Plant Biotechnology Journal (2007) **5**, pp. 646–656 doi: 10.1111/j.1467-7652.2007.00272.x

Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice

Se-Jun Oh[1], Chang-Woo Kwon[1], Dong-Woog Choi[2], Sang Ik Song[1] and Ju-Kon Kim[1,*]

[1]*School of Biotechnology and Environmental Engineering, Myongji University, Yongin 449-728, South Korea*
[2]*Department of Science Education, Chonnam University, Kwangju 500-757, South Korea*

Received 19 March 2007;
revised 8 May 2007;
accepted 9 May 2007.
**Correspondence* (fax 82-31-335-8249;
e-mail jukon@bio.mju.ac.kr)

Summary

C-repeat/dehydration-responsive element binding factors (CBF/DREBs) are a family of transcription factors that regulate freezing tolerance in *Arabidopsis*. As a step towards understanding the stress response of monocotyledonous plants, we isolated a barley gene *HvCBF4* whose expression is induced by low-temperature stress. Transgenic over-expression of *HvCBF4* in rice resulted in an increase in tolerance to drought, high-salinity and low-temperature stresses without stunting growth. Interestingly, under low-temperature conditions, the maximum photochemical efficiency of photosystem II in the dark-adapted state (F_v/F_m, where F_v is the variable fluorescence and F_m is the maximum fluorescence) in *HvCBF4* plants was higher by 20% and 10% than that in non-transgenic and *CBF3/DREB1A* plants, respectively. Using the 60K Rice Whole Genome microarray, 15 rice genes were identified that were activated by HvCBF4. When compared with 12 target rice genes of CBF3/DREB1A, five genes were common to both HvCBF4 and CBF3/DREB1A, and 10 and seven genes were specific to HvCBF4 and CBF3/DREB1A, respectively. Interestingly, HvCBF4 did not activate *Dip1* and *Lip5*, two important target genes of CBF3/DREB1A, in transgenic rice under normal growth conditions, but their expression was enhanced by HvCBF4 under low-temperature conditions. Our results suggest that *CBF/DREBs* of barley act differently from those of *Arabidopsis* in transgenic rice.

Keywords: AP2 transcription factor, CBF/DREB, HvCBF4, microarray, stress tolerance, transgenic rice plant.

Introduction

Environmental stresses, such as drought, high salinity and low temperature, influence the growth of plants and the productivity of crops. Plants are thought to have common mechanisms, not only in terms of the molecular and cellular response, but also in terms of the biochemical and physiological response, for adaptation to these stresses (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Kang *et al*., 2002). On exposure to such stresses, many genes are induced and function either as cellular protectants of stress-induced damage or as regulators of gene expression and signal transduction (Kang *et al*., 2002; Yu *et al*., 2002; Shinozaki *et al*., 2003).

C-repeat (*CRT*; Baker *et al*., 1994)/dehydration-responsive element (*DRE*; Yamaguchi-Shinozaki and Shinozaki, 1994) has been identified from the promoter regions of stress-inducible *Arabidopsis* genes, such as *cor15A*, *rd29A*, *kin1*, *cor6.6* and *cor47/rd17*. The *CRT/DRE* binding factors (CBF/DREBs) in *Arabidopsis* activate the transcription of genes with *CRT/DRE*s in their promoter regions (Stockinger *et al*., 1997; Gilmour *et al*., 1998; Liu *et al*., 1998). CBF/DREBs are key regulatory factors that function primarily in freezing tolerance by activating a battery of target genes (Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999; Fowler and Thomashow, 2002; Maruyama *et al*., 2004). CBF/DREBs are members of the AP2 superfamily and are identifiable by the presence of CBF/DREB signature motifs (PKK/RPAGRxKFxETRHP and DSAWR) directly flanking the AP2 domain (Jaglo *et al*., 2001). The expression of *Arabidopsis CBF1, 2, 3/DREB1A, B, C* is induced by low temperature (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998), whereas *Arabidopsis DREB2* (Liu *et al*., 1998) and *CBF4* (Haake *et al*., 2002) are not induced by low temperature, but by drought stress. Over-expression of *CBF/DREBs* in transgenic

Arabidopsis increases the transcript levels of stress-induced genes and enhances the tolerance to drought, high-salinity and freezing stresses (Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999; Haake *et al*., 2002). Thirty-eight target genes of CBF/DREBs have been identified using microarray analysis in *Arabidopsis* (Seki *et al*., 2001; Fowler and Thomashow, 2002; Maruyama *et al*., 2004). Most of these target genes contain CRT/DRE motifs in their promoter regions (Maruyama *et al*., 2004). *Arabidopsis CBF/DREB*s are also heterologously effective in canola (*Brassica napus*; Jaglo *et al*., 2001), tomato (*Lycopersicon esculentum*; Hsieh *et al*., 2002), tobacco (*Nicotiana tabacum*; Kasuga *et al*., 2004) and rice (Lee *et al*., 2004; Oh *et al*., 2005), up-regulating the corresponding target genes and enhancing stress tolerance in transgenic plants. *CBF/DREB* orthologues have been identified in canola, tomato, wheat, rye, barley and rice, and all are inducible by low-temperature treatment (Jaglo *et al*., 2001; Choi *et al*., 2002; Xue, 2002; 2003; Dubouzet *et al*., 2003; Skinner *et al*., 2005). These results suggest that *CBF/DREB*s play a significant role in the stress tolerance of monocotyledonous as well as dicotyledonous plants.

