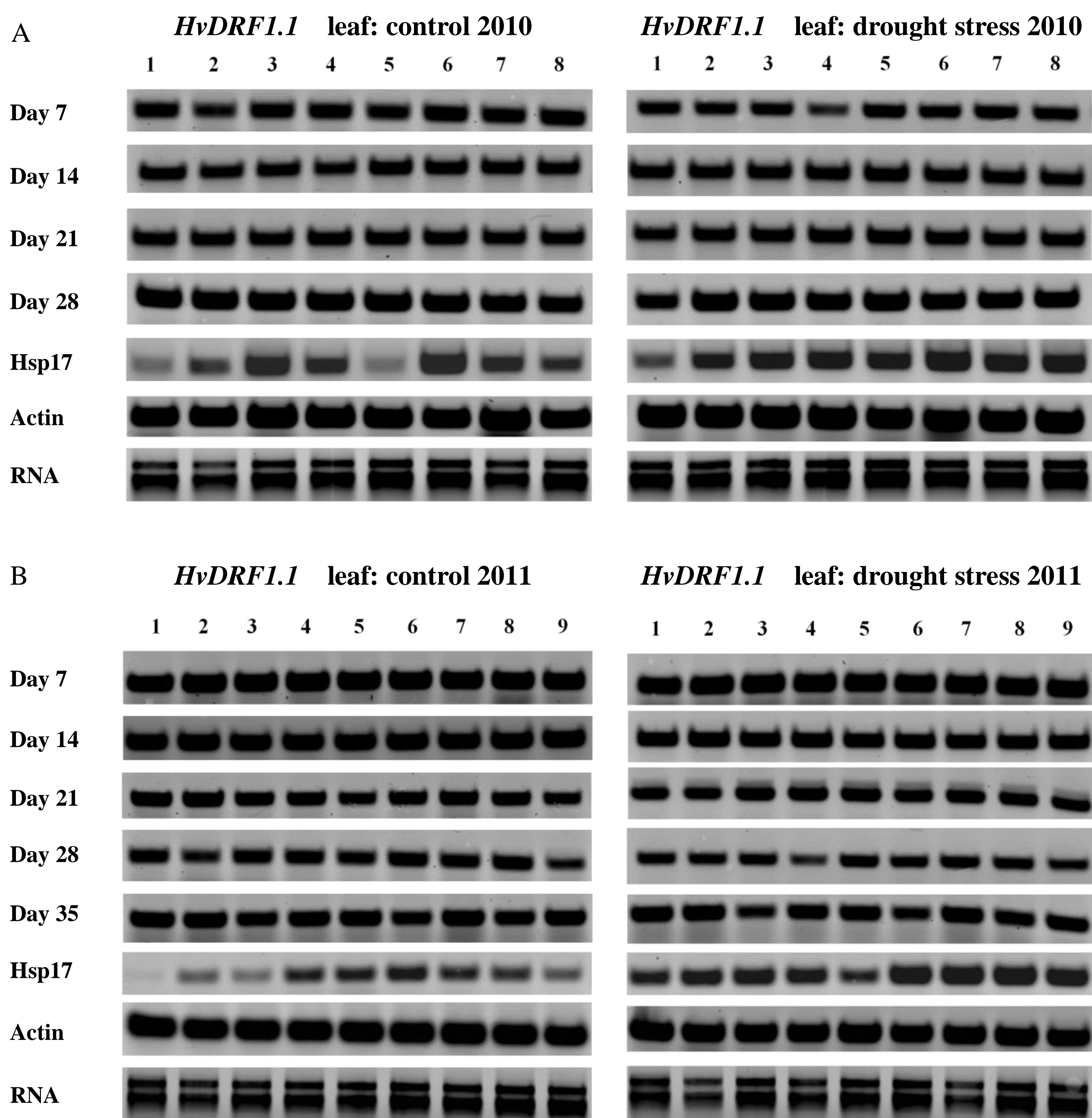


Fig. 18

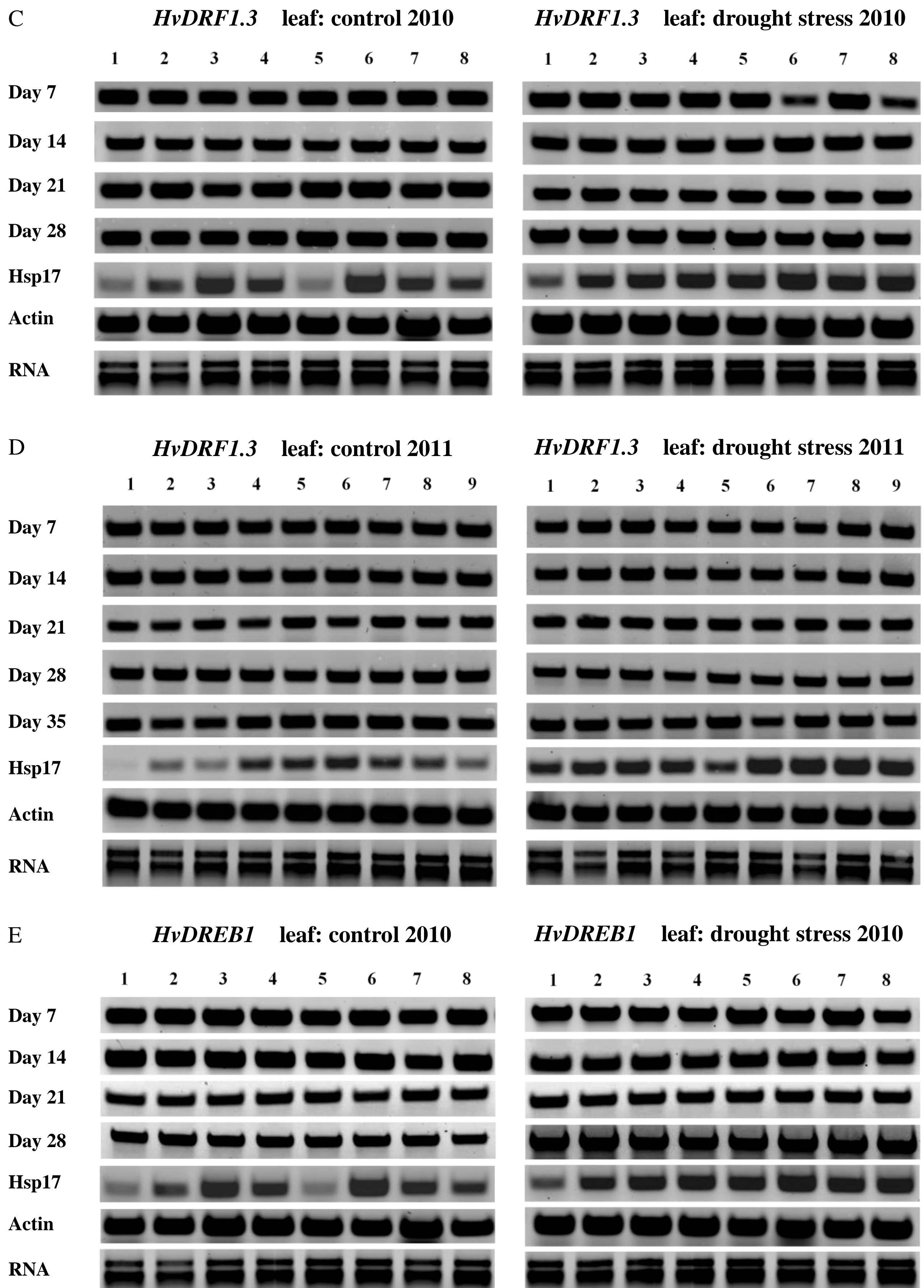


Fig. 18 continued

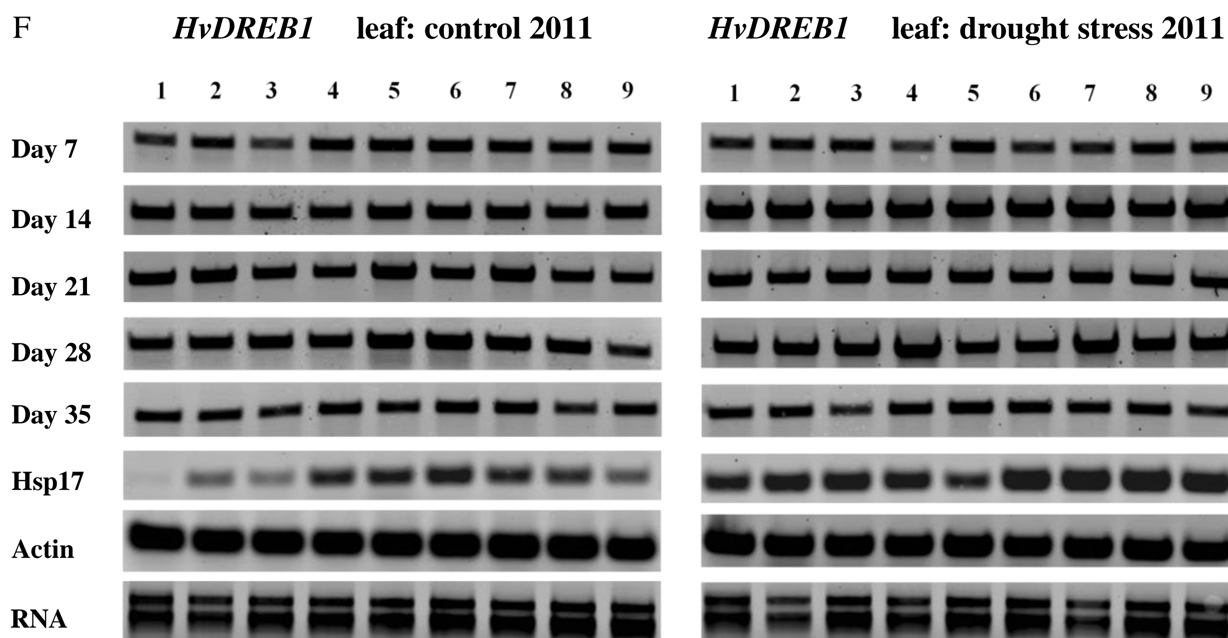


Fig. 18. Expression analysis of the *HvDRF1.1*, *HvDRF1.3* and *HvDREB1* genes in different barley cultivars by RT-PCR. (A) Expression of the *HvDRF1.1* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in the years 2010 and (B) 2011. (C). Expression of the *HvDRF1.3* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in the years 2010 and (D) 2011. (E). Expression of the *HvDREB1* gene in leaf tissues of barley plants grown under well-watered and progressive drought stress in a plastic tunnel in the years 2010 and (F) 2011. The control plants were watered regularly, while water was progressively withheld for drought-treated plants until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. Then the stressed plants were grown at permanent wilting point for another seven days. After the drought stress treatments, the plants were rewatered for one week. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

3.4.1.7 Tissue-dependent gene expression

In 2011, the influence of drought stress on barley *CBF/DREB* gene expressions was analyzed in leaf and root tissues from nine different barley cultivars. The expression level of *HvCBF1*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* varied in leaf and root tissues in nine different barley cultivars after exposure to drought for seven days (Fig. 19). The *HvCBF1*, *HvCBF3*, *HvCBF6* and *HvCBF11* genes displayed a higher expression level in root tissues than in leaf tissues. Their maximal expression level was observed in the stressed root tissues. Moreover, the expression of the *HvCBF1* and *HvCBF3* genes was down-regulated in the stressed leaf tissues, while an up-regulation of these two genes was recorded in the stressed root tissues. Unlike *HvCBF1* and *HvCBF3*, the *HvCBF6* genes were differentially expressed in leaf tissues of nine barley cultivars. Although an up-regulation of the *HvCBF6* genes in the leaf tissues of certain cultivars, its expression was in most cases down-regulated (described in 3.4.1.5). Furthermore, the *HvCBF6* expression was up-regulated in the stressed root tissues and

reached its maximal expression level. Similar to the *HvCBF6* expression pattern in leaf tissues, two different *HvCBF11* expression patterns were recorded in root tissues of nine different barley cultivars. Its expression was down-regulated in the cultivars *Isaria*, *Trumpf* and *Apex*, while an increased expression of the *HvCBF11* genes was observed in the other six barley cultivars. Compared to the root tissues, the *HvCBF11* expression was relatively low in leaf tissues of the control plants, except in the cultivars *Heils Franken* and *Apex*. Furthermore, the *HvCBF11* expression was reduced to a very low level after exposure to drought and it could hardly be detected. In contrast to the above-mentioned four genes, *HvCBF4* showed a higher expression level in leaf tissues than in root tissues.

