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Inducible and constitutive expression of *HvCBF4* in rice leads to differential gene expression and drought tolerance

T. LOURENÇO[1], N. SAIBO[1], R. BATISTA[1,2], C. PINTO RICARDO[1] and M.M. OLIVEIRA[1]*

ITQB-Universidade Nova de Lisboa, Laboratório de Genómica de Plantas em Stress,
Quinta do Marquês, 2784-505 Oeiras, Portugal[1]
Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida Padre Cruz, 1649-016 Lisboa, Portugal[2]

Abstract

The effects of the ectopic expression of a barley transcription factor (*HvCBF4*) under the control of a constitutive (maize Ubi1) or a stress-inducible (*Arabidopsis* RD29A) promoter in the abiotic stress response in rice (*Oryza sativa* L.) was investigated. The transformed plants were analyzed both at molecular and physiological level and the AtRD29A::*HvCBF4* plants were further analyzed using the *GeneChip®* rice genome array under control conditions. Only the plants constitutively expressing *HvCBF4* have shown increased survival to drought stress, but not to cold or high-salinity. These plants have also shown better photosynthetic capacity, as determined by chlorophyll fluorescence. Plants expressing AtRD29A::*HvCBF4* did not show increased survival to any of the stresses applied. However in the GeneChip® microarray, these plants have shown up-regulation of many stress-responsive genes (> 400) as compared to non-transformed plants. Interestingly, RT-PCR analysis revealed not only differential gene expression between roots and shoots, but also between transgenic lines with the different promoters. Our results indicate that different *HvCBF4* expression levels resulted in different transcriptomes and drought tolerance. Given that AtRD29A::*HvCBF4* plants did not show increased tolerance to any of the imposed stresses, we may conclude that this promoter may be inappropriate for rice transformation aiming for enhanced abiotic stress tolerance.

Additional key words: abiotic stress, chlorophyll fluorescence, microarray analysis, *Oryza sativa*, transcription factors, transformation.

Introduction

The impact of abiotic stresses such as drought, cold or high salinity seriously limits plant productivity worldwide and is responsible for crop losses by as much as 50 % (Boyer 1982). In order to respond to abiotic stresses, plants have evolved common mechanisms at molecular, cellular, and physiological level (Thomashow 1999). A variety of genes are induced upon abiotic stress, some of which function in gene expression regulation and signal transduction (Nakashima and Yamaguchi-Shinozaki 2006, Hu *et al.* 2010). The search and characterization of regulatory genes, such as the transcription factors, is therefore seen as a possible way to identify key modulators of the abiotic stress response. Transcription factors (TFs) can recognize and bind specific sequences (*cis*-motifs) in the promoter region of target genes activating or inhibiting target gene expression. Transcriptome analysis has revealed that one of the highest representative classes present in response to stress treatment are TFs (Agarwal and Jha 2010). Yamaguchi-Shinozaki and Shinozaki (1994) identified a dehydration-responsive element (DRE) core motif (A/GCCGAC) in the promoter of the stress inducible *Arabidopsis RD29A* gene. A similar motif was also found in the promoter of the *Arabidopsis cor15a* gene which was named C-repeat (CRT) (Baker *et al.* 1994). Promoter analyses have shown that several other stress-induced genes had the same DRE/CRT motif upstream the starting codon and that this motif was essential

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Abbreviations: Φ_{PS2} - efficiency of the photosystem 2 photochemistry; ETR - electron transfer rate; qP - photochemical quenching; qN - non-photochemical quenching; RWC - relative water content.

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* Corresponding author; fax: (+351) 214411277, e-mail: mmolive@itqb.unl.pt

for the binding of a specific class of TFs belonging to the AP2 (APETALA 2)/ERF (ethylene responsive factor) superfamily and were named as DREB (DRE binding) or CBF (CRT binding factor) (Stockinger *et al.* 1997, Gilmour *et al.* 1998, Liu *et al.* 1998). The expression of these genes is quickly and transiently induced by cold-stress but not by dehydration or salt stress and defines an abscisic acid (ABA) independent pathway for cold tolerance.

Since a single transcription factor can alter the expression of several genes, a transgenic approach with DREB1/CBF transcription factors was planned to facilitate coping with abiotic stresses (Wang *et al.* 2003, Zhang *et al.* 2004, Umezawa *et al.* 2006,). Transgenic *Arabidopsis* have been generated overexpressing the *DREB1A/CBF3*, *DREB1B/CBF1* and *DREB1C/CBF2*, and the plants have shown an increased tolerance not only to cold, but also to drought and salt stresses (Jaglo-Ottosen *et al.* 1998, Liu *et al.* 1998, Kasuga *et al.* 1999, Gilmour *et al.* 2000). Several target genes from the CBF regulon have been identified in *Arabidopsis* over the past recent years using different approaches, with either cDNA or *GeneChip* microarrays (Seki *et al.* 2001, Vogel *et al.* 2005). The majority of these genes have at least one DRE/CRT core *cis*-motif in the promoter region (*e.g.* late embryogenesis abundant, LEA, proteins). The overexpression of the *Arabidopsis DREB1/CBF* genes have been used to transform other plants. The generated plants (either dicots or monocots) have shown increased abiotic stress tolerance (Hsieh *et al.* 2002, Pellegrineschi *et al.* 2004, Bhatnagar-Mathur *et al.* 2007). These results also revealed that even cold sensitive species (*e.g.* rice) had a functional downstream CBF regulon (Oh *et al.* 2005).