We have demonstrated previously that *Arabidopsis CBF3/DREB1A* in transgenic rice increases tolerance to drought and high salinity, but with relatively low levels of tolerance to low-temperature exposure (Oh *et al*., 2005). These data are in direct contrast to *CBF3/DREB1A* in transgenic *Arabidopsis*, which functions primarily to enhance freezing tolerance. This is presumably because *Arabidopsis* plants that are capable of cold acclimatization have evolved differently from rice plants that are unable to undergo cold acclimatization (Jaglo *et al*., 2001). Barley (*Hordeum vulgare*) is an economically important monocotyledonous crop that acclimatizes to the cold and is relatively tolerant to low temperature. Barley has a large *CBF/DREB* family consisting of 20 genes (Skinner *et al*., 2005). Xue (2002, 2003) reported two *CBF/DREB* orthologues in barley. HvCBF1 is a cold inducible form which can bind to GCCGAC more efficiently than to ACCGAC, whereas CBF3/DREB1A binds equally well to both ACCGAC and GCCGAC in *Arabidopsis* (Xue, 2002). HvCBF2 is the other form that prefers GTCGAC as a binding motif to either GCCGAC or ACCGAC and, interestingly, its binding affinity is temperature dependent (Xue, 2003). Choi *et al*. (2002) also reported two other barley orthologues, *HvCBF4* and *HvCBF3*, whose function in the stress tolerance of plants remains unknown. These observations led us to examine whether *HvCBF4* from barley performs better than *CBF3/DREB1A* from *Arabidopsis* in the stress tolerance of transgenic rice. We set out to measure the transcript levels of *HvCBF4* in barley under various stress conditions, and generated transgenic rice plants that constitutively over-expressed *HvCBF4*. When compared with *CBF3/DREB1A* transgenic rice, *HvCBF4* showed similar levels of tolerance to drought and high salinity, but a higher level of tolerance to low temperature. We discuss the similarities and differences between *HvCBF4* and *CBF3/DREB1A* in transgenic rice plants.

Results and discussion

Signature motifs and expression of the barley *HvCBF4* gene

HvCBF4, a barley orthologue of *CBF/DREB*, has been isolated previously (Choi *et al*., 2002; GenBank accession no. AF298230). This gene encodes a protein of 225 amino acids with a conserved AP2 DNA binding domain followed by an acidic region. To examine the phylogenetic relationship of the CBF/DREB family, we compared the amino acid sequence of HvCBF4 with those of 13 barley, three *Arabidopsis* and two rice CBF/DREB orthologues (Gilmour *et al*., 1998; Dubouzet *et al*., 2003; Skinner *et al*., 2005). Members of the CBF/DREB family could be divided into three subgroups (I, II and III), with HvCBF4 in subgroup I and CBF3/DREB1A in subgroup III (Figure 1a). In the AP2 domain and flanking regions, HvCBF4 shares 80%–88%, 66%–72% and 68%–76% sequence identity with the members of subgroups I, II and III, respectively. Comparison of sequences spanning the AP2 domains and flanking regions led to the identification of six signature motifs through which each subgroup could be identified (Figure 1b). Signature motifs 1, 2 and 3 are specific to subgroups I, II and III, respectively.

Transcript levels of *HvCBF4* in barley were measured by RNA gel-blot analysis using total RNAs from leaf tissues of 14-day-old seedlings that had been exposed to drought, high salinity, abscisic acid (ABA) and low temperature (Figure 2). *HvDhn8* and *HvRbcS*, whose transcript levels increase or decrease in response to stress treatment, were included as controls to monitor when stress-induced damage occurred. *HvDhn8* is a barley orthologue of *Arabidopsis COR47* (Gilmour *et al*., 1992) and rice *Dip1* (Jang *et al*., 2003; Oh *et al*., 2005). As a result, expression of *HvCBF4* was induced within 2 h of exposure to low temperature and maintained until 6 h, but was not affected by drought, high-salinity or ABA treatment. The expression of *HvDhn8* and *HvRbcS* increased and decreased, respectively, with all treatments, except for low temperature to which *HvRbcS* is not responsive. The expression of *Arabidopsis CBF/DREB1* genes is induced within 15 min of exposure to low temperature (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998). Thus, the expression of *HvCBF4* is similar to that