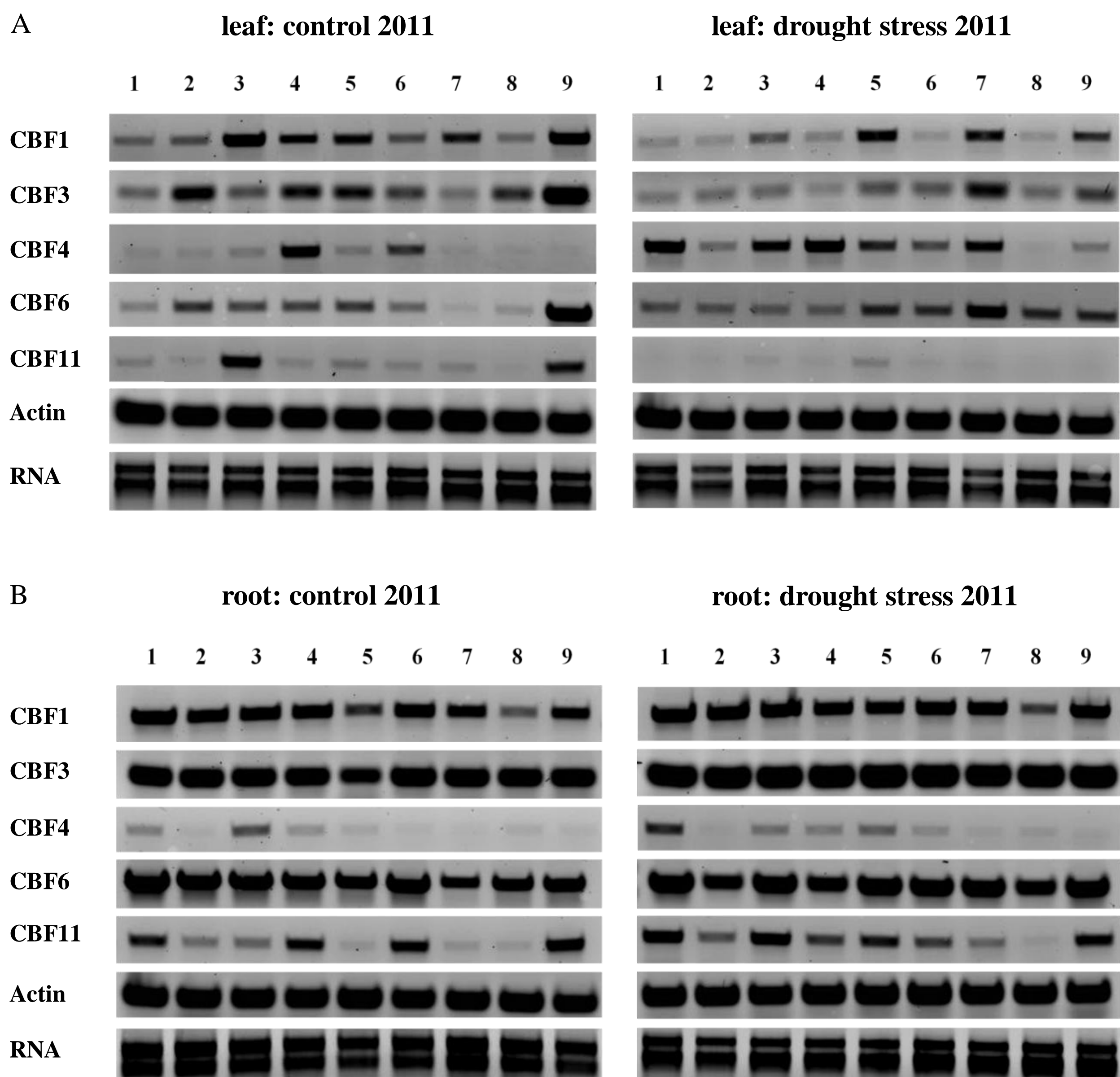


Fig. 19

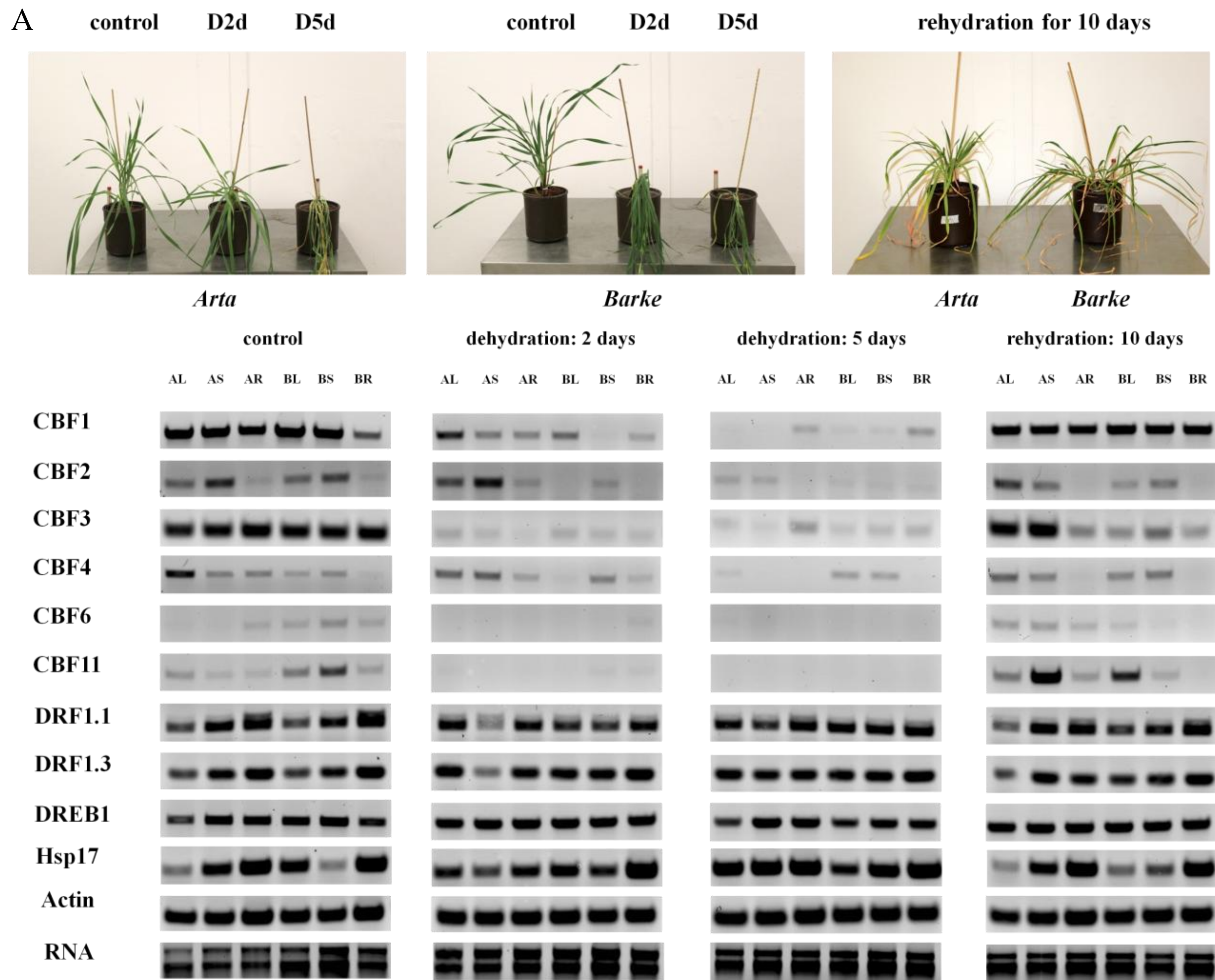
Fig. 19. Tissue-dependent *CBF/DREB* gene expression under progressive drought stress in different barley cultivars. (A) Expression of the *HvCBF1*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes in leaf tissues and (B) root tissues of barley plants after exposure to progressive drought stress for seven days in a plastic tunnel in 2011. Results were obtained from the following cultivars: 1.*Barke*; 2. *Eunova*; 3.*Heils Franken*; 4. *Isaria*; 5. *Pflugs Intensiv*; 6.*Trumpf*; 7. *Victoriana*; 8. *Wiebke*; 9. *Apex*.

3.4.2 Expression analysis of the barley *CBF/DREB* genes under drought stress conditions in the climate chamber

We have observed that rapid dehydration caused by high temperature had a negative influence on the *CBF/DREB* gene expression, including the *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes (described in 3.4.1). To validate these observations, rapid dehydration assays were performed by withholding watering barley plants grown in a climate chamber with constant temperature, humidity and light intensity. The cultivars *Arta* and *Barke* were selected for this study. The plants were grown in artificial granular substrate (Lamstedt Ton; LENI Gebr. Lenz GmbH, Bergneustadt, Germany), which could not hold water very well and a rapid dehydration was easily achieved by stopping the watering. Both control and stressed plants were watered regularly with nutrient solution (WUXAL Universaldünger, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany) before the stress experiments started. Then the control plants were watered normally and the drought-treated plants were stressed by withholding water for 5 days. For rehydration, the stressed plants were rewatered with the identical nutrient solution for 10 days until they had recovered. After exposure to drought for two days, the leaves of the cultivar *Arta* were unfolding in the stressed plants, while they were wilting in the *Barke*. With prolonged drought, no significant differences were observed in the stressed leaf tissues between the two cultivars on day 5 (Fig. 20A). To quantify the water balance in the leaf tissues, RWC was determined. After exposure to drought for two days, the leaf RWC was reduced to nearly 85% in the stressed *Arta* plants, while this value was about 55% in the cultivar *Barke*. However, it declined to nearly 25% in both cultivars on day 5. Moreover, the cultivar *Arta* showed a higher RWC value than *Barke* after rehydration (81% instead of 77%). Altogether, the leaf RWC assays revealed that the cultivar *Barke* was more susceptible to drought than the cultivar *Arta* (Fig. 20B).

The expression of the nine barley *CBF/DREB* genes were analyzed in the leaf, stem and root tissues by RT-PCR. In the cultivar *Barke*, a faint expression of the *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes were observed in all stressed tissue types after withholding water for two and five days, while the similar expression patterns were present in the cultivar *Arta* only after exposure to drought for five days. It should be noted that the *HvCBF6* and *HvCBF11* expression levels were relatively low in control plants of both

A



B

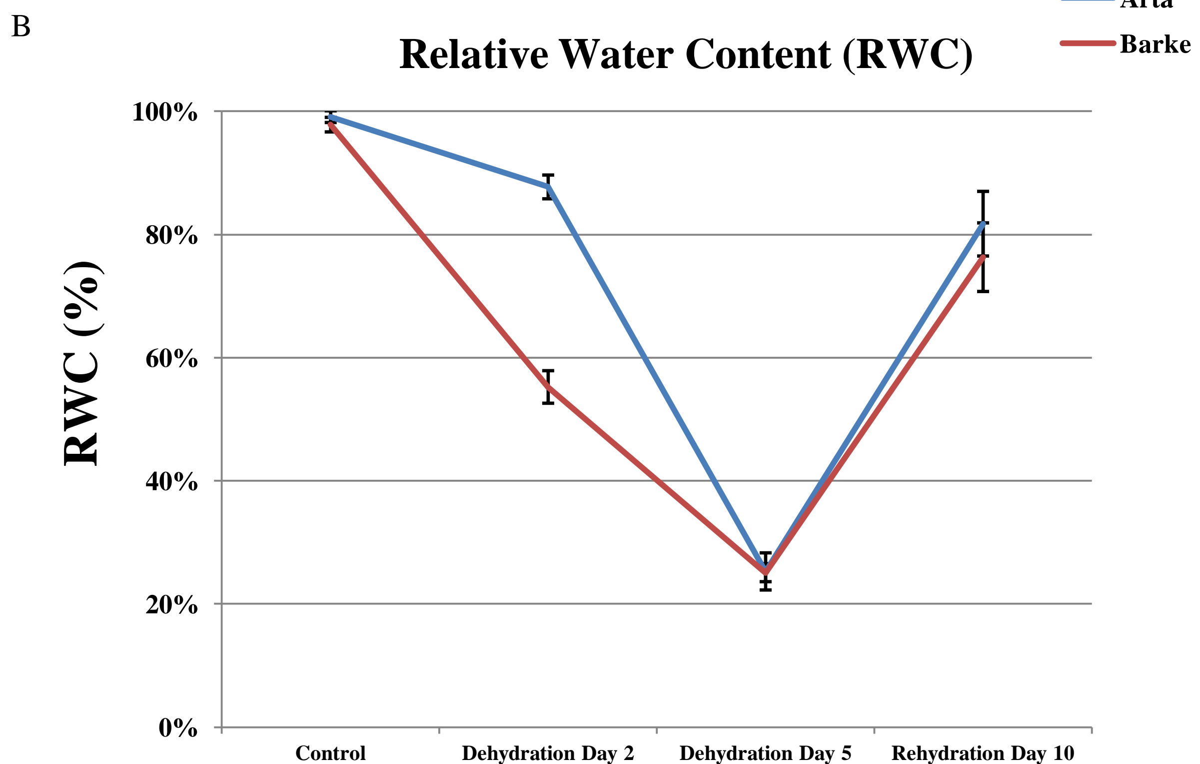


Fig. 20

Fig. 20. Expression analysis of the barley *CBF/DREB* genes under progressive drought stress in the climate chamber. (A) Expression analysis of the *CBF/DREB* genes in the barley cultivars *Arta* and *Barke* by RT-PCR. (B) Determination of the relative water content in the leaf tissues at different time points. Error bars represent standard deviations of an average of three independent measurements. AL: *Arta* leaf tissues; AS: *Arta* stem tissues; AR: *Arta* root tissues; BL: *Barke* leaf tissues; BS: *Barke* stem tissues; BR: *Barke* root tissues; D2d: Dehydration day 2; D5d: Dehydration day 5

cultivars and their expression was hardly detected in all tissues after exposure to drought. Unlike the above-mentioned two genes, a strong expression of the *HvCBF3* gene was observed in control plants of both cultivars. However, the *HvCBF3* gene expression was reduced to a low level in stressed plants after exposure to drought. It could hardly be detected in the leaf and stem tissues of the cultivar *Arta* after exposure to drought for five days. The negative influence of rapid dehydration on barley *CBF* gene expression was best demonstrated for the *HvCBF1* and *HvCBF4* genes. As shown in Fig. 20B, *Arta* possessed higher RWC than *Barke* during the early stage of drought stress (day 2), while this value was reduced to the same level at the end of the drought treatment (day 5). It indicated that *Barke* suffered from faster dehydration than *Arta* in the early stage of the drought stress. However, this process could be observed in *Arta* during the late drought stress procedure. Corresponding to these two different drought stress procedures, the repression of the *HvCBF1* and *HvCBF4* gene expression varied in both cultivars. During the early stage of drought stress, the expression level of the *HvCBF1* and *HvCBF4* genes was much higher in *Arta* than in *Barke*. In contrast, their expression was dramatically reduced in the cultivar *Arta* during the late drought stage. The *HvCBF1* expression was hardly detected in leaf and stem tissues in *Arta* after exposure to drought for five days. A similar phenomenon was observed in *HvCBF4* gene expression in the stem tissues of *Arta*. Compared to *Barke*, *Arta* is a drought-tolerant barley cultivar (Baum *et al.*, 2003). However, its superiority in drought tolerance was countervailed by a rapider dehydration procedure. All these observations revealed that rapid dehydration led to a reduction of the *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* transcripts.

3.5 Isolation of *CBF/DREB* promoters from barley

As described in 3.4.1 and 3.4.2, rapid dehydration led to a reduction of the *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* transcripts. Previous studies have revealed that promoter hypermethylation could repress the expression of the corresponding gene (Soppe *et al.*, 2000; Berdasco *et al.*, 2008; Suzuki and Bird, 2008). Moreover, it has also been shown that DNA methylation plays an important role in plant stress responses (see 1.4). In this study, we found that the cultivars *Heils Franken* and *Wiebke* showed completely different expression

patterns of the *HvCBF1* gene after exposure to drought for seven days in 2010. An up- and a down-regulation were observed in the cultivars *Wiebke* and *Heils Franken*, respectively (3.4.1.1A). The similar expression patterns were also recorded for *HvCBF3* in leaf and root tissues from several barley cultivars (see 3.4.1.7). One explanation for such phenomenon would be a differential hypermethylation pattern in the corresponding promoter. However, these promoter sequences were unknown in barley. The goal of this study was to isolate the promoter of the *HvCBF1* and *HvCBF3* genes from barley.