However, in several cases, the transgenic plants showed an unwanted dwarf phenotype with flowering retardation. The relation between *DREB1/CBF* genes and growth retardation is still unknown but the DREB1A/CBF3 downstream target gene *STZ* may be involved in growth retardation by negatively regulating the expression of photosynthesis and sugar metabolism related genes (Sakamoto *et al.* 2004). Rice plants overexpressing the *DREB1A/CBF3* gene and not showing stunting growth have already been reported (Oh *et al.* 2005). One strategy used to minimize the growth retardation effects is to use the stress-inducible *RD29A* gene promoter (Kasuga *et al.* 2004, Behnam *et al.* 2007, Pino *et al.* 2007) to drive the ectopic expression of *DREB1/CBF* genes.

Efforts have also been made to identify *DREB1/CBF* genes in other species than *Arabidopsis*. CBF regulon genes have been identified and isolated from *Brassica napus*, rice, wheat and barley, among others (Jaglo *et al.* 2001, Dubouzet *et al.* 2003, Skinner *et al.* 2005). Some of these genes have been overexpressed in *Arabidopsis* and the plants have shown an increased abiotic stress tolerance revealing a similar stress-response mechanism across-species (Dubouzet *et al.* 2003, Skinner *et al.* 2005). Dicots like *Arabidopsis* have 6 identified *DREB1/CBF* genes (Haake *et al.* 2002) while monocots like rice or barley have a larger gene family. Large *DREB1/CBF* gene families are typical of cereals irrespectively of the level of low-temperature tolerance (Skinner *et al.* 2005).

The translated protein of *HvCBF4* gene (accession number AF298230) is a 225 amino acids long transcription factor previously isolated from barley (*Hordeum vulgare* cv. Morex) which has a high tolerance to low-temperature (Choi *et al.* 2002). The HvCBF4 transcription factor belongs to the AP2/ERF superfamily and it has the typical signature motif of the DREB1/CBF family flanking each side of the AP2 (PKK/RPAGRxKFxETRHP and DSAWR). According to Skinner *et al.* (2005) the monocot DREB1/CBF transcription factors family can be divided into three subgroups. The HvCBF4 defines a subgroup of monocot DREB1/CBF with a typical response different from the other subgroups. The rice CBF member phylogenetically closer to HvCBF4 is OsDREB1B (Fig. 1). The expression of *HvCBF4* was found to be induced by cold stress but unaltered by salt, drought or ABA treatment (Skinner *et al.* 2005, Oh *et al.* 2007). HvCBF4 binds to DRE/CRT core motif in a cold-dependent manner (Skinner *et al.* 2005). Oh *et al.* (2007) transformed the barley *HvCBF4* gene into rice (*Oryza sativa* L., cv. Nakdong). The overexpression of this gene driven by the maize Ubi1 promoter in rice produced plants with increased abiotic stress tolerance without stunting growth.

In our work, we compared the effects of the ectopic expression of the *HvCBF4* transgene driven by either a constitutive promoter (maize ubiquitin promoter, Ubi1) or a stress-inducible one (*Arabidopsis RD29A* gene promoter). We have performed physiological analyses of the transgenic plants and analyzed one transgenic line for global gene expression changes using the *Affymetrix* rice whole genome *GeneChip*. The results comparing the two transgenic and the non-transformed (NT) lines are discussed.

Materials and methods

The *GATEWAY*® (*Invitrogen*, Carlsbad, USA) system was used to prepare the gene constructs used in this work. We cloned the *HvCBF4* cDNA from a plasmid kindly provided by Dr. Timothy Close (Choi *et al.* 2002) using *GATEWAY*® designed primers (GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAATGGACGTCGCCGACA and GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATTAGCAGTCGAACAAATAGCTC, the underlined regions are the *attB* regions for recombination on destiny *GATEWAY®* vector). The amplified *HvCBF4* cDNA was then cloned in plasmids derived from the pH7WG2 (University of Ghent, Belgium) having the maize Ubi (Christensen and Quail 1996) or the AtRD29A promoters instead of the CaMV 35S promoter (Saibo *et al.*, unpublished results) on the 5'-end of the *GATEWAY®* cassette. The Ubi::*HvCBF4* or the AtRD29A::*HvCBF4* gene constructs were then introduced in the appropriate *Agrobacterium tumefaciens* strain (LBA4404) using a freeze-thaw protocol.