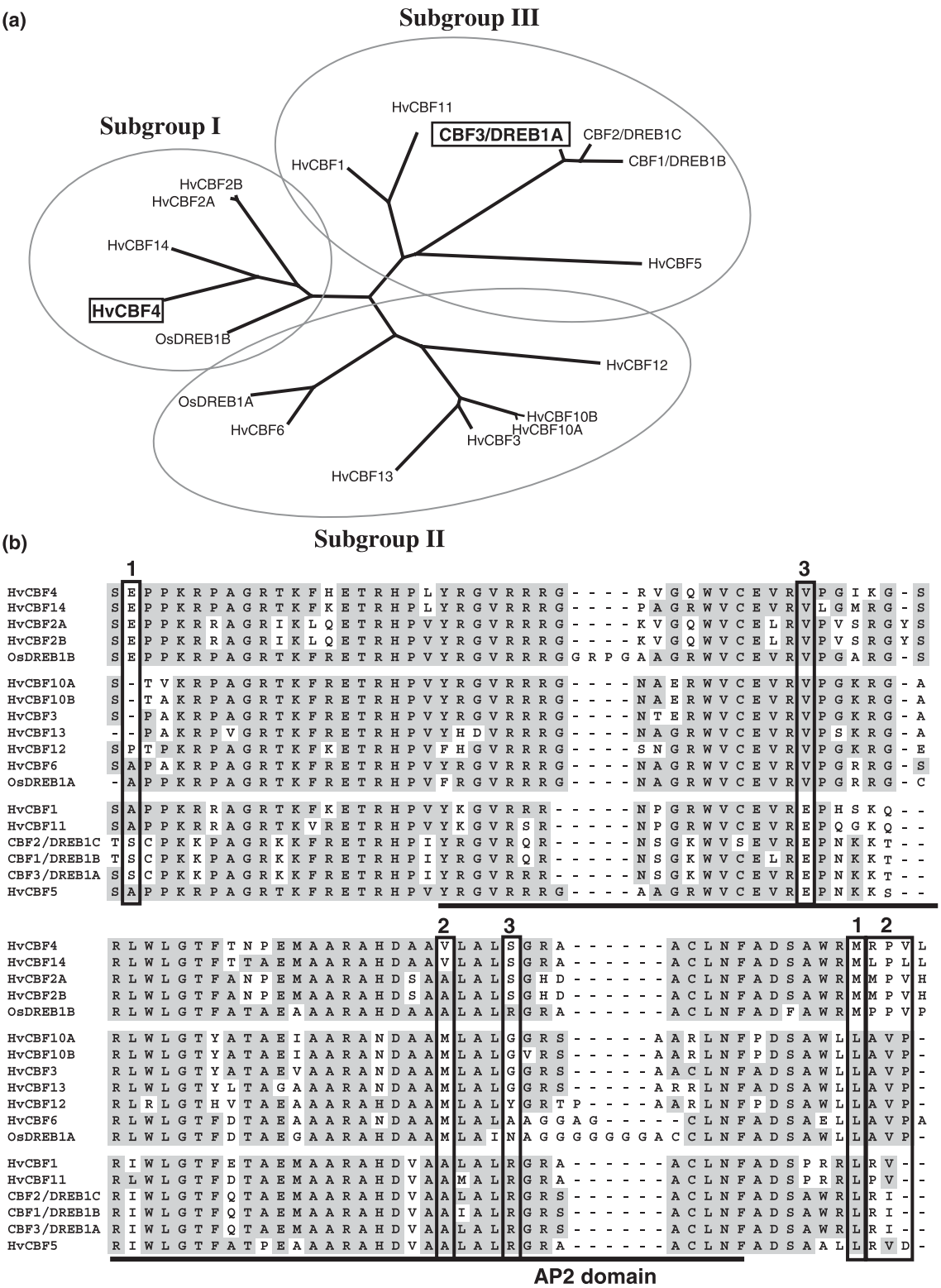

Figure 1 Comparison of C-repeat/dehydration-responsive element binding factor (CBF/DREB) family members from barley, rice and *Arabidopsis*. (a) Phylogenetic relationship of CBF/DREB family in barley, rice and *Arabidopsis*. Phylogenetic dendrogram was derived from an alignment of the deduced amino acid sequences of the 13 barley (Skinner *et al*., 2005), three *Arabidopsis* (Gilmour *et al*., 1998) and two rice (Dubouzet *et al*., 2003) CBF/DREBs. Circles indicate subgroups I, II and III. (b) Deduced amino acid sequences of the AP2 domains and their flanking regions of the CBF/DREB family members were aligned using the CLUSTAL W program. The AP2 DNA binding domain is shown. Signature motifs are indicated by boxes with numbers 1, 2 and 3 that are specific to subgroups I, II and III, respectively.

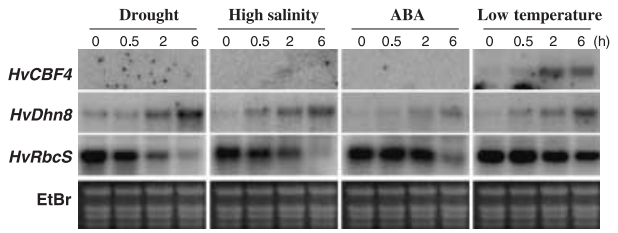

Figure 2 Expression of *HvCBF4* increases in response to low temperature in barely. Ten micrograms of total RNA from plants exposed to drought, high salinity (400 mM NaCl), abscisic acid (ABA) (100 μM) and low temperature (4 °C) for the indicated time points was blotted and hybridized to *HvCBF4* (AF298230), barley *Dhn8* (*HvDhn8,* AF181458) and barley *RbcS* (*HvRbcS*, U43493) gene-specific probes. Ethidium bromide (EtBr) staining of total RNA was used for equal loading of RNAs.

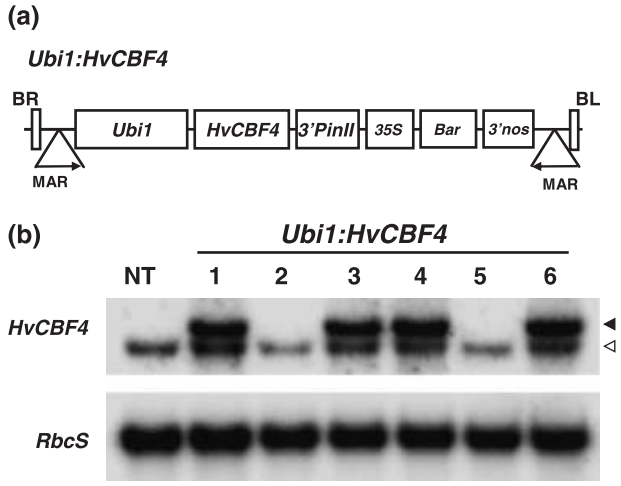

Figure 3 Production of *Ubi1:HvCBF4* transgenic rice plants. (a) *Ubi1:HvCBF4* consists of the maize *ubiquitin* promoter (*Ubi1*) linked to the *HvCBF4* coding region and the 3′-region of the potato *proteinase inhibitor II* gene (*3′PinII*), and a gene expression cassette that contains the *35S* promoter, the *bar* coding region and the 3′-region of the *nopaline synthase* gene (*3′nos*). The entire expression cassette is flanked by the 5′-*matrix attachment region* (*MAR*) of the chicken lysozyme gene (Phi-Van and Stratling, 1996). (b) RNA gel-blot analysis was performed using total RNAs from young leaves of six independent lines of *Ubi1:HvCBF4* plants and non-transgenic (NT) control plants. The blots were hybridized with the full-length *HvCBF4* gene (filled arrowhead) and reprobed with the rice *RbcS* gene (Kyozuka *et al*., 1993) for equal loading of RNAs. An open arrowhead indicates transcripts of an endogenous rice orthologue of HvCBF4.

of *Arabidopsis CBF/DREB1* in that it is induced only by low temperature; however, the time of induction is different.