3.5.1 Structure and sequence analysis of the *HvCBF1* promoter

The *HvCBF1* promoter of the cultivar *Apex* was isolated from the barley genomic DNA using the promoter specific primer pairs following the manual of GenomeWalker™ Universal Kit (Clontech, Saint-Germain-en-Laye, France). The isolated *HvCBF1* promoter was 823 bp in length. Nucleotide sequences of the obtained *HvCBF1* promoter are shown in Fig. 21. The recognition sequences for the restriction enzyme *Hpa*II (*Msp*I) are labelled with yellow square frames. To identify putative transcription factor binding sites and conserved plant *cis*-acting regulatory elements, the *HvCBF*1 promoter sequences were analyzed using the PLACE database (Prestridge, 1991; Higo *et al.*, 1999). Sequence analysis showed that a TATA-box was located 95 bp upstream from the translation initiation start codon ATG (Fig. 21). The

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  1 ACTATAGGGC ACGCGTGGTC GACGGCCCGG GCTGGTAACT AATAATGGTT TGTGCAAAAC
 61 ATTAATCATG ACATATGCAA ATTTGACCGG AAAGGAGCAA AAAGAACTCA TCGAGTCTTG
121 TTGGGTCACT CCGTTGAACA GGTCGTGCAC AACTCGTGCG ATGCCGGTCC CGTGCTTGTC
181 CGCGTCTGAA CGAGCTTTGA CGACTAACAT ACATCCACCG ACGACATCTG CGTTGGCGAA
241 AAGCTCTTCG AAATTCCTCA AACGGCCGTC AGAACCCCCT CTGTCCCAAC CTGGTCCAAC
301 AATGCGATCC TTGTCAACGG CTTGATGGAC GGGGTGCAGG GTCCTGGGTG CGACGACGGG
361 GGAGGGGGGA GGGTCACTTG GAAGCAGTAT TTGGGTACAA ATAGAATCAA CAGTCCTCTT
421 TTATTGACAG AAAATCACAC TATCCCACAG CGAGCTTTCT CTTTCAAGGC ACGTTGCTGT
481 CACAACTCGC AAGAGAGCCC CGGAGCACAG CAGACTGACC CAACCAGCCG GGCCCTCATC
541 CGTCCCATGC CTGCGCGTCG CGTGGGTCTC CATCCGCACC CTCGCGAGCT CCAGCTGGTT
601 CCGACCCAGT TAACGCCCCC GAGCCCAGCA CCGTCCCGAC CCATCAAGTC GCGCTACCTG
661 GCTGGCGCCT CCGGTGGCAC ACCGCGTTGA GCCGTGCACG CGGGGACGTC GCGCTCCACG
721 CGCTAAACTA TATAAACCGC CACCCTTCCC CTCCAAATCC AAACCACCTG CTTCCACTCC
781 CTCCCCCTGC TTACACTCCA GCAGCCAGCA TAGCCAGGCA GCCATGGACG TTGGT
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Fig. 21. Partial sequences of the *HvCBF1* promoter. The recognition sequences for the restriction enzyme *Hpa*II (*Msp*I) are labelled with yellow square frames. The TATA-box is labelled with a green square frame and the translation initiation start codon ATG is labelled in red.

Table 13. Putative *cis*-acting regulatory elements identified in the *HvCBF1* promoter by *in silico* analysis using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Element	Position (Strand)	Sequences	Description	References
ABRECE1HVA22	671 (--)	TGCCACCGG	CE1(coupling element 1) of barley HVA22 gene; possible binding site for nuclear bZIP protein; ABA responsive complex consists of a G-box, namely ABRE3 and CE1;	(Shen and Ho, 1995; Busk and Pages, 1998; Casaretto and Ho, 2003)
ABRELATERD1	470 (--)	ACGTG	ABRE-like sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in *Arabidopsis*;	(Simpson *et al.*, 2003; Nakashima *et al.*, 2006)
ABREOSRAB21	702 (--)	ACGTSSSC	"ABA responsive element (ABRE)" of wheat Em and rice rab21 genes; Proposed consensus sequence for the repeated motif (Em1a and Em1b) of wheat Em gene; S=C/G	(Marcotte *et al.*, 1989; Skriver and Mundy, 1990; Busk and Pages, 1998)
CAAT-Box	300 (+) 423 (--)	CAAT	common *cis*-acting element in promoter and enhancer regions	(Shirsat *et al.*, 1989)
CIACADIANLELHC	300 (+)	CAANNNNATC	Region necessary for circadian expression of tomato Lhc gene; N=A/G/C/T;	(Piechulla *et al.*, 1998)
DRECRTCOREAT	217 (+)	RCCGAC	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) cis-acting element found in many genes in plants; R=G/A;	(Dubouzet *et al.*, 2003; Qin *et al.*, 2004; Diaz-Martin *et al.*, 2005; Suzuki *et al.*, 2005)
MYB2CONSENSUSAT	315 (+) 131 (--) 607 (--)	YAACKG	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in *Arabidopsis*; Y=C/T; K=G/T;	(Abe *et al.*, 2003)
MYCCONSENSUSAT	72 (+) 225 (+) 375 (+) 592 (+) 765 (+) 72 (--) 225 (--) 375 (--) 592 (--) 765 (--)	CANNTG	MYC recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in *Arabidopsis*; Binding site of ATMYC2 (previously known as rd22BP1); N=A/T/G/C; MYC recognition sequence in CBF3 promoter; Binding site of ICE1 that regulates the transcription of CBF/DREB1 genes in the cold in *Arabidopsis*.	(Abe *et al.*, 2003; Chinnusamy *et al.*, 2003; Hartmann *et al.*, 2005; Lee *et al.*, 2005; Oh *et al.*, 2005; Agarwal *et al.*, 2006a)

The orientation of elements is indicated (+, forward; -, reverse).

analysis of the *HvCBF1* promoter revealed that numerous stress-related *cis*-acting regulatory elements are present in the *HvCBF1* promoter including three ABRE elements and one DRE/CRT element. One circadian element, two CAAT-boxes, three MYB and ten MYC recognition sequences are also identified in the *HvCBF1* promoter and are listed in Table 13.

3.5.2 Structure and sequence analysis of the *HvCBF3* promoter

The *HvCBF3* promoter of the cultivar *Apex* was isolated from the barley genomic DNA using the promoter specific primer pairs following the manual of GenomeWalker™ Universal Kit (Clontech, Saint-Germain-en-Laye, France). The isolated *HvCBF3* promoter was 553 bp in length. Nucleotide sequences of the *HvCBF3* promoter are shown in Fig. 22. The recognition sequences for the restriction enzyme *Hpa*II (*Msp*I) are labelled with yellow square frames. The putative *cis*-acting regulatory elements were identified using the PLACE database as described in 3.5.1. Several consensus eukaryotic regulatory domains such as a TATA-box, three ABRE elements and two DRE/CRT elements are present in the *HvCBF3* promoter (Table 14). The TATA-box is located 139 bp upstream from the translation initiation start codon ATG and labelled with a green square frame (Fig. 22). Similar to the *HvCBF1* promoter, one circadian element, five CAAT-boxes, one MYB and six MYC recognition sequences are also identified in the *HvCBF3* promoter and are listed in Table 14.