The phylogenetic trees were done using the *MEGA 4* software (http://www.megasoftware.net) (Tamura *et al.* 2007). The phylogenetic trees were generated through the Neighbour-Joining methodology on 1000 bootstrap replications.

For the production of transgenic rice (*Oryza sativa* L.) plants, we used cv. Nipponbare and a protocol based essentially on Hiei *et al.* (1994) with modifications (Rueb *et al.* 1994). Rice seeds were dehusked and treated with a solution of 1 g dm^{-3} of *Benlate* for 30 min at 50 ºC for elimination of possible fungal contamination. After two washes with sterile water, seeds were surface sterilized with ethanol (70 %, v/v) for 1 min with gentle shaking. The seeds were then washed twice with sterile water and further sterilized with a 2 % (v/v) sodium hypochlorite solution with 2 - 3 drops of *Tween® 20* (*Sigma*, St. Louis, USA) for 20 - 25 min with agitation. The seeds were then washed thoroughly with sterile water and placed on callus induction medium for 4 weeks (sub-cultured every 2 weeks). Embryogenic callus tissues were selected and co-cultivated with *Agrobacterium tumefaciens* (LBA4404 strain) for 3 d at 25 ºC in the dark. Then calluses were transferred to selection medium supplemented with 50 mg dm^{-3} of hygromycin B (*Duchefa*, Haarlem, The Netherlands) for 4 - 5 weeks at 28 ºC in darkness. Hygromycin-resistant callus were then transferred to regeneration medium for 2 - 3 weeks at 28 ºC under 12-h photoperiod (irradiance of 900 µmol m^{-2} s^{-1}). Regenerating plantlets (2 - 3 cm high) were transferred to glass tubes (capped with cotton) with plantlet development medium supplemented with 25 mg dm^{-3} of hygromycin B for 1 week at the same temperature and photoperiod. Hygromycin-resistant plantlets were transferred to fresh plantlet development medium without hygromycin B for another 2 weeks until a good rooting system developed. Plantlets were then transferred to pots filled with soil mixture (2:2:1, v/v/v, soil:turf:*Vermiculite*) and placed in the glasshouse. The plants were grown for further analysis and allowed to self-pollinate to retrieve progeny. As controls, non-transgenic rice cv. Nipponbare was used.

For PCR analysis the CTAB protocol (Doyle and Doyle 1987, Cullings 1992) was used to extract DNA from the leaf samples. The DNA was analyzed for its integrity using primers to amplify the rice *actin* gene. For determining if the plants had the T-DNA expression cassette integrated in its genome, we used the *HvCBF4* primers (Fw - GACCAA GTTCCACGAGACG and Rv - GCAGTCGAACAAATAGCTCCA) for the gene of interest, the *hpt*II gene primers (Fw - AATAGCTGC GCCGATGGTTTCTACA and Rv - AACATCGCCTCG CTCCAGTCAATG) for the selectable marker gene and the maize Ubi1 (Fw - TCTCGAGAGTTCCGCTCCAC and Rv - ATCTAGAACGACCGCCCAAC) or the AtRD29A (Fw - <u>CGTACGAAGCT</u>TGGAGGAGCC ATAGATGCAA and Rv - <u>CGGGATCCCGCTCTAGAG</u> CCAAAGATTTTTTTCTTTCCAATAG, cloning adaptors are underlined) primers for the promoter fragment. Only the plants with positive PCR amplification for all the components analyzed of the T-DNA expression cassette were grown and allowed to self-pollinate to retrieve T$_1$ progeny. Southern blotting analysis was used to confirm stable integration of the transgene using an Ubi1 fragment as probe (Fw - TCTCGAGAG TTCCGCTCCAC and Rv - ATCTAGAACGACCG CCCAAC) with the *Amersham* (Healthcare, UK) non-radioactive and chemiluminescent kit following the manufacturer's instructions.

Two-week old transgenic and not-transformed (NT) plants were used for the stress treatments. The seedlings were grown in Yoshida's medium (Yoshida *et al.* 1976) for 2 weeks at 28 ºC, a 12-h photoperiod, irradiance of 900 µmol m^{-2} s^{-1} and air humidity of 70 % in a climatic chamber (*Aralab*, Albarraque, Portugal). For cold stress, 2-week-old plantlets were transferred to fresh Yoshida's medium and placed in a climatic chamber at 10 ºC with an irradiance of 200 µmol m^{-2} s^{-1} for 4 d. After cold treatment, the plantlets were transferred back to 28 ºC and irradiance of 900 µmol m^{-2} s^{-1} for 1 week prior to evaluation of plant survival. For salt treatment, 2-week-old plantlets were transferred to Yoshida's medium supplemented with 100 mM of NaCl at 28 ºC (irradiance of 900 µmol m^{-2} s^{-1}) for 1 week. Plantlets were transferred to Yoshida medium for another week and plant survival was measured. The drought stress treatments were performed at irradiance of 900 µmol m^{-2} s^{-1} and watering was suspended for 12 d after which plants were again watered for 1 week.