Production and growth of *HvCBF4* transgenic rice plants

To study the role of *HvCBF4* in transgenic rice plants, a plasmid for rice transformation, *Ubi1:HvCBF4* (Figure 3a), was constructed in which the gene was under the control of the maize (*Zea mays*) ubiquitin1 promoter including its first intron (*Ubi1;* Christensen and Quail, 1996). Fifteen independent transgenic lines were produced using the *Agrobacterium*-mediated transformation method (Hiei *et al*., 1994). Their T_{1-4} seeds were collected. The copy numbers and integration events of the transgene in *Ubi1:HvCBF4* plants were determined by genomic Southern blots, which revealed that all were independent and that the copy numbers of the transgene varied from one to three. Expression levels of the transgene in six independent lines were examined by RNA-blot analysis using total RNAs from leaf tissues. As shown in Figure 3b, *HvCBF4* transcripts were detectable in four lines (1, 3, 4 and 6) at levels much higher than those in endogenous rice orthologues. The rice orthologues cross-hybridized with the *HvCBF4* probe may include genes with accession numbers of AK105599, XM483621 and AY327040. These share more than 85% nucleotide sequence identity with the AP2 region of *HvCBF4*. In addition, unlike the expression of *HvCBF4* in barley (Figure 2), their expression was detectable under normal growth conditions, as evidenced by DNA microarray experiments (Oh *et al*., 2005; Gene Expression Omnibus accession number of microarray data set is GSE2211). T_4 generations of the four homozygous lines were chosen for further analysis.

Transgenic over-expression of the *CBF/DREB* genes in *Arabidopsis* caused stunted growth under normal growth conditions (Kasuga *et al*., 1999; Gilmour *et al*., 2000; Dubouzet *et al*., 2003). Severe stunting, however, was not observed in our *Ubi1:CBF3/DREB1A* transgenic rice plants (Oh *et al*., 2005). To examine the growth phenotype of *Ubi1:HvCBF4* plants, their seeds, together with *Ubi1:CBF3/DREB1A* and non-transgenic (NT) seeds, were germinated, and their heights were measured following germination. *Ubi1:HvCBF4* plants showed normal vegetative growth and fertility, as in *Ubi1:CBF3/DREB1A* and NT plants, without notable differences in their heights (Figure S1, see 'Supplementary material'). However, it should be noted that stunting was observed in some transgenic lines with *HvCBF4* and *CBF3/DREB1A*, particularly in the earlier generations, such as T_0 and T_1. There was no correlation between stunting of transgenic lines and expression levels of *HvCBF4* and *CBF3/DREB1A*. We believe that stunting is caused by somaclonal variations that occur during tissue culture and transformation. This results in the normal growth of such transgenic plants as they are grown for more generations, as the somaclonal variations are diluted in successive generations by repeated selfing and selection. By contrast with our *Ubi1:CBF3/DREB1A* and *Ubi1:HvCBF4* rice plants (cv. Nakdong) that grew normally, *Ubi1:CBF3/DREB1A* and *Ubi1:OsDREB1A* rice plants (cv. Kita-ake) showed various levels of stunting (Ito *et al*., 2006). Although it cannot

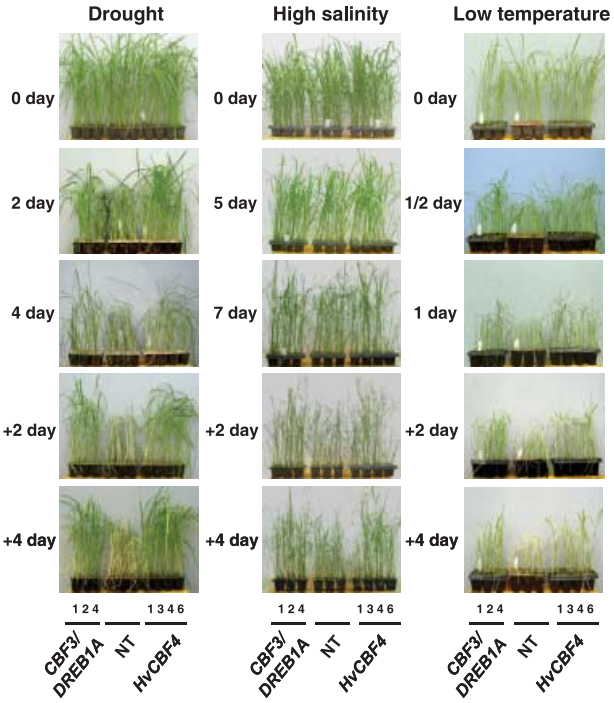

Figure 4 Appearance of plants during drought, high-salinity and low-temperature stresses. Three and four independent T_4 homozygous lines for *Ubi1:CBF3/DREB1A* and *Ubi1:HvCBF4*, respectively, and non-transgenic (NT) seedlings were grown in the glasshouse for 4 weeks and subjected to various stress treatments. For drought and high-salinity treatments, 4-week-old NT and transgenic seedlings were subjected to 4 days of drought and 7 days of 400 mM NaCl solution, respectively, followed by 4 days of watering in the glasshouse. For low-temperature treatment, 3-week-old NT and transgenic seedlings were incubated at 4 °C in a cold chamber under continuous light of 150 μmol/m²/s for 1 day, followed by 4 days of normal growth conditions in the glasshouse. Photographs were taken at the indicated time points; + followed by number of days denotes days of rewatering or recovery under normal growth conditions.

be ruled out that cultivars may grow differently, we believe that the discrepancy in stunting between the two transgenic rice groups with the same transgene reflects the different transgenic generations of the plants investigated.