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  1 ACTATAGGGC ACGCGTGGTC GACGGCCCGG GCTGGTATCA TGGAAGCATT TGGAAGATGG
 61 TATCACCAGA TACTCTCCCT CTCGGGGAAC AAGACGACCT CATTCAGCAG TGACCGCTGT
121 CTTCTCTTTC TGGCCGATCA GCCAGCGGAC CAATCAGGCA AGGCAATCAC CGCTGCATTA
181 ACACTGTTAA GCCAGAAGAA AGTTCGCTTT TTTCTTTTTC TTTTGAGAGG AGCAGGAAGT
241 TGCCTTTTTT GCTTAACACT GCAATGCCAA AAGCCCCCAC ACGCCCAGCA GGAGAAAAGT
301 CTCATGAACA CCACTTGATT TCATCCCATT GTCACCAGCT GTCCGGACAC CGCATCCCTA
361 CCGCCGTCCC AAGCGCGTTC ATACACTTCA ACCTCCAACA CCACGCATAC CTATAAATAT
421 GTCTCCCACA CTCTCGCTCA AGCTCAAGAA ATCATCTCAC ACTCCTCAGT CCTCAGTAAG
481 CTCAAGCACC AAGCTCAGAC TGTTCAAGAA GGAAGCCGCC TGCCAATCAC CCAGCACTCT
541 GCCGGTAGCC ATG
```

Fig. 22. Partial sequences of the *HvCBF3* promoter. The recognition sequences for the restriction enzyme *Hpa*II (*Msp*I) are labelled with yellow square frames. The TATA-box is labelled with a green square frame and the translation initiation start codon ATG is labelled in red.

Table 14. Putative *cis*-acting regulatory elements identified in the *HvCBF3* promoter by *in silico* analysis using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Element	Position (Strand)	Sequences	Description	References
ABRERATCAL	10 (+) 11 (--) 373 (--)	MACGYGB	"ABRE-related sequence" or "Repeated sequence motifs" identified in the upstream regions of 162 Ca(2+)-responsive upregulated genes; M=C/A; Y=T/C; B=T/C/G;	(Kaplan *et al.*, 2006)
CAAT-Box	151 (+) 164 (+) 262 (+) 524 (+) 328 (--)	CAAT	common *cis*-acting element in promoter and enhancer regions	(Shirsat *et al.*, 1989)
CIACADIANLELHC	159 (+)	CAANNNNATC	Region necessary for circadian expression of tomato Lhc gene; N=A/G/C/T;	(Piechulla *et al.*, 1998)
CBFHV	18 (+) 18 (--)	RYCGAC	Binding site of barley CBF1, and also of barley CBF2; CBF = C-repeat (CRT) binding factors; CBFs are also known as dehydration-responsive element (DRE) binding proteins (DREBs); R=A/G; Y=C/T;	(Xue, 2002; Svensson *et al.*, 2006)
MYBCORE	184 (+)	CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2, both isolated from *Arabidopsis*; ATMYB2 is involved in regulation of genes that are responsive to water stress in *Arabidopsis*; A petunia MYB protein (MYB.Ph3) is involved in regulation of flavonoid biosynthesis; R=A/G; N=A/T/G/C;	(Luscher and Eisenman, 1990; Urao *et al.*, 1993; Solano *et al.*, 1995)
MYCCONSENSUSAT	47 (+) 312 (+) 336 (+) 47 (--) 312 (--) 336 (--)	CANNTG	MYC recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in *Arabidopsis*; Binding site of ATMYC2 (previously known as rd22BP1); N=A/T/G/C; MYC recognition sequence in CBF3 promoter; Binding site of ICE1 that regulates the transcription of CBF/DREB1 genes in the cold in *Arabidopsis*.	(Abe *et al.*, 2003; Chinnusamy *et al.*, 2003; Hartmann *et al.*, 2005; Lee *et al.*, 2005; Oh *et al.*, 2005; Agarwal *et al.*, 2006a)

The orientation of elements is indicated (+, forward; -, reverse).

3.6 DNA methylation assays in the isolated *HvCBF1* promoter

3.6.1 Pre-digestion with *Dra*I significantly increased the resolution of the methylation-sensitive Southern blot in barley.

The methylation status in the isolated *HvCBF1* promoter was analyzed using methylation sensitive Southern blotting. Total genomic DNA from the control samples during the first week of drought stress in 2010 was isolated from the barley cultivars *Barke*, *Eunova*, *Pflugs Intensiv*, *Trumpf* and *Victoriana* then digested with *Hpa*II and *Msp*I, respectively. The putative restriction sites of the enzyme *Hpa*II (*Msp*I) are indicated in the isolated *HvCBF1* promoter in Fig. 22. The digested DNA fragments were blotted to an N^+ nylon membrane and hybridized using a radioactive ^{32}P-labelled PCR product obtained from the isolated *HvCBF1* promoter. The putative restriction sites of *Hpa*II (*Msp*I) as well as the digestion products are indicated in the utilized radioactive probe (Fig. 23A). As shown in Fig. 23C, a smear of high-molecular weight was hybridized to the probe in the *Hpa*II digested samples, which suggested an inefficient digestion by the enzyme *Hpa*II. In contrast, the separation of the *Msp*I digested DNA fragments was much better on the same blot. However, the obtained hybridization signals were also smeary and distributed mainly over 2 kb on the gel. These results suggested on the one hand the occurrence of DNA methylation in the isolated *HvCBF1* promoter and on the other hand an inefficient single digestion with either *Hpa*II or *Msp*I in barley. The incomplete digestion of the DNA with *Hpa*II (*Msp*I) could be due to the large genome size of the DNA. Therefore, a double digestion with the combination of *Dra*I and *Hpa*II (*Msp*I) was performed. *Dra*I recognizes the sequence 5´-TTTAAA-3´and is methylation insensitive. As shown in Fig. 23B, a double digestion with the combination of *Dra*I and *Hpa*II (*Msp*I) increased the resolution of the DNA fragments. Compared to the single digestion with *Hpa*II (*Msp*I), the separation of the digested fragments was well-distributed and the hybridization signals are clear. Differences in *Hpa*II and *Msp*I treated samples were observed, which indicated the occurrences of DNA methylation in the isolated *HvCBF1* promoter.

A

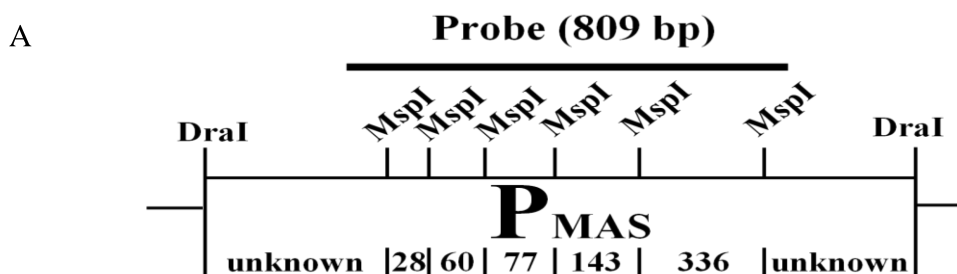

Fig. 23.

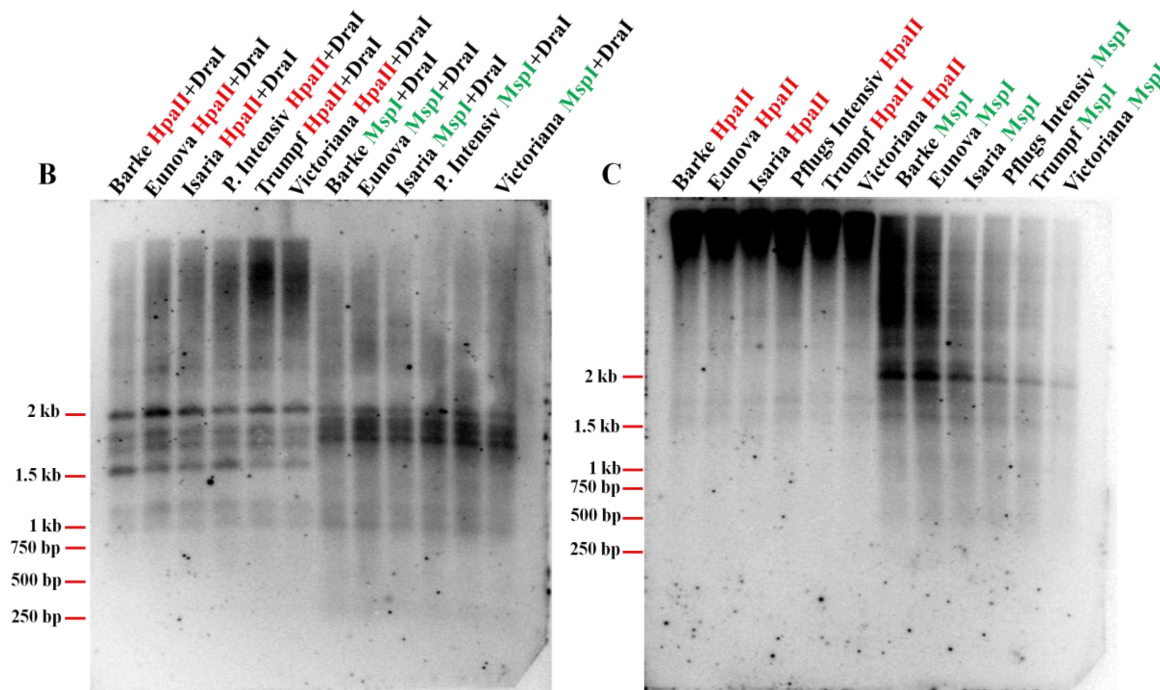

Fig. 23. Southern blot analysis to detect the methylation status of the isolated *HvCBF1* promoter. (A) Putative *Hpa*II (*Msp*I) restriction sites and the obtained digestion products were indicated in the radioactive ^{32}P-labelled probe. (B) Southern blot analysis was performed using double digestion in combination of *Dra*I and *Hpa*II (*Msp*I). (C) Southern blot analysis was performed using *Hpa*II and *Msp*I.

3.6.2 No differences in methylation status were found in the isolated *HvCBF1* promoter between control and stressed barley plants.

As described in the 3.6.1, the occurrence of DNA methylation was observed in the isolated *HvCBF1* promoter in the control samples during the first week of drought stress. The cultivars *Heils Franken* and *Wiebke* showed completely different expression pattern of the *HvCBF1* gene after exposure to drought for seven days in 2010. It could be due to the differences in methylation status of the *HvCBF1* promoter. Methylation sensitive Southern blot was performed as described in the 3.6.1. For this study, total genomic DNAs of the cultivars *Heils Franken* and *Wiebke* were isolated from the control and drought stress treated samples during the first week of drought stress in 2010. As shown in Fig. 24A, the *Hpa*II treated control and drought samples were characterized with four intensive bands of a molecular weight of about 1.4 kb to 2 kb. Two additional bands were also present in the *Hpa*II treated samples with a molecular weight around 1 kb. These two bands were also confirmed in the *Msp*I treated samples. Four characteristic bands with higher molecular weight from about 1.7 kb to 2 kb were observed in the *Msp*I treated samples. One single band with the molecular weight about 1.3 kb was only present in the *Msp*I treated samples. Altogether, the differences in the methylation status of the isolated *HvCBF1* promoter suggested that both cytosines in the

recognition sequences 5´-CCGG-3´ from *Hpa*II (*Msp*I) are methylated. However, a comparison of the methylation status between the control and drought-treated samples in the cultivars *Heils Franken* and *Wiebke* showed that no differences in methylation status are present in the *HvCBF1* promoters. These observations revealed that the hypermethylation in this part of the *HvCBF1* promoter was not responsive for the repression of the *HvCBF1* gene expression.

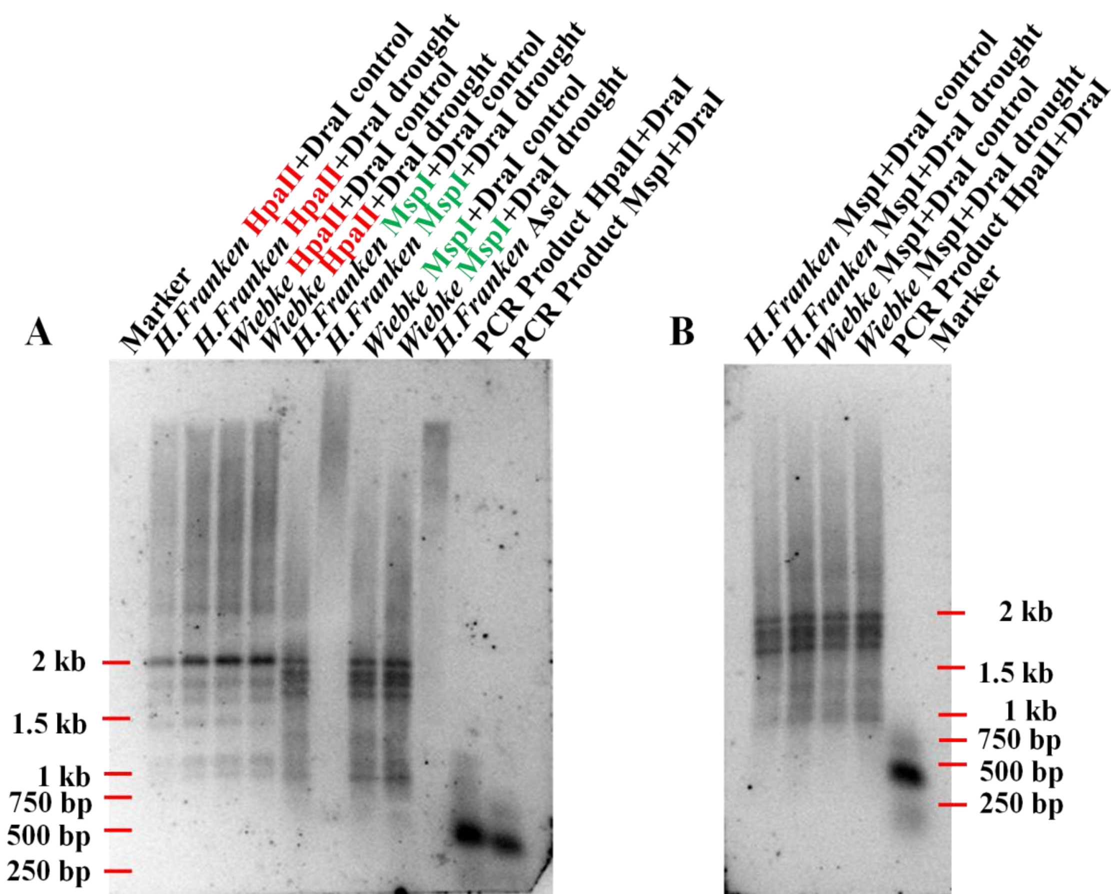

Fig. 24. Analysis of methylation status in the isolated *HvCBF1* promoters from control and drought-treated barley plants. (A) Southern blot analysis was performed using double digestion in combination of *Dra*I and *Hpa*II (*Msp*I) in the cultivars *Heils Franken* and *Wiebke*. Genomic DNAs from the control and drought samples in the first week of drought stress experiment in 2010 were ultilized. (B) Repetition of the methylation sensitive Southern blotting using *Dra*I and *Msp*I.

3.7 Analysis of the root system architecture in barley with a non-invasive method

3.7.1 Optimization of sterilization procedures for barley seeds

The efficiency of different seed surface sterilization methods is shown in Fig. 25. To find the best method, the following criteria were chosen, namely the contamination-, germination- and survival rate. A big challenge of this experiment was to get rid of the contamination. As

shown in Fig. 25, the contamination rate (represented by blue column) was significantly reduced after the removal of husks. The contamination rate declined from over 90% to 10%. This step was therefore considered to the most crucial step of this experiment. In case of germination rate (represented by green column), sterilization with 35% H_2O_2, chlorine gas or 70% ethanol combined with 35% H_2O_2 demonstrated similar germination rate. However, the seed germination was totally inhibited, if they were soaked in sterile ddH_2O overnight before treated with 35% H_2O_2 for 10 min. Besides high germination rate and low contamination rate, the survival rate should also be taken into account. In this case, sterilization with 35% H_2O_2 for 10 min was the best choice. Altogether, the most efficient method for barley seed surface sterilization is using 35% H_2O_2 for 10 min after the removal of husks.

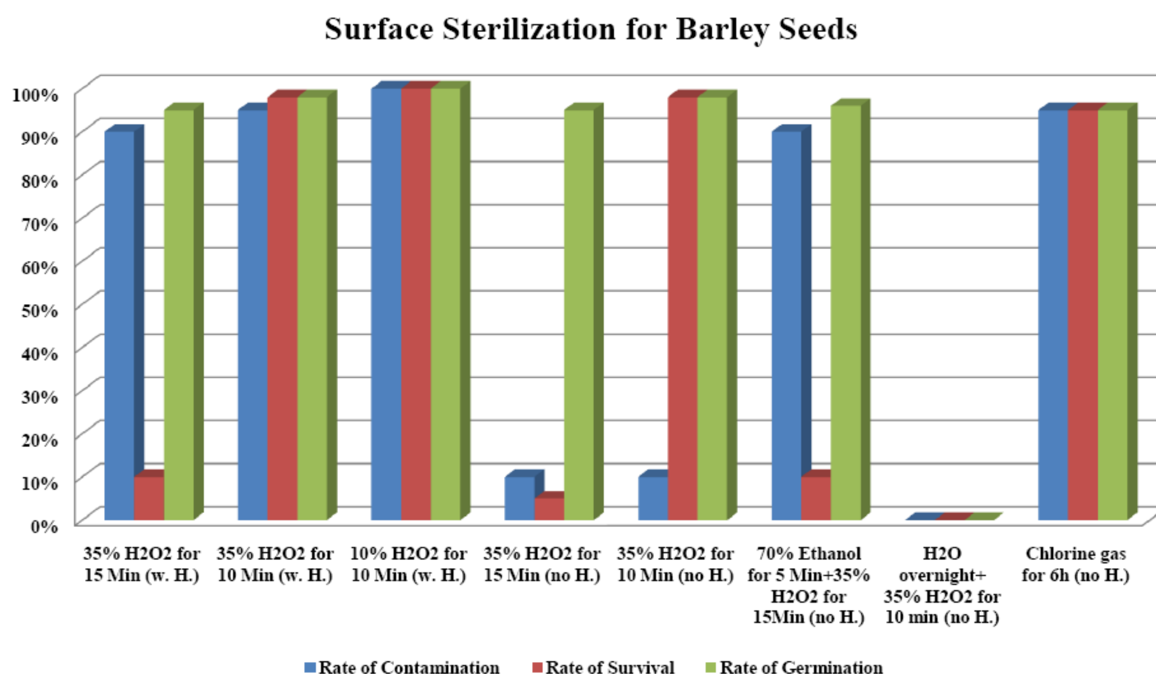

Fig. 25. Optimization of sterilization procedures for barley seeds. The efficiency of different seed surface sterilization methods was demonstrated by contamination-, germination- and survival-rate (in percentage). w. H.: grains with husks; no H.: grains without husks.

3.7.2 Hoagland ́s media were more suitable for the RSA analysis in the cultivar Morex.

In the literature, no standard medium for hydroponic cultivation of barley plants was reported. Hoagland ́s and Yoshida ́s media are successfully used in Prof. Benfey ́s lab for hydroponic cultivation of corn and rice, respectively. Therefore, these media were tested for the cultivation of the variety *Morex* in this study. The root system of 15-day-old and 21-day-old seedlings grown in Hoagland ́s and Yoshida ́s media were shown in Fig. 26. Media with equal volume and identical hardness were used. As shown in Fig. 26A, the root system developed much better in Hoagland ́s media than in Yoshida ́s media. Seedlings cultivated with