For the growth measures, transgenic (from both lines) and NT plants were grown as described above for two weeks and then transferred to soil mixture and to the glasshouse. The plants were allowed to self-pollinate and growth measurements were determined afterwards.

For chlorophyll fluorescence analyses, 2-week old transgenic and NT plants were used. Fluorescence measurements were made on the youngest fully expanded leaf during the drought-stress treatment using a *Mini-PAM* (*Walz*, Effetrich, Germany) and data retrieved using the *Da-Teach* software. The selected leaf was dark-adapted for 6 min after which a measuring light was switched on giving the minimum fluorescence (F$_0$). A pulse of saturating light was then applied allowing the measurement of maximum fluorescence (F$_m$) in the dark-adapted state giving also maximum quantum yield of the photosystem (PS) 2 (determined as F_v/F_m ratio, where variable fluorescence $F_v = F_m - F_0$). In this system, an actinic light is applied at 120 and 600 µmol m^{-2} s^{-1} for 5 min after which a saturating flash is again applied allowing the determination of the F_v'/F_m' ratio (efficiency of the PS 2 photochemistry). After each flash of saturating light, a pulse of far-red light is given to empty the PS 2 reaction centres. With this protocol we were able to measure the electron transport rate (ETR), the photochemical quenching (qP) and non-photochemical quenching (qN).

The leaf relative water content (RWC) was measured on the youngest fully expanded leaf analysed using a previously described protocol (Barr and Weatherley 1962).

Two independent sets of six seedlings from the transgenic line and NT control were used for extraction with the RNeasy Plant *Mini* kit (*Qiagen*, Hilden, Germany) following the manufacturer's instructions. The RNA was processed for *GeneChip*® hybridization in the *Affymetrix* core facility located at the Instituto Gulbenkian de Ciência (Oeiras, Portugal). The rice array used has to query 51.279 probe transcripts from 2 rice subspecies (*japonica* and *indica*). The *GeneChip*® hybridization was performed in duplicate.

The data generated in this experiment was analysed using the *Partek* genomics suite software. The *Affymetrix CEL* files were imported using robust multi-chip average (RMA) method which includes $\log_2$ transformation of data. For the identification of differentially expressed genes we used the analysis of variance (*ANOVA*) and the false discovery rate (FDR) with a $P < 0.05$ threshold.

Four plantlets were then dried at room temperature on bench top for 2 h for drought stress treatment. Total RNA from roots and shoots separately was extracted using *TRIZOL*® (*Invitrogen*) reagent following the manufacturer's protocol. For cDNA synthesis we further treated the total RNA with DNAse in the *RNA-EZ* columns from *Qiagen*.

For cDNA synthesis we used 500 ng of total RNA using the *Invitrogen* cDNA synthesis kit following the manufacturer's instructions using oligo-dT as primer for first-strand synthesis. PCR was performed using the following conditions: 95 ºC for 5 min for denaturing cDNA, 25 - 28 cycle of 95 ºC for 1 min, 53 - 57 ºC for 1 min and 72 ºC for 1 min. A final extension step was made for 5 min at 72 ºC. The rice *actin* gene cDNA was used as control.

The list of primers used for target gene expression by RT-PCR is available in Table 1. Each PCR was repeated twice for validation.

Table 1. Primer pairs were designed using the *Primer 3* software using the longest gene cDNA available in the public database. Fw - left-forward primer, Rv - the right-reverse primer.

Affy ID	Locus	Gene name	Primer pair
Os.51923.1.S1_at	Os12g05440	cytochrome P_{450}	Fw - TCAGCTACGAGCACCTGAAGC Rv - AACGCGTCTGATCTTCACTGG
Os.54944.1.S1_at	Os02g52670	AP2/ERF domain	Fw - CGAGGGGAGCAGGTACAG Rv - GCGGGGAACAGTAGTAAAGG
OsAffx.20377.1.S1_x_at	Os10g25620	putative NAM	Fw - CAACGTCGTACATGCTGGAA Rv - CGTCTCCATCACCTCCATCT
OsAffx.27442.1.s1_at	Os06g03670	transcription factor CBF1	Fw - CCATGATGATGCAGTACCAG Rv - AAAATCTCCATTAATTTCTCCTACAG
Os.4181.1.s1_at	Os01g67980	cysteine proteinase precursor	Fw - GCGTTCGAGTACATCAAGCA Rv - ATCGATCGACCTCACCTCAC
Os.6092.1.S1_at	Os02g44230	trehalose-6-phosphate phosphatase	Fw - CTGAACATTTGAAGTGCAACG Rv - AGGACATTTTGCCATCCAAG
Os.49249.1.S1_at	Os02g52710	α-amylase precursor	Fw - CACCACCAAGGGCATCCT Rv - TCGTGGACAATTGCATCC
Os.51718.1.S1_at	Os11g26780	Rab16b-dehydrin	Fw - AAGCTCCAGCTCGTCGTCT Rv - ACAAAGCTTGCAATGGCATC
Os.6863.1.S1_at	Os12g14440	Jacalin 1	Fw - CTTGTTCAGGGCAACCAGAT Rv - TGCCATAGCAAACTGTCCTG
Os.23518.1.A1_at	Os02g36190	cytochrome P450	Fw - TCCCAGTTACCGACGAAATC Rv - GCCAAATGGAAGGAATTCAA
Os.4614.1.S1_a_at	Os02g40260	leucine-rich repeat protein	Fw - ATTCTAAGGCGCTCCTCCTC Rv - GCAGTTGTTCCTGACGTTGA
Os.11398.1.S1_at	Os10g05980	proline-rich protein	Fw - CTAGTTCTCCTCGGCGTTTG Rv - ACGATCTTGGATGGCTCTTG