Stress tolerance in *Ubi1:HvCBF4* plants

To investigate whether the over-expression of *HvCBF4* was correlated with stress tolerance in transgenic plants, 4-week-old NT and transgenic seedlings were subjected to drought, high-salinity and low-temperature stresses (Figure 4). After stress treatments, the plants of each line showed wilting and rolling of leaves with a concomitant loss of chlorophyll. Overall, NT plants exhibited leaf rolling and other stress-induced symptoms earlier than did transgenic lines. NT plants were affected by 2 days of drought stress, whereas *Ubi1:HvCBF4* and *Ubi1:CBF3/DREB1A* plants were affected similarly by 4 days of drought stress. Both transgenic lines recovered faster than NT plants on removal of stress. For example, after 4 days of drought stress and subsequent watering for 4 days, the growth of both transgenic lines was almost identical to that of non-stressed control plants, and 98% survived (Table 1). In contrast, the growth of drought-stressed NT plants was severely inhibited, and over 94% of the plants never recovered and finally died. Similarly, both transgenic lines responded significantly better to high-salinity and low-temperature treatments than did NT plants. *Ubi1:HvCBF4*

Table 1 Survival of *Ubi1:HvCBF4* and *Ubi1:CBF3/DREB1A* plants under stress conditions

	Plants	Total¶	Survival**	Survival rate (%)††
Drought*§				
NT		108	7	6
CBF3/DREB1A	1	54	52	96
	2	54	54	100
	4	54	53	98
HvCBF4	1	54	53	98
	3	54	54	100
	5	54	54	100
	6	54	52	93
High salinity†§				
NT		108	11	10
CBF3/DREB1A	1	54	49	91
	2	54	52	96
	4	54	51	94
HvCBF4	1	54	47	87
	3	54	52	96
	5	54	51	94
	6	54	50	93
Low temperature‡§				
NT		108	4	4
CBF3/DREB1A	1	54	44	81
	2	54	46	85
	4	54	49	91
HvCBF4	1	54	47	87
	3	54	46	85
	5	54	46	85
	6	54	50	93

*Four-week-old soil-grown plants withheld water for 4 days, followed by watering for 4 days and results scored.
†Four-week-old soil-grown plants treated by 400 mM NaCl for 7 days, followed by watering for 4 days and results scored.
‡Three-week-old soil-grown plants exposed to low temperature for 1 day, followed by normal growth conditions for 4 days and results scored.
§Plants were considered as dead if there was no regrowth after 4 days of rewatering or the return of normal growth conditions. NT represents non-transgenic control plants that had not been transformed.
¶Total number of plants used in each assay.
**Number of surviving plants.
††Percentage of surviving plants (survived/total × 100).

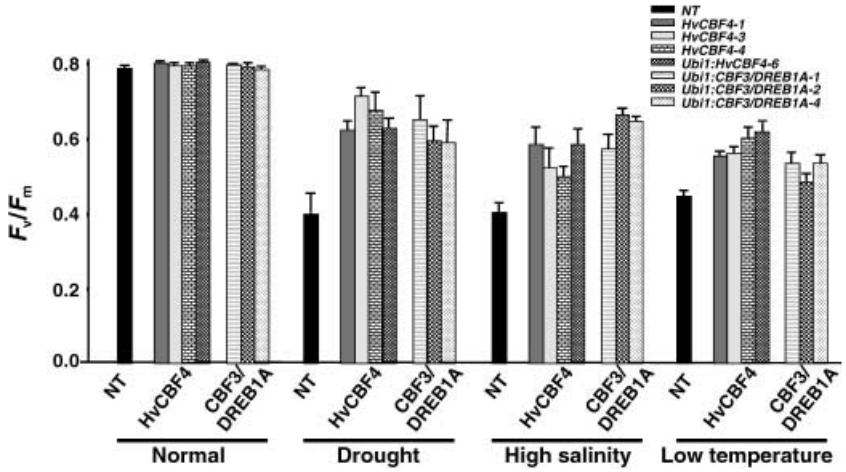

Figure 5 Changes in chlorophyll fluorescence (maximum photochemical efficiency of photosystem II in the dark-adapted state, F_v/F_m, where F_v is the variable fluorescence and F_m is the maximum fluorescence) during drought, high-salinity and low-temperature stresses. Three and four independent T_4 homozygous lines for *Ubi1:CBF3/DREB1A* and *Ubi1:HvCBF4*, respectively, and non-transgenic (NT) seedlings grown on Murashige–Skoog (MS) medium for 14 days were subjected to various stress conditions as described: for drought stress, the seedlings were air dried for 2 h at 28 °C and, for high-salinity stress, the seedlings were exposed to 400 mM NaCl for 2 h at 28 °C. For low-temperature stress, the seedlings were exposed to a temperature of 4 °C for 6 h. After treatment, F_v/F_m was measured using a pulse-modulated fluorometer (mini-PAM, Walz, Germany). All experiments were carried out under continuous light of 150 μmol/m²/s. Each data point represents the mean ± standard error of triplicate experiments (n = 6).

and *Ubi1:CBF3/DREB1A* plants showed survival rates of over 91% and 87%, respectively, whereas less than 10% of NT plants survived under such stress conditions. The enhanced stress tolerance of *Ubi1:HvCBF4* plants was further verified by measuring changes in chlorophyll fluorescence. After leaf discs of 14-day-old transgenic and NT seedlings had been exposed to drought, high-salinity and low-temperature stresses, the reduction in the maximum photochemical efficiency of photosystem II in the dark-adapted state (F_v/F_m, where F_v is the variable fluorescence and F_m is the maximum fluorescence) was measured. Levels of F_v/F_m were approximately 30% higher in both *Ubi1:HvCBF4* and *Ubi1:CBF3/DREB1A* plants than in NT plants under drought and high-salinity conditions (Figure 5). Interestingly, under low-temperature conditions, F_v/F_m levels in *Ubi1:HvCBF4* plants were higher by 20% and 10% than those of NT and *Ubi1:CBF3/DREB1A* plants, respectively. Together, these results indicate that the over-expression of barley *HvCBF4* and *Arabidopsis CBF3/DREB1A* in rice confers increased tolerance to drought, high-salinity and low-temperature stresses, and that the tolerance to low temperature is greater in plants over-expressing *HvCBF4*.