Hoagland ́s media possessed a deeper and thicker root system than seedlings cultivated with Yoshida ́s media. In Fig. 26B and 26C, the root system of 21-day-old seedlings was shown in the above-mentioned media. Seedlings grown in Yoshida ́s media had abnormal root shape. Many seminal roots possessed no root hairs, compared to those grown in Hoagland ́s media. Moreover, a shallow root system was observed in the seedlings grown in Yoshida ́s media, whereas seedlings grown in Hoagland ́s media possessed a deep root system. Due to the time limit, only the root growth of the variety *Morex* had being checked so far. It appeared that the variety *Morex* grew better in Hoagland ́s media than in Yoshida ́s media.

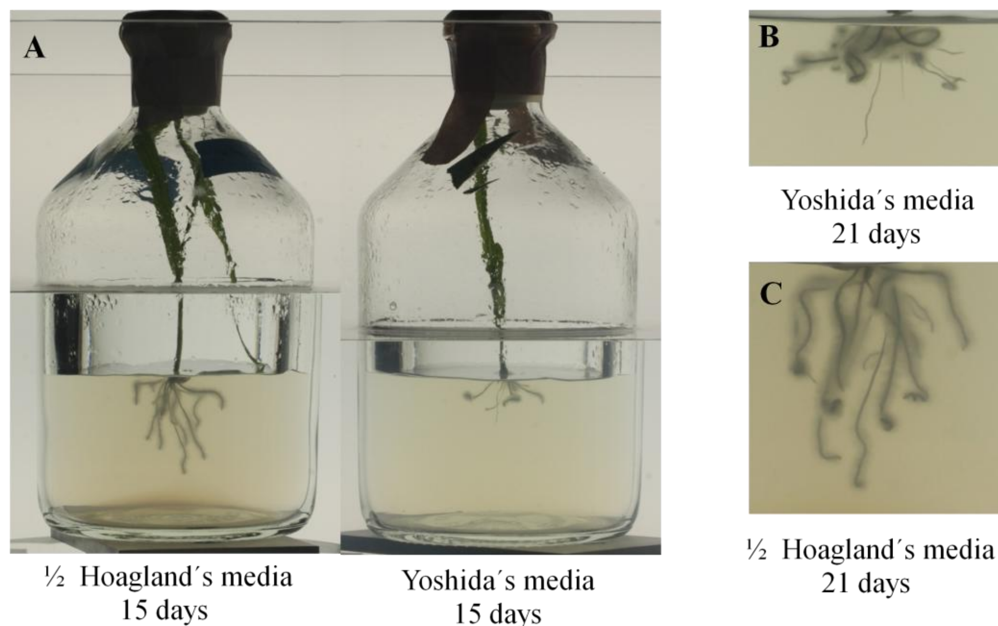

Fig. 26. Comparison of barley ́s root growth in two different hydroponic media. (A) Roots of 15-day-old *Morex* seedlings grown in Hoagland ́s and Yoshida ́s media; Roots of 21-day-old Morex seedlings grown in Hoagland ́s (B) and Yoshida ́s media (C).

3.7.3 Seedlings with 2-3 cm long primary roots and about 1 cm long coleoptiles are most suitable for transfer to the gel system.

After the pre-germination treatment, the germinated seedlings were transferred to the gel system. Seedlings grown in Hoagland ́s media for 17 days were shown in Fig. 27. When the pre-germinated seedlings were plunged deeply in the media, its development was arrested (Fig. 27A). Moreover, the transfer timing was another criterion for a better development of the seedlings. If the seedling was too young, its development was also partially inhibited after transfer. As shown in Fig. 27B, one-day-old seedlings were transferred to the gel system and grown in the Hoagland ́s media for 17 days. A narrow and shallow root system was observed. If the seedlings were too old, the roots had already well developed and the primary root was too long to be transferred into the gel. Moreover, the long primary root was very susceptible

to damage. If the long primary roots were not totally plunged into the gel, they grew only around the gel surface and did not grow downwards into the gel (data not shown). The best timing for transfer was two days after germination. At this stage, the two-day-old seedlings possessed 2-3 cm long primary roots and 1 cm long coleoptiles. As shown in Fig. 27C, two-day-old seedlings were transferred to the gel system and grown in the Hoagland ́s media for 17 days. Compared to the one-day-old seedlings, the two-day-old seedlings developed much better after transfer. A deeper and wider root system has developed. Altogether, seedlings with 2-3 cm long primary roots and about 1 cm long coleoptiles are most suitable for transfer to the gel system.

Fig. 27. Determination of criteria for the seedling transfer. (A) One-day-old pre-germinated seedlings were plunged deeply into the Hoagland ́s media. Its development was arrested. (B) One-day-old seedlings with short primary roots were plunged into the Hoagland ́s media. The roots developed abnormal after 17 days. (C) Two-day-old seedlings with 2-3 long primary roots and 1cm long coleoptiles were transferred into the Hoagland ́s media. The seedlings grew better with normal root development.

3.7.4 Aeration and moisture play an important role in root morphology.

To better observe the root morphology, seedlings were grown in cylinders containing Hoagland ́s media instead of bottles. Seedlings were grown in the same media and under equal conditions. Compared to bottles, the seedlings developed less root hairs in cylinders. Moreover, a deeper and larger root system had being developed in the cylinder than in the bottles (Fig. 28). The only difference in the cultivation conditions was the seal materials for the two different containers. The bottles were sealed with a sponge stopper whereas the cylinders were sealed with a plastic autoclave membrane. As shown in Fig. 28, the moisture content was much higher in the cylinder than in the bottles. A lot of condensation water was

present on the inner wall of the cylinders, whereas the condensation water was hardly observed on the inner wall of the sponge sealed bottles. Such observations suggested better aeration in the sponge sealed bottles. Such differences in the cultivation conditions led to the great diversity in the root morphology. Therefore, the levels of aeration and moisture are critical for the development of the barley root system architecture and root morphology. Aerated conditions with lower moisture content allowed the development of abundant root hairs in barley.

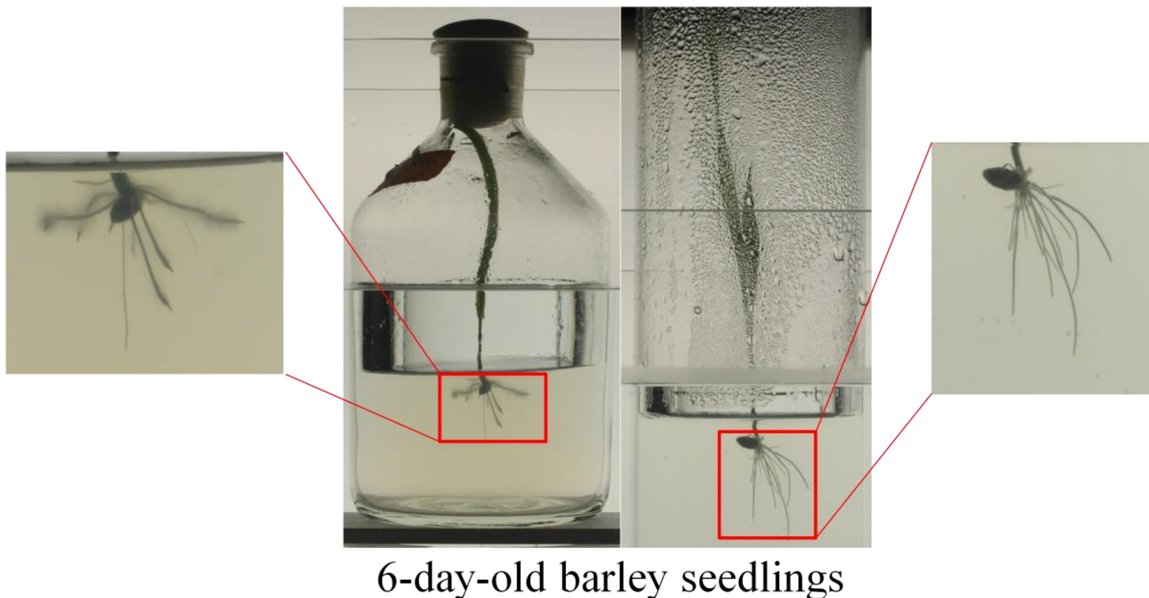

Fig. 28. Root morphology was affected by aeration and moisture content. 6-day-old seedlings were grown in glass bottles and glass cylinders containing the equal volume of Hoagland ́s media under identical growth conditions. The seedlings grown in the glass bottles were better aerated and possessed abundant root hairs whereas seedlings grown in the glass cylinders with high moisture content had less root hairs.

3.7.5 Technical difficulties in image thresholding and reconstructions hampered the further analysis of barley root system architecture.

After imaging, the original images were cropped and converted to a binary format using the GiaRoot software. Forty images of each plant were converted into 2D format. Based on the obtained data, important values were automatically calculated, including the total root length, the root number and the average root width and so on. As shown in Fig. 29A, the barley cultivar *Morex* was hydroponically cultivated for 21 days. Its root system was characterized with seminal roots containing hundreds and thousands of penicillate root hairs. As this root system was converted to the 2D format, the GiaRoot software considered each seminal root with the root hairs to a thick single root (Fig. 29 B). Therefore, the acquired data could not be used to characterize barley root system architecture of the cultivars with abundant root hairs.

Further settings on the sensitivity and the precision of the program are necessary.

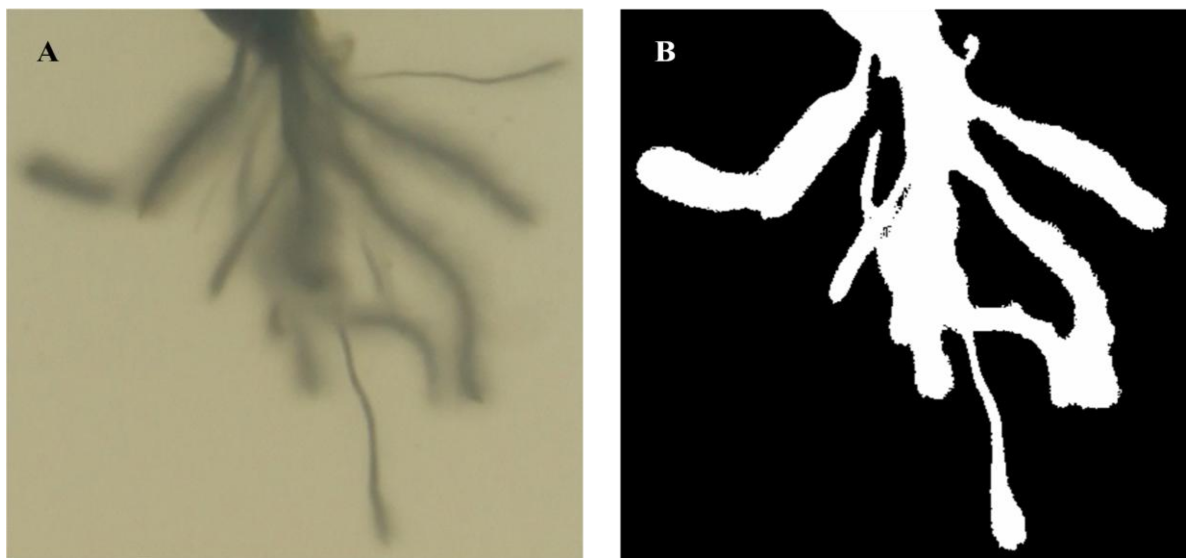

Fig. 29. Conversion of the original root images to 2D format. (A) One of the 40 acquired root images from the cultivar *Morex*; (B). The original image was converted to 2D format using the GiaRoot software. Because of the technical limitation of the software, each seminal root with its root hairs was considered to a thick single root.

4. DISCUSSION

Drought is one of the greatest global environmental constraints for agriculture. The current knowledge about stress-responsive genes in barley and other crops is mainly based on studies carried under controlled laboratory conditions. This information does not often account for the complexity and instability of the environmental factors under field conditions. In this study, we have considered these issues by comparing the expression patterns of stress-related transcription factors *CBF/DREB* in nine different barley cultivars under field conditions. These nine barley cultivars are evolved at different time points. The oldest cultivar *Heils Franken* was released in 1895, while the newest cultivar *Victoriana* originated from 2007. Certain cultivars are genetically related (see 3.2.1). We compared *CBF/DREB* expression patterns under progressive drought conditions in the field to assess the impact of environmental factors on drought responses in these nine barley cultivars at the molecular level.

As described in 1.3, the function of *CBF/DREB*s in response to drought has been well studied in the model plant *Arabidopsis thaliana* and some important crop plants. But the situation in barley is less clear. The published data about *CBF/DREB* transcription factors focused mainly on their functions in cold stress (Choi *et al.*, 2002; Skinner *et al.*, 2005; Francia *et al.*, 2007; Stockinger *et al.*, 2007). A few studies have focused on drought stress responses and were rather conducted under laboratory conditions (Xue and Loveridge, 2004; Oh *et al.*, 2007; Xu *et al.*, 2009). We believe that monitoring the expression of these transcription factors in field grown plants should bring further insights into how plants react to fluctuating environmental conditions, particularly in the actual contest of climatic changes. Moreover, it has been shown in several studies that modulation of the *CBF/DREB* gene expression by transgenic approaches can improve plant stress tolerance (Hsieh *et al.*, 2002; Umezawa *et al.*, 2006; Chen *et al.*, 2007; Oh *et al.*, 2007; Zhao *et al.*, 2007; Xiao *et al.*, 2009; Bihani *et al.*, 2010; Morran *et al.*, 2010; Mallikarjuna *et al.*, 2011). However, detrimental effects due to constitutive expression of *CBF/DREB* transcription factors were recorded in various transgenic plants. These include growth retardation, reduced fruit and seed numbers and fresh weight under normal growth conditions (Hsieh *et al.*, 2002; Umezawa *et al.*, 2006; Chen *et al.*, 2007; Oh *et al.*, 2007; Zhao *et al.*, 2007; Xiao *et al.*, 2009; Bihani *et al.*, 2010; Morran *et al.*, 2010; Mallikarjuna *et al.*, 2011). DNA cytosine methylation has been identified to be involved in regulation of gene expression without any detrimental effects demonstrated in the

transgenic plants (see 1.6). In this study, the effect of DNA methylation in the promoter region on the *CBF/DREB* gene expression was also considered.

4.1 *CBF/DREB* genes show extensive sequence conservation except for a few SNPs among different barley cultivars.

Barley contains a large family of at least 20 *CBF* genes and three *DREB* genes (Xue and Loveridge, 2004; Skinner *et al.*, 2005; Nayak *et al.*, 2009; Xu *et al.*, 2009). In this study, 10 putative drought stress inducible *CBF/DREB* genes were isolated from nine different barley cultivars using gene specific primers. The multiple sequence alignment of the isolated barley *CBF* genes with their cDNAs indicated that no intron sequences were present in the obtained *CBF* homologues (see 3.2.1-3.2.6). In contrast, intron sequences have been identified in the isolated barley *DREB* genes (see 3.2.7-3.2.9). A 96-bp long intron has been newly identified in the isolated barley *DRF2* genes. Furthermore, sequence alignments showed that the coding region of the *CBF/DREB* genes was conserved and the features of the sequences can be summarized in the following four categories:

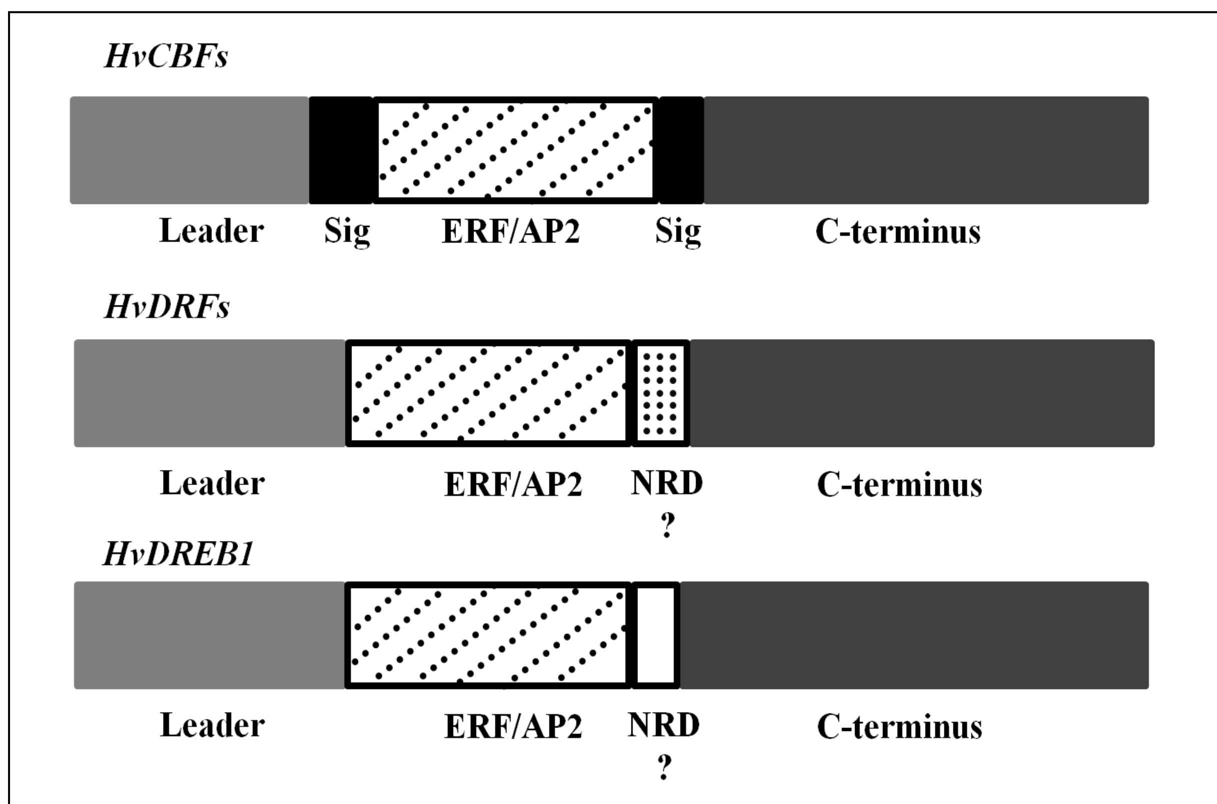

Fig. 30. Domain structure of barley *CBF/DREB genes*. The leader, AP2/ERF and C-terminal domain are noted. In *HvCBFs*, AP2/ERF domain is bracketed with conserved *CBF* signature motifs (Sig), which are indicated as black blocks. Instead of the signature motifs, potential negative regulatory domains (NRD) adjacent to the ERF/AP2 domain were identified in *HvDRFs* and *HvDREB1*, respectively.