Results

To study the effect of the *HvCBF4* expression in rice plants, we cloned the gene in order to have either constitutive or inducible expression. For this purpose, we used either a constitutive promoter, maize ubiquitin 1 promoter including its first intron (maize Ubi1) or a stress-inducible promoter (RD29A promoter from *Arabidopsis*) (Fig. 2*A*). Transgenic homozygous T_1 plants from each genetic construct were allowed to self-pollinate and their progeny was used on subsequent tests. The expression levels of the transgene were analysed on the T_2 plants from each line using RT-PCR (Fig. 2*B*). Both lines expressed the transgene, but the AtRD29A::*HvCBF4* line only showed increased expression levels after a few hours of stress imposition

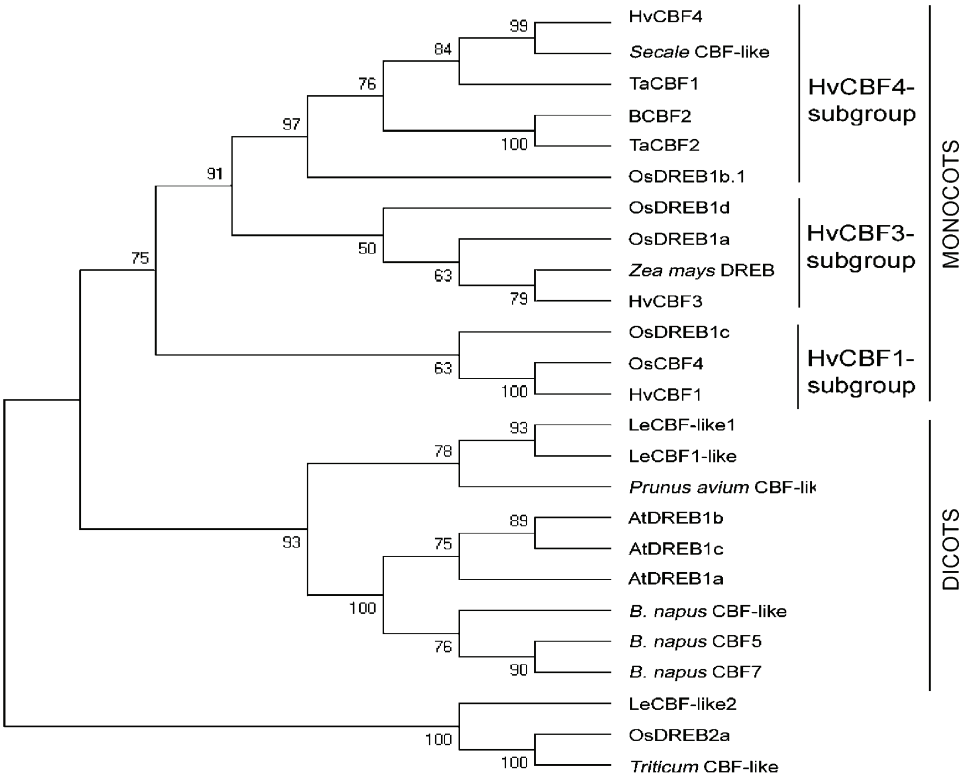

Fig. 1. Phylogenetic analysis between DREB1/CBF AP2-domain amino acids sequences. The phylogenetic tree was generated using the Neighbour-Joining methodology using a refined alignment of the DREB1/CBF proteins (accession numbers: HvCBF4 (AAK01088), *Secale*-CBF like (AAL35761), TaCBF1 (AAL37944), BCBF2 (AAM13419), TaCBF2 (AAX28961), OsDREB1b.1 (AAX28958), OsDREB1d (AAX23721), OsDREB1a (AAN02486), *Zea mays* DREB (AAN76804), HvCBF3 (AAX23692), OsDREB1c (AAP92125), OsCBF4 (BAD29237), HvCBF1 (AAL84170), *Lycopersicum esculentum* CBF-like 1 (AAS77819), *Lycopersicum esculentum* CBF1-like (AAS77820), *Prunus avium* CBF-like (BAD27123), AtDREB1b (NP_567721), AtDREB1c (BAA33436), AtDREB1a (BAA33434), *Brassica napus* CBF-like (AAL38242), *Brassica napus* CBF5 (AAM18958), *Brassica napus* CBF7 (AAM18959), LeCBF-like 2 (AAN77051), OsDREB2a (AAN02487) and *Triticum aestivum* CBF-like (ABA08424)). The monocot and dicot DREB1/CBF amino acids sequences are phylogenetic separated (*vertical bars*) and monocot functional subgroups (according to Skinner *et al*. 2005) are also shown in *vertical bars*. As outliers 3 DREB2 proteins (DREB1/CBF related but without typical signature motifs) were included in the alignment.