HvCBF4 and CBF3/DREB1A activate different groups of target genes in rice

To identify the genes that are up-regulated by HvCBF4 in rice plants, we performed gene expression profiling on the *Ubi1:HvCBF4* plants in comparison with NT plants under normal growth conditions. Profiling was conducted using the 60K Rice Whole Genome microarray (GreenGene Biotech, Yongin, South Korea; Oh *et al.*, 2005). RNA samples from leaf tissues of 14-day-old transgenic and NT seedlings were used to generate cyanine-5 (Cy5) and Cy3-labelled complementary DNA (cDNA) probes, respectively, which were then hybridized to the microarray. Expression analyses with three biological replicates identified 15 different genes with 1.6-fold greater induction in transgenic plants than in NT plants (Table 2; Table S1, see 'Supplementary material'). We have previously identified 12 target genes of *CBF3/DREB1A* in rice (Oh *et al*., 2005). HvCBF4 or CBF3/DREB1A-induced expression of the candidate genes was subsequently confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 6, lanes 1–3). Our results revealed that HvCBF4 activates 15 rice genes, including *aminotransferase* (*ATase*), *cytochrome P450* (*CytP450*), *chromatin assembly factor-I* (*CA factor-I*), *proline-rich protein* (*Pro-rich*) and *leucine-rich repeat protein* (*LRR*). Five of these genes, *Jacalin1*, *Jacalin2* (*Jac2*), *Bowman Birk trypsin inhibitor 1* (*BBTI 1*), *lipoxygenase* (*LOX*) and *acetyltransferase*, are commonly activated by both HvCBF4 and CBF3/DREB1A. To investigate whether the target genes are stress inducible and/or further induced by the corresponding factors under stress conditions, we measured the transcript levels of the target genes (five HvCBF4-specific, two CBF3/DREB1A-specific and two common genes) by RT-PCR

Table 2 List of genes up-regulated in *HvCBF4* and *CBF3/DREB1A* plants under normal growth conditions. Numbers in bold represent the genes induced 1.6-fold or more in *HvCBF4* and *CBF3/DREB1A* plants

Gene name	Accession number*	DRE1†	DRE2‡	ABRE§	HvCBF4¶	CBF3/DREB1A¶
Jacalin2 (*Jac2*)	AK101991	1	0	0	**15.08**	**3.32**
Jacalin1 (*Jac1*)	AK066682	0	0	4	**13.14**	**3.57**
Bowman Birk trypsin inhibitor 1 (*BBTI 1*)	AK065846	0	0	0	**2.72**	**1.83**
Lipoxygenase	AJ270938	0	1	2	**2.31**	**3.11**
Acetyltransferase	AK065433	0	2	4	**2.17**	**3.46**
Aminotransferase (*Atase*)	XM_468881	1	2	0	**8.67**	1.04
Leucine-rich repeat (*LRR*)	AK104310	0	1	0	**6.15**	–1.13
Cytochrome P450 (*Cyt P450*)	AK070167	1	3	0	**4.38**	–1.37
Unknown	AK103314	0	0	0	**3.55**	–1.23
Proline-rich protein (*Pro-rich*)	AK058887	0	1	0	**2.59**	–1.12
Unknown	AK074005	2	2	3	**2.22**	–1.56
β-Ketoacyl-CoA synthase	AK101377	0	0	0	**2.19**	–1.21
Chromatin assembly factor-I (*CA factor-I*)	AK109038	0	2	0	**2.15**	–1.04
α-L-Fucosidase	AK109462	0	0	0	**2.12**	–1.03
Inter-specific proline-rich protein	AK058562	0	0	0	**2.00**	–1.23
Lip5	AB011368	2	0	0	–1.09	**2.35**
Glutelin	AK107238	0	1	0	1.41	**2.18**
Hsp70	AK072830	1	0	0	–1.13	**2.03**
Receptor kinase containing LRR repeats	AK119823	1	1	0	–1.04	**1.68**
Dip1	AY587109	2	0	0	1.11	**1.66**
Bowman Birk trypsin inhibitor 2	AK105455	1	0	3	1.23	**1.65**
PP2C	XM463364	0	1	2	1.19	**1.65**

*GenBank accession numbers for full-length cDNA sequences of corresponding genes.

†DRE1 (G/ACCGAC), ‡DRE2 (G/ATCGAC) or §ABRE (C/TACGTGG/T) sequence observed in the promoter regions within 1 kb upstream of the ATG start codon.

¶Each number represents the mean fold induction in transcript levels of transgenic plants relative to those of non-transgenic plants under normal growth conditions.

using total RNAs from transgenic and NT seedlings that were exposed to drought, high-salinity and low-temperature stresses (Figure 6, lanes 4–12). In NT plants, eight of 15 HvCBF4 target genes were induced by low-temperature stress, and four, *Lip5*, *Dip1*, *BBTI 1* and *Jac2*, were induced by all stresses. Interestingly, levels of *CytP450*, *Jac2*, *Lip5* and *Dip1* transcripts were higher in *Ubi1:HvCBF4* plants than in *Ubi1:CBF3/DREB1A* plants under low-temperature conditions. In particular, levels of *Lip5* and *Dip1* transcripts in *Ubi1:HvCBF4* plants were higher than those in NT plants under low-temperature conditions. These results may account for the higher level of tolerance of *Ubi1:HvCBF4* plants than *Ubi1:CBF3/DREB1A* plants to low temperature. Two important target genes of CBF3/DREB1A, *Lip5 and Dip1*, however, were not activated in *HvCBF4* plants under normal growth conditions. These results are similar to those obtained in *Arabidopsis* over-expressing *35S:HvCBF4*, which did not activate CBF3/DREB1A target genes such as *cor15a* and *cor47*, *Arabidopsis* orthologues of *Lip5* and *Dip1*, under normal growth conditions (Skinner *et al*., 2005). The latter was thought to be because HvCBF4 and HvCBF2, members of subgroup I, have low-temperature-dependent DNA binding activity (Xue, 2003; Skinner *et al*., 2005). It is similarly possible that HvCBF4 binds to the promoters of *Lip5* and *Dip1* in a low-temperature-dependent manner, resulting in enhanced expression of the genes. Xue (2003) reported that HvCBF2, a member of the HvCBF4 subgroup (Figure 1), interacts not only with G/aCCGAC (DRE1), but also with G/aTCGAC (DRE2). When the copy numbers of the core conserved sequence elements in promoter regions of the rice target genes were searched, multiple copies were found to be located within 1 kb upstream of the ATG start codon (Table 2). HvCBF4 target genes, such as *ATase*, *LRR*, *CytP450*, *Pro-rich* and *CA factor-I*, carry more DRE2 than DRE1, whereas CBF3/DREB1A target genes, such as *Lip5* and *Dip1*, carry more DRE1 than DRE2 (Table 2). It would be interesting to determine whether HvCBF4 binds to DRE1 and/or DRE2 in a low-temperature-dependent manner. Overall, our results demonstrate that HvCBF4 enhances tolerance to low temperature by activating target genes that are different from those of CBF3/DREB1A in transgenic rice.