i) Highly conserved N-terminus.

The N-terminus of the barley *CBF/DREB* genes defines the region from the ATG translation start codon to the beginning of the AP2/ERF DNA binding domain. This region contains the NLS consensus motif. Only one SNP was found in this region in *HvCBF6* and *HvDREB1*, and two SNPs in *HvCBF3*, *HvDRF1* and *HvDRF2*.

ii) Highly conserved AP2/ERF domain.

In plants, *CBF/DREB* genes were characterized with the AP2/ERF domain, which is a DNA-binding domain containing approximately 60 conserved amino acid residues (Jofuku *et al.*, 1994; Okamuro *et al.*, 1997; Stockinger *et al.*, 1997; Liu *et al.*, 1998; Shen *et al.*, 2003). The AP2/ERF domain is highly conserved in all obtained *CBF/DREB* DNA sequences with the exception of certain SNPs (see 3.2). Previous studies revealed that valine at position 14 (V14) and glutamic acid at position 19 (E19) are conserved in the AP2/ERF domain and play an important role in the determination of DNA-binding specificity (Liu *et al.*, 1998; Sakuma *et al.*, 2002). In this study, V14 was identified in all obtained *CBF/DREB* DNA sequences, whereas E19 was only found in the isolated *HvCBF1*, *HvDREB1* and *HvDRF1* genes. Dubouzet *et al.* (2003) have shown a conserved valine at position 14 but not glutamic acid at position 19 in the *OsDREB1A*, *OsDREB1B* and *OsDREB1D* genes in rice. The divergence in the conservation of amino acids in the DNA-binding domain explains to some extent the difference in the DNA-binding specificity (Liu *et al.*, 1998; Sakuma *et al.*, 2002). Two non-synonymous mutations, phenylalanine108 to serine108 (F108S) and asparagine89 to serine89 (N89S) have been identified in the AP2/ERF domain of *HvCBF2* and *HvCBF3* in the cultivar *Isaria*, respectively (see Fig. 3B and 4B). Because serine is usually a substrate for kinases for phosphorylation, these two non-synonymous mutations may affect the DNA-binding activity of *CBF2* and *CBF3* genes via post-translational modification in the cultivar *Isaria*. This possibility should be further validated using gel mobility shift assays.

iii) Highly conserved *CBF* signature motifs

Previous studies revealed that the AP2/ERF domain of the *CBF* genes is bracketed with conserved *CBF* signature motifs PKK/RPAGRxKFxETRHP and DSAWR in *Arabidopsis*, rapeseed, wheat, rye, and tomato (Jaglo *et al.*, 2001). Similar motif structures were also described by Skinner *et al.* (2005) for barley. The signature

motifs are generally conserved in the barley *CBF* genes isolated in this study. A few variations were however seen for some genes. These could be summarized as follows: P/AKRxAGRT/IKxxETRHP and DSAWR (*HvCBF2* and *HvCBF4*) vs. DSP (*HvCBF1*) vs. DSAW (*HvCBF3*) vs. DSAE (*HvCBF6*) vs. DSE (*HvCBF11*) (see Fig. 2B-7B and 30). The function of these motifs is still unknown. These motifs were not present in the isolated *HvDREB1*, *HvDRF1* and *HvDRF2* genes (see Fig. 8B, 9B, 9C, 10B and 30).

iv) highly conserved Ser- and Thr-rich region in *HvDRF*s

A Ser- and Thr-rich 98-amino acid region, which is located immediately downstream of the AP2/ERF domain, was identified in *HvDRF1.1*, *HvDRF1.3* and *HvDRF2*. Comparative sequence analysis showed that this region was highly conserved among the above-mentioned *HvDRFs* (see Fig. 9B, 9C, 10B and 30). Another Ser- and Thr-rich 53-amino acid region adjacent to the AP2/ERF domain was identified in *HvDREB1* (see Fig. 8B). The function of these domains is still unclear. Similar domains have been identified in *AtDREB2A* and *GmDREB2A*. The functions of these genes have also been investigated (Sakuma *et al.*, 2006; Mizoi *et al.*, 2012). In *Arabidopsis*, deletion of the negative regulatory domain (NRD) of *AtDREB2A*, a Ser- and Thr-rich 30-amino acid region adjacent to the AP2/ERF domain, led to a constitutive expression of *AtDREB2A* and conferred tolerance to drought stress (Sakuma *et al.*, 2006). Similar results have been shown by Mizoi *et al.* (2012) in soybean. Whether the identified Ser- and Thr-rich regions also correspond to negative regulatory domains of barley *HvDRF*s and *HvDREB1* should be further validated using deletion mutants (deletion of the Ser- and Thr-rich region) and comparing the transactivation activity of these mutants with wild-type protein in barley protoplasts.

v) conserved C-terminus

Comparative sequence analysis of the C-terminus reveals that the C-terminus regions of the isolated *CBF/DREB* genes have a moderate level of sequence similarity among different barley cultivars. Numerous SNPs are present in this region (Fig. 2A-10A). The percentage of genetic divergence has been conducted using the p-distance model and listed in Table 3-11. As shown in Table 3-11, a high percentage of genetic divergence has been recorded for the cultivar *Arta*, which is well-known for its high drought tolerance (Li *et al.*, 2006a; Hossain *et al.*, 2012). Whether such SNPs are important for drought tolerance is unclear.

However, trait-associated SNPs have been reported in several important crops. In rice, a C/A transversion in the second exon of the *GS3* (*grain size 3*) gene resulted in enhanced grain length in *Oryza sativa* but not in other Oryza species (Fan *et al.*, 2009). In maize, a phenylalanine insertion in DGAT1-2 (an acyl-CoA: diacylglycerol acyltransferase) at position 469 (F469) is associated with the increased oil and oleic acid contents. The *DGAT1-2* allele with F469 is ancestral, whereas the allele without F469 is a more recent mutant selected by domestication or breeding (Zheng *et al.*, 2008). Allele-specific PCR assays have been developed to enable the direct selection of allele-specific SNP-markers. In rapeseed, allele-specific C/T or G/A transitions of FAD3 desaturase genes led to increased linolenic acid content. These two allele-specific SNP markers can be identified by using specific PCR amplifications with locus-specific primers (Mikolajczyk *et al.*, 2010; Bocianowski *et al.*, 2011). In foxtail millet, a synonymous A/G transition in the foxtail *DREB2* gene is associated with dehydration tolerance. The A/G transition can be identified using the allele-specific PCR systems, which allow to distinguish drought-tolerant from drought-sensitive foxtail millet cultivars (Lata *et al.*, 2011). Such observations suggest that drought-tolerance-associated allele-specific SNP-markers may exist among the identified SNPs in barley *CBF/DREB* genes. These SNPs identified in *CBF/DREB* genes of the cultivar *Arta* represent therefore a good start for future gene trait association studies in this drought-tolerant barley cultivar. Allele-specific PCRs have been done here in this work to investigate whether a T/C transition at position 1279 in the *HvDREB1* gene identified in the cultivar *Arta* is also present in the other seven drought-tolerant barley cultivars, including *IG128216*, *IG32973*, *IG31903*, *IG31424*, *K10877*, *Tadmor* and *Unumli-Arpa* (see Fig. S1 in appendix). This T/C transition was not present in the tested drought-tolerant cultivars. It is likely that this transition is unique for *Arta*, as that was the case for some other genes listed above (Fan *et al.*, 2009; Zheng *et al.*, 2008; Lata *et al.*, 2011). Validations of the other putative drought-tolerance-associate SNP markers are planned.

Most of the SNPs identified in this study lead to non-synonymous mutations (see 3.2). Whether these non-synonymous mutations affect protein structure, binding affinities or cause post-translational modification is unclear. However, McKhann *et al.* (2008) showed that non-synonymous mutations at positions 452 and 578 of

the barley *CBF3* gene affect protein structure. These two non-synonymous mutations fall into a region between two hydrophobic clusters described by Wang *et al*. (2005) and play crucial roles for *HvCBF3* transactivation. Notably, some of the SNPs identified in this study result in the variation of the amino acid codon for serine or threonine e.g. a G/A transition at position 358 of the *HvCBF2* gene in the cultivar *Wiebke* (see 3.2.2). These amino acids could be phosphorylated and lead to changes in the regulation functions of the proteins (Burnett and Kennedy, 1954; Cozzone, 1988; Ciesla *et al.*, 2011). It has been reported that phosphorylation is likely to be necessary for protein activation under drought stress conditions, thereby promoting the DNA-binding activity of several transcription regulators (Busk and Pages, 1998; Kagaya *et al.*, 2002; Lata and Prasad, 2011). The phosphorylation of threonine in the *PgDREB2A* gene of pearl millet did not bind to the DREs and the phosphorylation of the *PgDREB2A* gene negatively regulates its DNA-binding activity (Agarwal *et al.*, 2007). Such observations indicated that stress could change the activity of the protein via post-translational modification by phosphorylation. The post-translational modification could be therefore one source of divergences in gene expression among different barley cultivars. The findings therefore represent a valuable source of information for future post-translational regulation studies in barley *CBF/DREB* genes. It should be, however, noted that it is difficult to validate the effect of specific SNPs on the gene expression by using the data collected by gene expression studies with the small number of accessions (McKhann *et al.*, 2008). Therefore, larger gene expression studies using a larger number of accessions (more than 50 accessions) should be considered.

4.2 Phylogenetic distribution of the isolated barley *CBF/DREB* genes agrees with their responses to drought.

As described in 3.3, the isolated ten barley *CBF/DREB* genes could be phylogenetically grouped into *HvCBF1*-, *HvCBF4*- and *HvDREB1*-subgroups. These three subgroups together with other *CBF/DREB* genes from *Arabidopsis*, rice, maize, sorghum and wheat formed the reconciled tree. We found a demarcation, which divided these 29 *CBF/DREB* genes from monocot and dicot plants into two clusters. The *HvDREB1*-subgroup forms the cluster I, whereas the *HvCBF1*- and *HvCBF4*-subgroups compose the cluster II. When comparing the expression patterns of ten barley *CBF/DREB* genes from plants grown under progressive drought stress in the field or in the controlled climate chamber, two clusters could also be seen.