Table 2. Growth and agronomic characteristics of transgenic and NT mature plants. Plants were grown to maturity and self-pollinate. After seed setting, plants were harvested for growth and agronomical measurements (means ± SE, $n \geq 5$).

Plant line	Plant height [cm]	Panicle length [cm]	Filled grains	Empty grains	Total flowers
Ubi::*HvCBF4*	53.40±2.07	12.56±0.86	32.40±5.59	7.60±4.93	40.00±3.39
AtRD29A::*HvCBF4*	69.80±6.34	14.80±1.15	60.80±18.13	14.60±12.76	77.40±27.77
NT	67.30±4.18	14.20±0.91	59.80±8.17	2.00±1.41	61.80±8.47

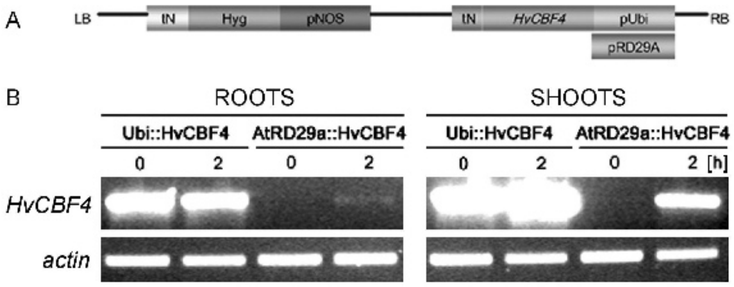

Fig. 2. Production of transgenic rice (*Oryza sativa* L., cv. Nipponbare) plants. *A* - Two genetic constructs were used for transformation of rice plants with different promoters. RB - right border; LB - left border; pUbi - maize ubiquitin promoter for constitutive expression of the trangene of interest; pRD29A - *Arabidopsis* stress-inducible promoter from *RD29A* gene; HvCBF4 - transgene of interest from barley; tN - *nopaline synthase* gene terminator; pNOS - *nopaline synthase* gene promoter; Hyg - *hpt*II gene selectable marker for antibiotic resistance to hygromycin B. *B* - Reverse-transcriptase PCR in the T_2 homozygous lines expressing the *HvCBF4* gene. Total RNA was extracted from 2-week-old seedlings (according to the experimental procedures) under normal conditions (0 h) or after 2 h of drought stress. The seedlings were also separated in roots and shoots for total RNA extraction. The rice *actin* gene was used as internal control of the RT-PCR.

Table 3. Parameters of chlorophyll *a* fluorescence and RWC [%] in transgenic and NT plants during development of water stress. Means ± SD, $n \geq 4$.

Parameters	0 d	2 d	3 d	4 d	6 d	8 d	9 d	11 d
Ubi:: *HvCBF4*								
Φ_{PS2}	0.308±0.054	0.425± 0.006	0.415±0.048	0.456±0.035	0.454±0.028	0.443±0.031	0.409±0.010	-
ETR	77.225±13.49	107.000±1.390	103.767±11.39	114.570±5.450	114.425±0.111	111.419±5.717	103.050±2.475	-
qP	0.575±0.091	0.732±0.012	0.692±0.056	0.717±0.011	0.790±0.036	0.766±0.027	0.747±0.010	-
qN	0.711±0.033	0.604±0.004	0.596±0.094	0.588±0.046	0.620±0.044	0.619±0.032	0.656±0.028	-
RWC	97.0 ±0.8	98.0 ±1.8	-	99.0 ±1.4	100.0	95.0 ±1.4	-	86.0±5.7
AtRD29A:: *HvCBF4*								
Φ_{PS2}	0.346±0.083	0.406±0.060	0.397±0.042	0.383±0.010	0.353±0.033	0.326±0.011	0.252±0.024	-
ETR	87.050±20.96	102.375±15.18	100.138±11.08	96.575±2.555	88.900±8.276	82.125±2.869	82.125±2.237	-
qP	0.583±0.126	0.713±0.076	0.720±0.056	0.731±0.014	0.655±0.048	0.657±0.031	0.536±0.013	-
qN	0.512±0.025	0.598±0.069	0.669±0,019	0.727±0.045	0.667±0.029	0.728±0.033	0.753±0.009	-
RWC	96. 0 ±2.2	100.0	-	96.0 ±5.0	94.0 ±4.1	60.0 ±5.8	-	18.0±8.7
NT								
Φ_{PS2}	0.316±0.053	0.343±0.035	0.345±0.048	0.407±0.020	0.370±0.030	0.323±0.034	0.315±0.004	-
ETR	79.686±13.22	86.450±8.952	86.950±12.20	102.600±4.937	93.233±7.577	81.425±8.538	79.375±1.047	-
qP	0.599±0.082	0.644±0.047	0.706±0.077	0.725±0.033	0.702±0.037	0.617±0.056	0.663±0.037	-
qN	0.668±0.061	0.658±0.058	0.679±0.076	0.626±0.010	0.670±0.046	0.712±0.014	0.724±0.018	-
RWC	96.0 ±3.7	96.0 ±2.5	-	96.0 ±3.5	80.0 ±3.2	69.0 ±3.3	-	49.0±6.0