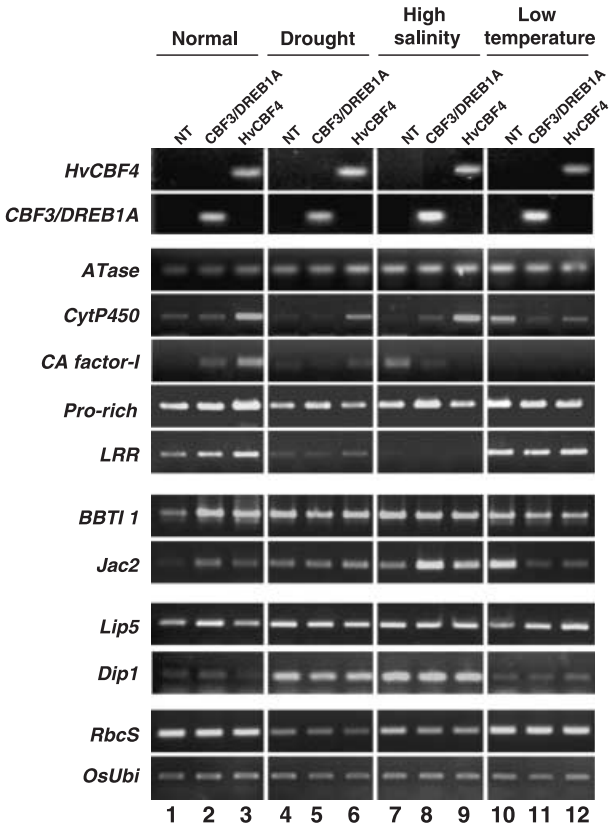

Figure 6 Induction of stress-related genes in *Ubi1:CBF3/DREB1A* and *Ubi1:HvCBF4* plants under normal and stress conditions. T_4 homozygous lines for *Ubi1:HvCBF4*, *Ubi1:CBF3* and non-transgenic (NT) seedlings were grown in the glasshouse for 14 days. Transgenic and NT plants were then treated for 2 h with drought (seedlings were excised and air dried for 2 h) or high salinity (400 mM NaCl) in the glasshouse, and with low-temperature stress (4 °C) in a cold chamber under continuous light of 150 μmol/m^2/s. Transcript levels of *CBF3/DREB1A*, *HvCBF4* and their target genes were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) using gene-specific primer pairs of the corresponding genes (Table 2; Table S2). The rice *ubiquitin* gene, *OsUbi* (Kim *et al*., 1994), was used as equal loading control. See text and Table 2 for definition of gene abbreviations.

Experimental procedures

Expression levels of *HvCBF4* gene in barley

Barley (*Hordeum vulgare* cv. Morex) seeds were germinated in soil and grown in the glasshouse (16 h light/8 h dark cycle) at 22 °C. For high-salinity and ABA treatments, 14-day-old seedlings were transferred to nutrient solution containing 400 mM NaCl or 100 μM ABA for the indicated time course in the glasshouse under continuous light of approximately 1000 μmol/m^2/s. For drought treatment, 14-day-old seedlings were excised and air dried for the indicated time course under continuous light of approximately 1000 μmol/m^2/s. For low-temperature treatments, 14-day-old seedlings were exposed at 4 °C in a cold chamber for the indicated time course under continuous light of 150 μmol/m^2/s. The preparation of total RNA and RNA gel-blot analysis were performed as reported previously (Jang *et al*., 2002). The probes for *HvDhn8*, *HvRbcS* and *HvCBF4* were RT-PCR amplified from barley total RNA using primer pairs of the corresponding genes with the RT-PCR system (Promega, WI, USA), according to the manufacturer's instructions. The primer pairs were as follows: *HvCBF4* probe DNA, forward primer (5′-GACGAGGAGCAGTGGTTTGG-3′) and reverse primer (5′-GAATCGGATGAGATCCTCCT-3′); *HvRbcS* probe DNA, forward primer (5′-GCATCATCGGATTCGACAAC-3′) and reverse primer (5′-TAGCCACCGATAGCATTCAC-3′); *HvDhn8* probe DNA, forward primer (5′-CCGCGTCTGTTCAGACTCAC-3′) and reverse primer (5′-ACAAGACAGAGGAGGAGGAC-3′). Hybridization signals were captured using a phosphor image analyser (FLA 3000, Fuji, Tokyo, Japan).

Plasmid construction and transformation of rice

The expression plasmid, *Ubi1:HvCBF4*, contains the *35S:bar* cassette for herbicide-based selection and a pair of matrix attachment region sequences from chicken lysozyme gene for stable expression of the transgene (Phi-Van and Stratling, 1996; Oh *et al*., 2005). The *ubiquitin1* promoter, together with its intron (*Ubi1*), was used to drive constitutive expression (Christensen and Quail, 1996). The *HvCBF4* cDNA clone was kindly provided by Dr D.W. Choi (Choi *et al*., 2002). The *Ubi1:HvCBF4* plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating, and embryogenic (*Oryza sativa* cv. Nakdong) calli from mature seeds were transformed as described previously (Jang *et al*., 1999).