Cluster one represents the constitutively expressed barley *CBF/DREB* genes and cluster two represents the drought inducible genes. The constitutively expressed barley *CBF/DREB* genes coincided with the *HvDREB1*-subgroup of the reconciled tree and the drought inducible genes phylogenetically belonged to the *HvCBF1*- and *HvCBF4*-subgroup, respectively (Fig. 11B). We thus found an agreement between the phylogenetic distribution of barley *CBF/DREB* genes in the reconciled tree and their responses to drought. The published data of studies of these 29 *CBF/DREB* genes are consistent with these foundings (Choi *et al.*, 2002; Haake *et al.*, 2002; Knight *et al.*, 2004; Skinner *et al.*, 2005; Agarwal *et al.*, 2006b; Qin *et al.*, 2007a; Qin *et al.*, 2007b; Nayak *et al.*, 2009; Bihani *et al.*, 2010; Matsukura *et al.*, 2010; Lata and Prasad, 2011; Mallikarjuna *et al.*, 2011). Differences were, however, seen for the constitutively expressed *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes compared with the information of up-regulation of these genes upon drought in the literature. These are discussed in section 4.3.

4.3 Age and physiological state of plants may lead to the different expression patterns of the *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes.

We observed a constitutive expression for the *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes upon drought in the field or in the controlled climate chamber grown plants, which is contrary to the results on *HvDREB1* and *HvDRF1.1/HvDRF1.3* reported by Xu *et al*. (2009) and Xue and Loveridge (2004), respectively. It has been shown in these studies that *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes are up-regulated upon drought. Such deviations could be due to the differences in plant materials and stress treatments. In this study, we used four-week-old barley seedlings grown in soil in a plastic greenhouse tunnel, in which the growth conditions varied according to external climate conditions. Water was progressively withheld for drought treatments until the water content in the pots declined to the permanent wilting point (15% VWC) within 21 days. To perform the drought experiment under controlled laboratory conditions, we used 4-week-old plants grown in a growth chamber under approximately 120-150 µE m^{-2} s^{-1} light intensity at 22 ℃ with a day/night cycle of 8/16h. Water was withheld for two days or five days by drought treated plants. In contrast, Xu *et al.* (2009) utilized 10-day-old barley seedlings grown hydroponically at 25 ℃ for the drought assays, in which barley seedlings were air-dried on filter paper for up to 48 hours. Similarly, 9-day-old barley seedlings were used by Xue and Loveridge (2004) in their studies. Barley seedlings were grown in temperature-controlled growth rooms and allowed to dry at room temperature for rapid drought treatment (2-5 hours). Big differences in experimental results caused by using different plant materials and stress treatments have been shown in previous studies. Sakuma *et al*. (2002) and Haake *et al*. (2002) showed different results on the

responsiveness of *CBF4/DREB1D* to ABA in *Arabidopsis*. Haake *et al*. (2002) reported that *AtCBF4/DREB1D* is induced rapidly by ABA, while the observation from Sakuma *et al*. (2002) was completely opposite to Haake *et al*. (2002). Haake *et al*. (2002) utilized 10-day-old seedlings grown on filter paper and the ABA treatment was done by transferring the filter papers from the plate and placing them in Gamborgs B5 liquid medium plus 100 µM ABA. In contrast, Sakuma *et al*. (2002) used 3-week-old plants grown hydroponically in solution containing 100 µM ABA under dim light. Indeed, *AtCBF4/DREB1D* was found to be induced rapidly by ABA in another independent study reported later (Knight *et al*., 2004). Knight *et al*. (2004) reported that induction of *AtCBF4/DREB1D* genes is dependent on the context and that the age and physiological state of plants influence the fluxes through the different branches of the signalling network. Knight *et al*. (2004) indicated that water relations are likely to be very different between the plants grown on soil or tissue culture during growth period and this may affect the responsiveness of *AtCBF4/DREB1D* to ABA. Such observations suggest that differences in age and physiological state of plants have great influence on experimental results.

4.4 Barley cultivars respond to drought at different time points.

In this study, the expression of six barley *CBF* genes, including *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* upon drought in leaf tissues of plants grown in the field has been investigated in eight different barley cultivars over two successive years: 2010 and 2011. The analysis indicated that *HvCBF1*, *HvCBF2*, *HvCBF6* and *HvCBF11* show differential timings of expression within barley cultivars (Fig.12A, 13A, 16A and 17A). In the cultivars *Heils Franken*, *Pflugs Intensiv* and *Wiebke*, changes in expression level of the above-mentioned four genes are observed immediately after exposure to drought. In contrast, changes in the expression levels of these four genes are recorded later in the cultivar *Victoriana* than in the above-mentioned three cultivars. Therefore, the cultivars *Heils Franken*, *Pflugs Intensiv* and *Wiebke* are clustered into one group with sensitivity to early drought, while the cultivar *Victoriana* composes another group with sensitivity to late drought. The timing in the drought sensitivity has been difficult to define for other cultivars, because of the variations in the expression patterns of these *CBF* genes. Changes in *HvCBF3* and *HvCBF4* expression levels emerge immediately in all eight barley cultivars after exposure to drought (Fig. 14A and 15A). It suggests that these genes are likely to play a role in the early stage of drought stress.

4.5 DNA methylation within the *CBF1* promoter does not trigger a reduction of *HvCBF1* transcripts.

As described in 3.4, rapid dehydration led to a reduction of the *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* transcripts. Previous studies have revealed that promoter hypermethylation represses the expression of the corresponding gene (Eden and Cedar, 1994; Ng *et al.*, 1999; Newell-Price *et al.*, 2000; Bird, 2002; Berdasco *et al.*, 2008). It is generally assumed that the repression of gene expression is greater, if the promoter itself is modified (Newell-Price *et al.*, 2000). In this study, we found that the promoter of the *HvCBF1* gene was hypermethylated (see Fig. 24). But the hypermethylated promoter does not trigger a reduction of *HvCBF1* transcripts. No differences in DNA methylation patterns were observed in the promoter of both control and stressed plants. A study by Curradi *et al.* (2002) demonstrated in *Xenopus* that, in contrast to the common thought, promoter methylation does not always lead to a transcriptional repression of the corresponding gene expression. The existence of a competition between transactivators and methyl-binding proteins for the establishment of an open chromatin conformation is rather crucial for the transcriptional activities (Curradi *et al.*, 2002).

4.6 Establishment of a non-invasive system to analyze barley root architecture

In this work, a new approach has been developed to analyze the barley root architecture in a non-invasive way. The techniques allow to observe barley root growth in three dimensions under different growth conditions for several weeks. The nutrient composition of growth media can be easily adjusted without destroying the root. This new approach is much cheaper and more efficient to acquire images than some other non-invasive imaging methods such as magnetic resonance imaging (Van As, 2007) or laser scanning (Fang *et al.*, 2009). Laser scanning requires longer imaging time, while magnetic resonance imaging is very costly and time-consuming because a pre-treatment of demagnetization for plant growth media is necessary. We have performed some experiments to better study and understand the barley root system architectures. First, a new protocol has been developed for barley seed surface sterilization. We found that the most efficient method for barley seed surface sterilization is incubation of barley seeds in 35% H_2O_2 for 10 min after the removal of seed husks (Fig. 25). Second, the optimum hydroponic media for *in vitro* cultivation of barley plants have been determined. As shown in Fig. 26, the root system development of barley plants grown in Hoagland ́s media is much better than in Yoshida ́s.

The development of root hairs causes some problems for image conversions. For image

conversion of acquired root pictures to a 2D format, the GiaRoot software considered each seminal root with the root hairs to a thick single root (Fig. 29 B). Therefore, the acquired data could not be used to characterize barley root system architecture of the cultivars with many root hairs. Therefore, some improvements of this system are required for the analysis of barley root system architecture. Better image quality and updates of current GiaRoot software

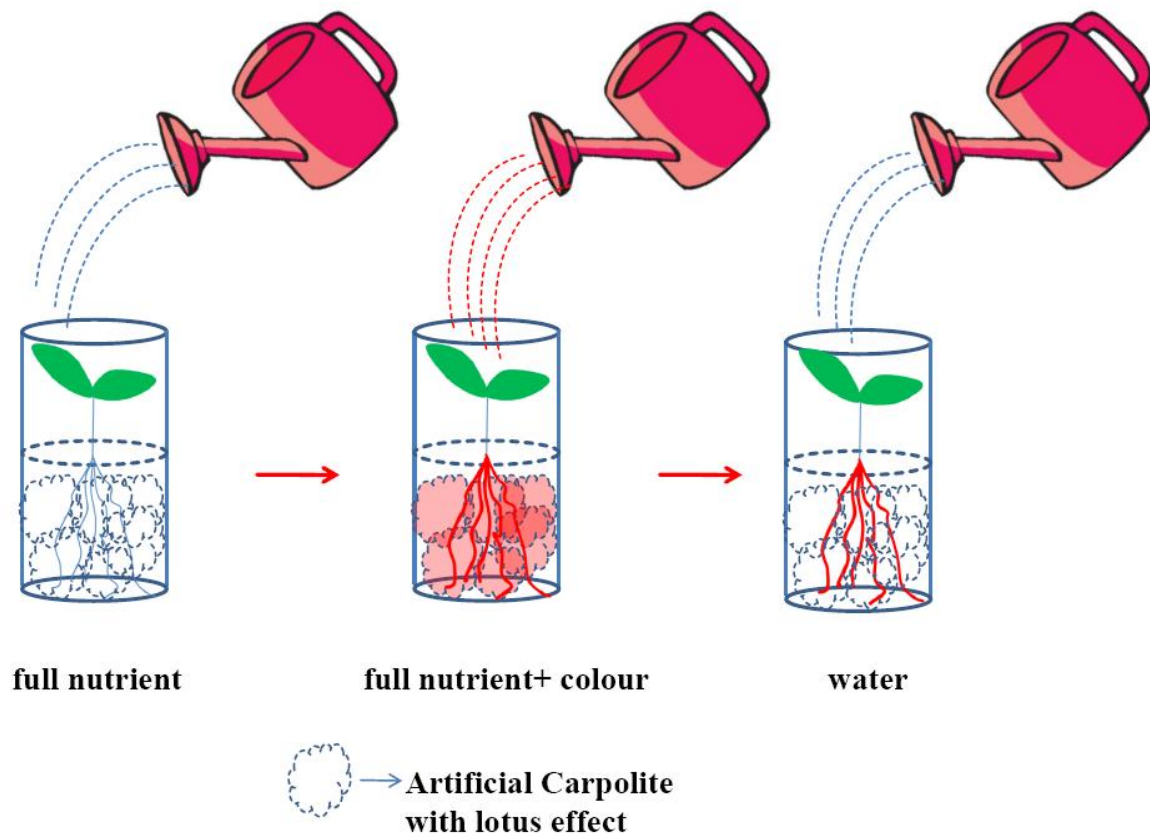

Fig. 31. Study of barley root system architecture using an open cultivation system. Barley plants are grown in the glass bottles containing artificial carpolites. Special transparent coating materials with lotus effect are coated on the surface of the glass bottles and the artificial carpolites. Plants are watered with full nutrient regularly. Several days before imaging they are watered with full nutrient supplies containing staining dyes. Before imaging, plants are watered with normal water. Lotus effect allows to wash off the staining dyes easily from the surface of the carpolites and glass bottles, whereas plant roots are stained.

are necessary. The image quality can be improved by using cameras with higher resolution, which helps to better distinguish plant root hairs from the background. In contrast, an update of software is much more challenging than acquiring pictures with improved resolution, depending on the camera. It will take a lot of time to get rid of the technical barriers. Notably, we found that the development of root hairs can be inhibited under bad aeration and higher