(*e.g*. drought) (Fig. 2*B*). However, a low but consistent leaky-expression, of the *HvCBF4* driven by the AtRD29A promoter in unstressed plants, could be observed when the amplification program included 35 cycles (data not shown).

In the Ubi::*HvCBF4* transgenic plants we observed a stunted growth (Table 2). The AtRD29A::*HvCBF4* plants also showed some growth reduction at seedling stage probably due to the "leaky-expression" of the promoter. However, during vegetative growth and seed setting conditions AtRD29A::*HvCBF4* and NT plants did not show significant differences in plant height and number of fertile seeds (Table 2). The reduced growth observed at seedling stage of Ubi::*HvCBF4* transgenic line was also observed during vegetative growth and seed setting (Table 2).

In order to understand if the transgenic rice plants expressing the *HvCBF4* gene were more tolerant to abiotic stress, we subjected two week-old seedlings to drought, high-salinity and cold stress. No significant differences were observed when comparing survival rates of transgenic *versus* NT lines, under cold or high-salinity (data not shown). However, after 12 d of drought stress, the transgenic line Ubi::*HvCBF4* revealed increased drought tolerance when compared to NT. Interestingly, the AtRD29A::*HvCBF4* line showed lower tolerance to drought than the NT line. This behaviour was corroborated by the leaf relative water content (RWC)