Growth measurements

Transgenic and NT rice (*O. sativa* cv. Nakdong) seeds were germinated in half-strength Murashige–Skoog (MS) solid medium in a growth chamber in the dark at 28 °C for 3 days, transplanted into soil pots and grown in the glasshouse (16 h light/8 h dark cycle) at 28–30 °C. Each pot (5 × 5 × 6 cm) was filled with nursery soils (Bio-media, Kyeongju, South Korea) and planted with six seedlings. The height of the plants was determined over time. Each experiment was repeated three times with three or four independent transgenic lines.

Stress tolerance of plants grown in soil

Transgenic and NT rice (*O. sativa* cv. Nakdong) seeds were germinated in half-strength MS solid medium in a growth chamber in the dark at 28 °C for 3 days, transplanted into soil and grown in the glasshouse (16 h light/8 h dark cycle) at 28–30 °C. All transgenic and NT lines were grown in pots (5 × 5 × 6 cm; six plants/pot). For drought and high-salinity stress, 4-week-old NT and transgenic seedlings were subjected to 4 days of drought and 7 days of 400 mM NaCl solution, respectively, followed by 4 days of watering in the glasshouse. For low-temperature treatment, 3-week-old NT and transgenic seedlings were incubated at 4 °C in a cold chamber under continuous light of 150 μmol/m^2/s for 1 day, followed by 4 days of normal growth conditions in the glasshouse. The numbers of plants that survived or continued to grow were scored.

Chlorophyll fluorescence under stress conditions

Transgenic and NT rice (*O. sativa* cv. Nakdong) seeds were germinated and grown in half-strength MS solid medium for 14 days in a growth chamber (16 h light at 150 μmol/m^2/s, 8 h dark at 28 °C). The green parts of approximately 10 seedlings were cut by scissors before stress treatments *in vitro*. For low-temperature stress, the seedling parts were incubated in 4 °C water for up to 6 h under continuous light of 150 μmol/m^2/s. For high-salinity stress, the seedling parts were incubated in 400 mM NaCl for 2 h at 28 °C under continuous light of 150 μmol/m^2/s and, for drought stress, they were air dried for 2 h at 28 °C under continuous light of 150 μmol/m^2/s. The F_v/F_m values of transgenic and NT plants were measured with a pulse-modulated fluorometer (mini-PAM, Walz, Germany), as described previously (Jang *et al*., 2003; Oh *et al*., 2005).

60K Rice Whole Genome DNA chip analysis

Expression profiling was conducted with the 60K Rice Whole Genome microarray. This information can be found at www.ggbio.com/rice60kchip.html (GreenGene Biotech), and has been reported previously by Oh *et al*. (2005). Total RNA (100 μg) was prepared from leaf tissues of 14-day-old transgenic and NT rice (*O. sativa* cv. Nakdong) seedlings (5–10 plants each), as reported previously (Jang *et al*., 2002), and messenger RNA (mRNA) was purified from total RNAs using a Qiagen Oligotex column (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Preparation of fluorescent probes, hybridization, image scanning and statistical analysis of microarray data have been described previously by Oh *et al*. (2005). To assess the reproducibility of microarray analysis, each experiment was repeated three times, including dye swapping with three independent lines.

RT-PCR analysis

Transgenic and NT rice (*O. sativa* cv. Nakdong) seeds were germinated on soil and grown in the glasshouse (16 h light/8 h dark cycle). For drought stress, 14-day-old seedlings were excised and air dried in the glasshouse under continuous light of approximately 900–1000 μmol/m^2/s. For high-salinity stress, 14-day-old seedlings were grown in a nutrient solution, 0.1% (v/v) Hyponex (Hyponex, Busan, South Korea), for 2 days and then transferred to fresh nutrient solution containing 400 mM NaCl in the glasshouse under continuous light of approximately 900–1000 μmol/m^2/s. For low-temperature stress treatment, 14-day-old seedlings were exposed to a temperature of 4 °C in a cold chamber under continuous light of 150 μmol/m^2/s. After harvesting at 2 h of exposure, total RNA was extracted as reported previously (Jang *et al*., 2002).

For the detection of the transcript levels of target genes, RT-PCR was performed with the cDNA synthesis system for RT-PCR (Invitrogen), according to the manufacturer's instructions. PCR products were amplified using gene-specific primers designed from the coding sequence of each gene by Primer Designer 4 software (Sci-ed Software, Cary, NC, USA). RT-PCR was carried out using primer pairs (listed in Table S2, see 'Supplementary material') at a final concentration of 10 pM each and 2 μL (equivalent to 5 ng of total RNA) of cDNA as template. PCR was performed at 95 °C for 10 min for denaturing of template cDNAs, followed by 20–25 cycles of 94 °C for 30 s, 57 °C for 30 s and 68 °C for 1 min. Each PCR product was resolved on a 2% agarose gel. To validate our RT-PCR results, we repeated the experiment twice with independently prepared total RNA.

Acknowledgements

This work was supported by the Ministry of Science and Technology through the Crop Functional Genomics Centre (grant to J.-K.K. and S.I.S.), by the Biogreen21 Programme (grant to J.-K.K. and S.I.S.) and by the Korea Science and Engineering Foundation through the Plant Metabolism Research Centre at Kyung-Hee University (grant to J.-K.K.).

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Growth characteristics of *Ubi1:CBF3/DREB1A* and *Ubi1:HvCBF4* plants. (a) Growth phenotypes of three or four independent T_4 homozygous lines for *Ubi1:CBF3/DREB1A*, *Ubi1:HvCBF4* and non-transgenic (NT) control plants at the indicated days after germination (DAG). (b) Average height of *Ubi1:CBF3/DREB1A*, *Ubi1:HvCBF4* and NT plants. Plants grown in the glasshouse during the same time course as shown in (a) were harvested and their heights were measured. Each data point represents the mean ± standard deviation of triplicate experiments with corresponding lines.

Table S1 List of genes that are up-regulated in HvCBF4 and CBF3/DREB1A plants under normal growth conditions. Microarray data sets of three biological replicates are included

Table S2 List of primers used for reverse transcriptase-polymerase chain reaction (RT-PCR), shown in Figure 6

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