humidity conditions (Fig. 28). But such isolated artificial growth conditions cannot mimic the natural conditions for plant growth and development. Suggestions for improvement of the current platform are to use an open system for plant cultivation, which can better mimic the natural conditions for plant growth and development. As shown in Fig. 31, barley plants can be grown in a glass pot containing transparent artificial carpolites. Special transparent coating materials with lotus effect are coated on the surface of the glass bottles and the artificial carpolites. Barley plants are watered regularly with full nutrient supplies. Several days before imaging, plants are watered with full nutrient supplies containing staining dyes. Before Imaging, plants are watered with regular tap water. The lotus effect allows to wash off the staining dyes easily from the surface of the glass bottles and the artificial carpolites, while plant roots are stained. It helps to distinguish stained plant roots from the background easily. With this method, it is not necessary to remove any husks from barley seeds, which can hurt the embryos during the preparation. Furthermore, no growth media need to be prepared and autoclaved. Most importantly, an open system can better mimic the natural growth conditions for plants than the current isolated system.

4.7 Conclusions and perspectives

In this study, ten barley *CBF/DREB* transcripts are isolated from nine different barley cultivars using gene-specific primers. The multiple sequence alignment of barley *CBF/DREB* genes with the published cDNA sequence from NCBI indicates the absence of introns in the isolated barley *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* genes, which is consistent with the current knowledge about barley *CBF* genes. In contrast, a 96-bp long intron has been newly identified in the barley *DRF2* genes. Comparative sequence analyses showed extensive sequence conservation with few SNPs among different barley cultivars. However, the functions of the identified SNPs are still unknown. According to the recent research results, such SNPs may play a role in post-translational regulation of *CBF/DREB* gene transactivation or be exploited in breeding programs as trait-associated selection marker. Multiple sequence alignment revealed that the AP2/ERF domain of *CBF/DREB* genes has high sequence similarity among both monocot and dicot species. Moreover, the isolated barley *CBF/DREB* genes were phylogenetically grouped into *HvCBF1*-, *HvCBF4*- and *HvDREB1*-subgroups. We found that the phylogenetic distribution of barley *CBF/DREB* genes in the reconciled tree coincided with their responses to drought. This is consistent with reports in the literature.

The expression patterns of the ten candidate barley *CBF/DREB* genes upon drought have been

analyzed by RT-PCR in barley plants grown in the field or under controlled laboratory conditions. By analyzing the *CBF* expression patterns upon drought in leaf tissues of plants grown in the field, we found that barley cultivars respond to drought at different time points. Monitoring the expression of *HvCBF1*, *HvCBF2*, *HvCBF3*, *HvCBF4*, *HvCBF6* and *HvCBF11* transcripts upon drought in the field in the year 2011 revealed that rapid dehydration triggered the reduction of the above-mentioned transcripts. This was further validated by experiments performed under controlled laboratory conditions. By analyzing of methylation patterns in the isolated *HvCBF1* promoter, we found that hypermethylation of barley *HvCBF1* promoter did not trigger a reduction of *HvCBF1* transcripts. Interactions between all DNA-binding factors determine the final outcome. In contrary to the published data in the literature, we observed a constitutive expression of *HvDREB1*, *HvDRF1.1* and *HvDRF1.3* genes upon drought in leaf tissues of barley plants grown in the field or under controlled conditions. This could be due to the age and physiological state of the plants.

Finally, a more efficient and non-invasive method to analyze the barley root architecture has been developed with help and permissions from Prof. Dr. Philip Benfey, Duke University Durham NC, USA. Some suggestions for improvement of this platform have been made.

5. APPENDICES

5.1 Protein sequences for phylogenetic analysis in FASTA format

AtCBF1/DREB1B (BAA33435)

MNSFSAFSEMFGSDYEPQGGDYCPTLATSCPKKPAGRKKFRETRHPIYRGVRQRNSGKWVSEVREPNK
KTRIWLGTFQTAEMAARAHDVAALALRGRSACLNFADSAWRLRIPESTCAKDIQKAAAEAALAFQDET
CDTTTTNHGLDMEETMVEAIYTPEQSEGAFYMDEETMFGMPTLLDNMAEGMLLPPPSVQWNHNYDG
EGDGDVSLWSY

AtCBF2/DREB1C (BAA33436)

MNSFSAFSEMFGSDYESPVSSGGDYSPKLATSCPKKPAGRKKFRETRHPIYRGVRQRNSGKWVCELREP
NKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTCAKEIQKAAAEAALNFQD
EMCHMTTDAHGLDMEETLVEAIYTPEQSQDAFYMDEEAMLGMSSLLDNMAEGMLLPSPSVQWNYNF
DVEGDDDVSLWSY

AtCBF3/DREB1A (BAA33434)

MNSFSAFSEMFGSDYESSVSSGGDYIPTLASSCPKKPAGRKKFRETRHPIYRGVRRRNSGKWVCEVREP
NKKTRIWLGTFQTAEMAARAHDVAALALRGRSACLNFADSAWRLRIPESTCAKDIQKAAAEAALAFQ
DEMCDATTDYGFDMEETLVEAIYTAEQSENAFYMHDEAMFEMPSLLANMAEGMLLPLPSVQWNHNH
EVDGDDDDVSLWSY

AtCBF4/DREB1D (AED96156)

MNPFYSTFPDSFLSISDHRSPVSDSSECSPKLASSCPKKRAGRKKFRETRHPIYRGVRQRNSGKWVCEVR
EPNKKSRIWLGTFPTVEMAARAHDVAALALRGRSACLNFADSAWRLRIPETTCPKEIQKAASEAAMAF
QNETTTEGSKTAAEAEEAAGEGVREGERRAEEQNGGVFYMDDEALLGMPNFFENMAEGMLLPPPEVG
WNHNDFDGVGDVSLWSFDE

AtDREB2A (BAA36705)

MAVYDQSGDRNRTQIDTSRKRKSRSRGDGTTVAERLKRWKEYNETVEEVSTKKRKVPAKGSKKGCM
KGKGGPENSRCSFRGVRQRIWGKWVAEIREPNRGSRLWLGTFPTAQEAASAYDEAAKAMYGPLARLN
FPRSDASEVTSTSSQSEVCTVETPGCVHVKTEDPDCESKPFSGGVEPMYCLENGAEEMKRGVKADKHW
LSEFEHNYWSDILKEKEKQKEQGIVETCQQQQQDSLSVADYGWPNDVDQSHLDSSDMFDVDELLRDL
NGDDVFAGLNQDRYPGNSVANGSYRPESQQSGFDPLQSLNYGIPPFQLEGKDGNGFFDDLSYLDLEN

AtDREB2B (BAA36706)

MAVYEQTGTEQPKKRKSRARAGGLTVADRLKKWKEYNEIVEASAVKEGEKPKRKVPAKGSKKGCMK
GKGGPDNSHCSFRGVRQRIWGKWVAEIREPKIGTRLWLGTFPTAEKAASAYDEAATAMYGSLARLNFP
QSVGSEFTSTSSQSEVCTVENKAVVCGDVCVKHEDTDCESNPFSQILDVREESCGTRPDSCTVGHQDMN
SSLNYDLLLEFEQQYWGQVLQEKEKPKQEEEEIQQQQQEQQQQQLQPDLLTVADYGWPWSNDIVNDQ
TSWDPNECFDINELLGDLNEPGPHQSQDQNHVNSGSYDLHPLHLEPHDGHEFNGLSSLDI

AtDREB2C (AEC09816)

MPSEIVDRKRKSRGTRDVAEILRQWREYNEQIEAESCIDGGGPKSIRKPPPKGSRKGCMKGKGGPENGIC
DYRGVRQRRWGKWVAEIREPDGGARLWLGTFSSSYEAALAYDEAAKAIYGQSARLNLPEITNRSSSTA
ATATVSGSVTAFSDESEVCAREDTNASSGFGQVKLEDCSDEYVLLDSSQCIKEELKGKEEVREEHNLAV
GFGIGQDSKRETLDAWLMGNGNEQEPLEFGVDETFDINELLGILNDNNVSGQETMQYQVDRHPNFSYQ
TQFPNSNLLGSLNPMEIAQPGVDYGCPYVQPSDMENYGIDLDHRRFNDLDIQDLDFGGDKDVHGST

OsDREB1A (AAN02486)

MCGIKQEMSGESSGSPCSSASAERQHQTVWTAPPKRPAGRTKFRETRHPVFRGVRRRGNAGRWVCEV
RVPGRRGCRLWLGTFDTAEGAARAHDAAMLAINAGGGGGGGACCLNFADSAWLLAVPRSYRTLADV

RHAVAEAVEDFFRRRLADDALSATSSSSTTPSTPRTDDDEESAATDGDESSSPASDLAFELDVLSDMGW
DLYYASLAQGMLMEPPSAALGDDGDAILADVPLWSY

OsDREB1B (AAN02488)
MEVEEAAYRTVWSEPPKRPAGRTKFRETRHPVYRGVRRRGGRPGAAGRWVCEVRVPGARGSRLWLG
TFATAEAAARAHDAAALALRGRAACLNFADFAWRMPPVPASAALAGARGVRDPVAVAVEAFQRQSA
APSSPAETFANDGDEEEANKDVLPVAAAEVFDAGAFELDDGFRFGGMDAGSYYASLAQGLLVEPPAA
GAWWEDGELAGSDMPLWSY

OsDREB2 (AAN02487)

MERGEGRRGDCSVQVRKKRTRRKSDGPDSIAETIKWWKEQNQKLQEENSSRKAPAKGSKKGCMAGK
GGPENSNCAYRGVRQRTWGKWVAEIREPNRGRRLWLGSFPTALEAAHAYDEAARAMYGPTARVNFA
DNSTDANSGCTSAPSLMMSNGPATIPSDEKDELESPPFIVANGPAVLYQPDKKDVLERVVPEVQDVKTE
GSNGLKRVCQERKNMEVCESEGIVLHKEVNISYDYFNVHEVVEMIIVELSADQKTEVHEEYQEGDDGF
SLFSY

OsDREB2A (AFB77198)

MLFRFVSCNVQLCGIIELPHWVRKKRTRRKSDGPDSIAETIKWWKEQNQKLQEENSSRKAPAKGSKKG
CMAGKGGPENSNCAYRGVRQRTWGKWVAEIREPNRGRRLWLGSFPTALEAAHAYDEAARAMYGPTA
RVNFADNSTDANSGCTSAPSLMMSNGPATIPSDEKDELESPPFIVANGPAVLYQPDKKDVLERVVPEVQ
DVKTEGSNGLKRVCQERKTMEVCESEGIVLHKEVNISYDYFNVHEVVEMIIVELSADQKTEVHEEYQE
GDDGFSLFSY

OsDREB2B (Q5W6R4)

MTVDQRTTAKAIMPPVEMPPVQPGRKKRPRRSRDGPTSVAETIKRWAELNNQQELDPQGPKKARKAP
AKGSKKGCMKGKGGPENTRCDFRGVRQRTWGKWVAEIREPNQQSRLWLGTFPTAEAAACAYDEAAR
AMYGPMARTNFGQHHAPAASVQVALAAVKCALPGGGLTASKSRTSTQGASADVQDVLTGGLSACEST
TTTINNQSDVVSTLHKPEEVSEISSPLRAPPAVLEDGSNEDKAESVTYDENIVSQQRAPPEAEASNGRGEE
VFEPLEPIASLPEDQGDYCFDIDEMLRMMEADPTNEGLWKGDKDGSDAILELGQDEPFYYEGVDPGML
DNLLRSDEPAWLLADPAMFISGGFEDDSQFFEGL

OsDREB2C (Q84ZA1)

MEMDIGEGESCCGRRKQQQQQNISSSKSRKCCPLRRSRKGCMKGKGGPENQRCPFRGVRQRTWGKWV
AEIREPNRGARLWLGTFNTALDAARAYDSAARALYGDCARLNLLLAAATAGAPPAAATPSVATPCSTN
DDSNNSSSTTHQQQLTTMLQLDDDNYTLQPSSSDQEDFETYVTRLPKAEDFGLEGFQEVPLDVLDEAG
GGISIWDLSICPADFMATAATTTAKSS

OsERF3 (BAB16083)

MAPRAATVEKVAVAPPTGLGLGVGGGVGAGGPHYRGVRKRPWGRYAAEIRDPAKKSRVWLGTYDTA
EEAARAYDAAAREFRGAKAKTNFPFASQSMVGCGGSPSSNSTVDTGGGGVQTPMRAMPLPPTLDLDLF
HRAAAVTAVAGTGVRFPFRGYPVARPATHPYFFYEQAAAAAAAEAGYRMMKLAPPVTVAAVAQSDS
DSSSVVDLAPSPPAVTANKAAAFDLDLNRPPPVEN

OsDBF1 (AAP56252)

MAAAIEGNLMRALGEAPSPQMQKIAPPPFHPGLPPAPANFSSAGVHGFHYMGPAQLSPAQIQRVQAQL
HMQRQAQSGLGPRAQPMKPASAAAPAAAAARAQKLYRGVRQRHWGKWVAEIRLPRNHPRLWLGTF
DTAEEAALTYGQAAYRLRGDAARLNFPDNAASRGPLDAAVDAKLQAICDTIAASKNASSRSRGGAGR
AMPINAPLVAAASSSSGSDHSGGGDDGGSETSSSSAAASPLAEMEQLDFSEVPWDEAEGFALTKYPSYE
IDWDSLLNNN

ZmDREB1A (ACG40680)

MDAAGSFSDYSSGTPSPVAAAGGGDDFGGSGSGSSSYMTVSSAPPKRRAGRTKFKETRHPVYKGVRRR
NPGRWVCEVREPHGKQRIWLGTFETAEMAARAHDVAALALRGRAACLNFADSPRLLRVPPTGSGHDEI
RRAAAVAADQFRPAPDQGNVAAEEEAADTPPPDALPSVTMQSVDDDPYCIIDDRLDFGMQGYLDMAQ
GMLIDPPPMAGSSTSGGGGDDDDDDGEVKLWSY

ZmDREB1C (ACO72991)

MTLDQNHAMPMQPPALQPGRKKRPRRSRDGPTSVAAVIQRWAERNKHLEYEESEEAKRPRKAPAKGS
KKGCMKGKGGPDNTQCGYRGVRQRTWGKWVAEIREPNRVDRLWLGTFPTAEDAARAYDEAARAMY
GDLARTNFPGQDATTSAQAALSSTSAQAAPTAVEALQTGTSCESTTTSNHSDIASTSHKLEASDISSYLK
EKCPAGSCGIQDGTPIVADKEVFGPLEPITNLPDGGDGFDIGEMLRMMESDPHNAGGADAGMGQPWYL
DELDSSVLESMLQPEPEPEPEPFLMSEEPDMFLAGFESAGFVEGLERLN

ZmDREB2A (ACG47772)

MEELGDAGQGPQGDASGALVRKKRMRRKSTGPDSIAETIRWWKEQNQRLQDESGSRKAPAKGSKKGC
MMGKGGPENVNCVYRGVRQRTWGKWVAEIREPNRGRRLWLGSFPTAVEAAHAYDEAAKAMYGPKA
RVNFSESSADANSGCTSALSLLASSVPAAALQRSDEKVETEVESVETEEVVHEVKTEAKNGDLGSVKVT
CVIQSAEETVLHKEGDVSYDYFNVEDAVEMIIVELNADKKFEAHEEYLDGDDGFSLFAY

ZmDBF1 (AAM80486)

MQFIQAQLHLQRNPGLGPRAQPMKPAVPVPPAPAPQRPVKLYRGVRQRHWGKWVAEIRLPRNRTRLW
LGTFDTAEQAALAYDQAAYRLRGDAARLNFPDNAESRAPLDPAVDAKLQAICATIAAASSSSKNSKAK
SKAMPINASVLEAAAASPSNSSSDEGSGSGFGSDDEMSSSSPTPVVAPPVADMGQLDFSEVPWDEDESF
VLRKYPSYEIDWDALLSN

ZmDBF3 (NP_001105651)

MDTAGLVQHATSSSSTSTSASSSSSEQQSRKAAWPPSTASSPQQPPKKRPAGRTKFRETRHPVFRGVRRR
GAAGRWVCEVRVPGRRGARLWLGTYLAAEAAARAHDAAILALQGRGAGRLNFPDSARLLAVPPPSAL
PGLDDARRAALEAVAEFQRRSGSGSGAADEATSGASPPSSSPSLPDVSAAGSPAAALEHVPVKADEAVA
LDLDGDVFGPDWFGDMGLELDAYYASLAEGLLVEPPPPPAAWDHGDCCDSGAADVALWSYY

TaDBF (AAZ08560)

MATTVDWRSYRPDLPAAMYRMVDSRDQVMHAFAPPTAQGAAPTISFAFPCPGADQSAGLLRGATYLT
PAQILQLQSQLHHVRRAPGAPMAAVGQPMKRHGVAALPARPATKLYRGVRQRHWGKWVAEIRLPRN
RTRLWLGTFDTADEAALAYDAAAFRLRGESARLNFPELRRGGEHHGPPLDAAIDAKLRSICHGEDMPQ
SQSNETPAPTPTLTPISFPDVKSEPVCSVSESSSSADGEVSSCSDVVPEMQLLDFSEAPWDESLLRKYPSLE
IDWDAILP

SbDREB2 (ACA79910)

MELGDATAGQGAQGDAASGALVRKKRMRKKSTGPDSIAETIKWWKEQNQKLQDESGSRKAPAKGSK
KGCMTGKGGPENVNCVYRGVRQRTWGKWVAEIREPNRGRRLWLGSFPTAVEAAHAYDEAAKAMYG
PKARVNFSDNSADANSGCTSALSLLASSVPVATLQRSDEKVETEVESVETEVHEVKTEGNDDLGSVHV
ACKTVDVIQSEKSVLHKAGEVSYDYFNVEEVVEMIIIELNADKKIEANEEYHDGDDGFSLFAY

5.2 Sequence analysis of barley *DREB1* genes in drought-tolerant barley cultivars

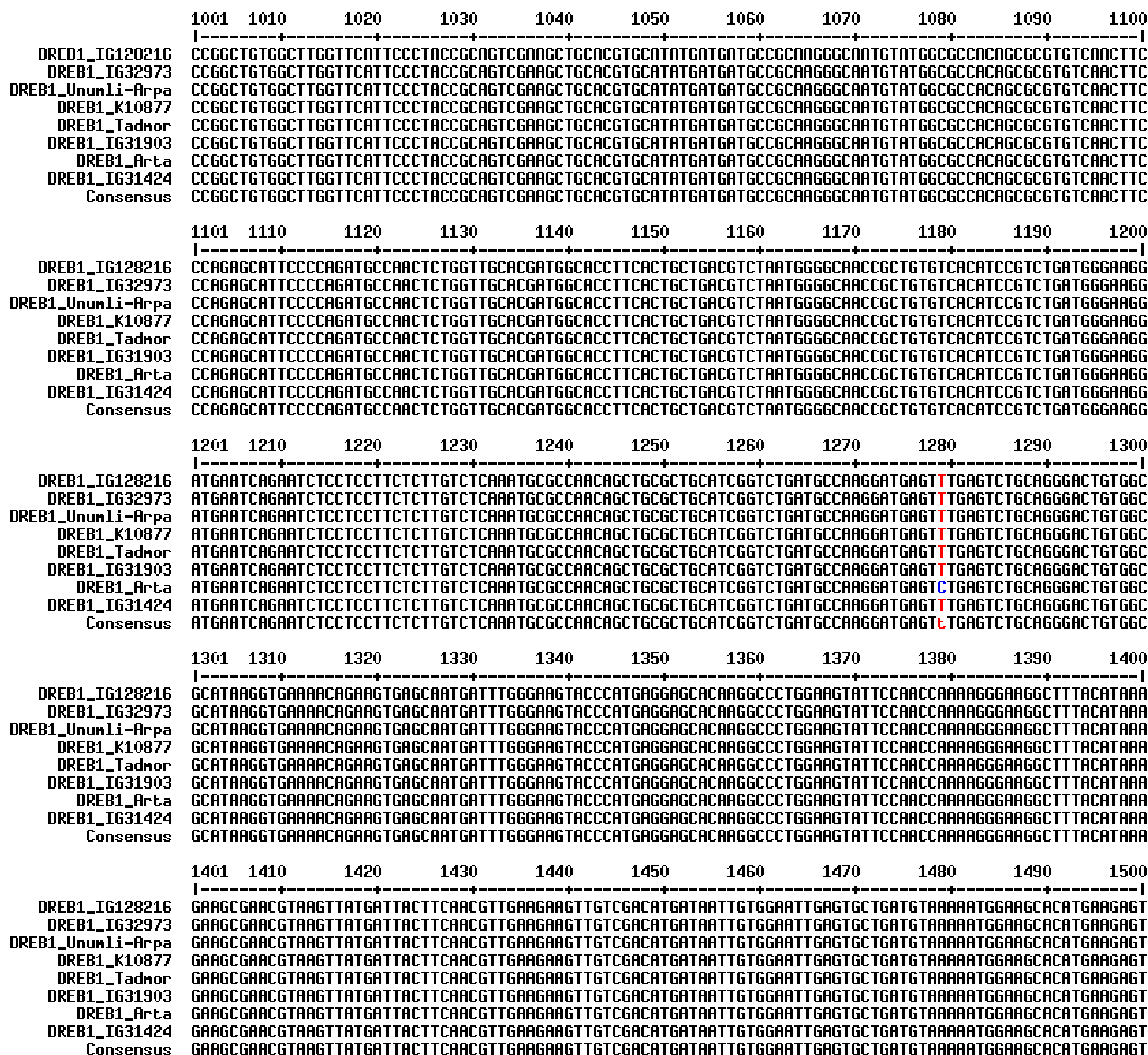

Fig. 32. Comparison of *DREB1* genomic DNA sequences in different barley cultivars using the Multialign software. Black indicates high consensus, red indicates low consensus. Blue is chosen as neutral colour.

6. REFERENCES

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