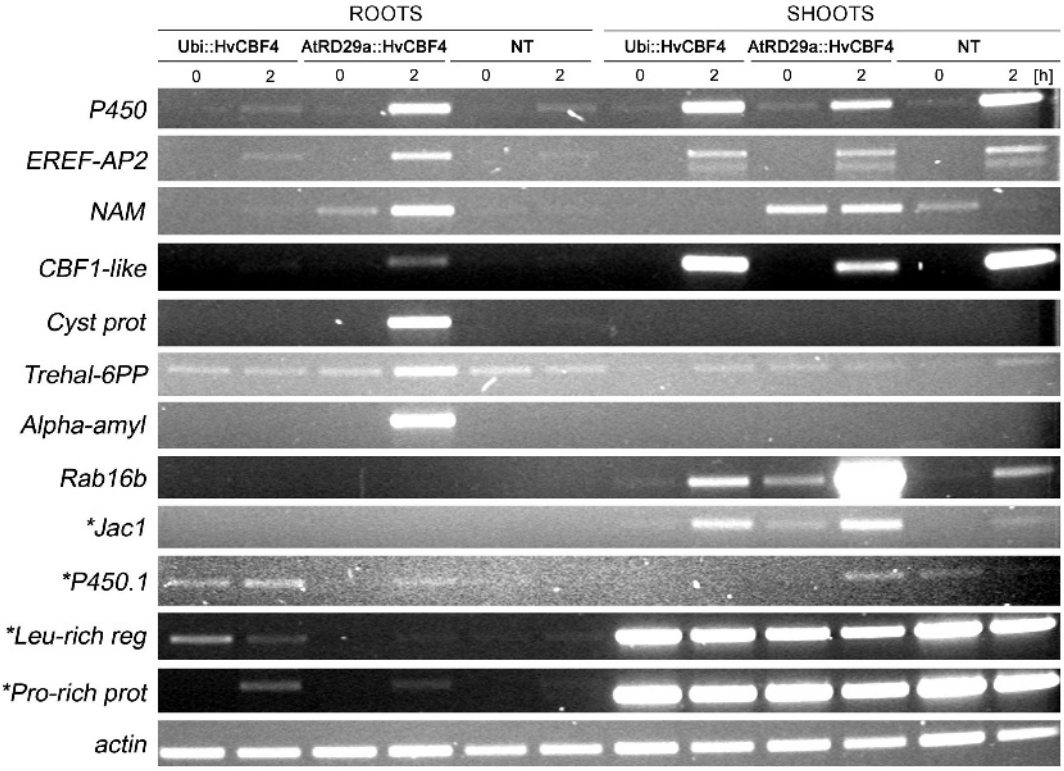

Fig. 3. Gene expression analyses between transgenic and NT plants under normal and drought stress conditions. The changes in gene expression were analyzed using target gene specific primer pairs for PCR using the cDNA prepared. The genes accession numbers used in this analysis were: *cytochrome P450* (AK064287), *EREF-AP2* gene domain (AK107146), *no-apical meristem* (*NAM*) (no full-length cDNA in database, affymetrix code OsAffx.20377.1.S1_x_at), *CBF1-like* gene (AY327040), *cysteine proteinase precursor* (D76415), *trehalose-6-phosphate phosphatase* gene (AK103391), *alpha-amylase precursor* gene (AK101744), *Rab16b-dehydrin* gene (AK063517) *Jacalin 1* gene (AK066682), *cytochrome P450.1* (AK070167), *leucine-rich repeat protein* gene (AK104310) and *proline-rich protein* gene (AK058887). The genes marked with an *asterisk* were described in the work of Oh and colleagues as direct targets of HvCBF4. As control, PCR was performed using specific primer pairs for the rice *actin* gene.

measurements (Table 3). These results agree with the visual observations of the leaves. The leaves of AtRD29A::*HvCBF4* plants started to show drought symptoms later than those of NT, but the transgenic plants showed more leaves wilted and rolled by the end of the drought stress. On the other hand, Ubi::*HvCBF4* plants showed a survival rate 70 % higher than NT and a RWC close to 85 % by the end of the drought stress assay (Table 3). By visual observation, the leaves of this transgenic line showed drought symptoms much later than those of NT or other transgenic line. After rehydration, the Ubi::*HvCBF4* leaves showed recovery from drought stress symptoms within hours, while most of the NT and AtRD29A::*HvCBF4* plants failed to survive.

Transgenic and NT plants were also evaluated for the integrity of their PS 2 during drought stress by measuring changes in chlorophyll fluorescence. At normal growth conditions, minor differences were observed between transgenic lines and NT plants in terms of the maximum quantum yield of PS 2 (F_v/F_m) (data not shown). However, under drought stress, differences were already clearly visible (Table 3), with Ubi::*HvCBF4* plants showing a higher quantum yield than NT or AtRD29A::*HvCBF4* plants (Table 3). In line with these results were changes in several other parameters, such as ETR, qP and qN (Table 3). During stress, both NT and AtRD29A::*HvCBF4* plants revealed a higher amount of closed reaction centres and thus lower qP values. Moreover, Ubi::*HvCBF4* plants consistently showed lower heat dissipation (qN) as compared to the other two lines, revealing a better adaptation to drought stress.

Changes in the expression profile of the transgenic plants *versus* NT plants were analyzed using the rice whole genome *GeneChip*® array (*OMNIBUS* accession numbers: Nipponbare control plants GSM304478 and GSM304485; AtRD29A::*HvCBF4* transgenic plants GSM304497 and GSM304646). Transgenic AtRD29A::*HvCBF4* plants were used to monitor, in the absence of imposed drought stress, the putative effect of the HvCBF4 leaky-expression on the alteration of the expression profile of rice genes. Ubi::*HvCBF4* rice plants had been previously used in transcriptomic experiments by Oh (2007). In order to identify differentially expressed genes (with a cut-off $P < 0.05$), a $\log_2$ transformation was performed and genes with a 2-fold difference in expression (as compared to the controls) were selected for further analyses. More than 500 genes were considered differentially expressed (with a 2-fold change after $\log_2$ transformation) and most of them (over 450) were up-regulated in the absence of stress imposition. We also analyzed the promoter region of the genes with altered expression pattern in search of *cis*-acting motifs. The majority of the genes had not only DRE 1 and/or DRE 2 (Xue 2003) *cis*-acting motifs, but also ABA responsive elements (ABRE), although no correlation could be established between the number/type of *cis*-motifs and the fold-change observed. The closest *cis*-motifs to the ATG starting codon were also analyzed for these genes.

In order to validate the results obtained with the *GeneChip*®, we also used RT-PCR to analysed the expression level of several genes showing up-regulation. For comparison, we also used genes that showed up-regulation in the microarray analysis conducted by Oh *et al.* (2007) on transgenic Ubi::*HvCBF4* rice plants (cv. Nakdong). The expression of the selected genes was compared in the transgenic rice lines and NT plants, under control and imposed drought conditions (2 h of drought stress) in roots and shoots (Fig. 3). Interestingly, we found that *HvCBF4* plants did not activate the same genes when constitutively expressed (driven by Ubi1) as when under control of stress-inducible AtRD29A (Fig. 3). However, the Ubi::*HvCBF4* plants we produced, did in fact activate the expression of some genes previously described by Oh *et al.* (2007; *P450*, *Leu-rich repeat*, *Pro-rich protein*). The transgenic plants with the stress-inducible promoter AtRD29A indeed activated the genes that showed up-regulation in the *Affymetrix* gene chip, an effect especially evident after 2 h of drought. The transgenic and NT plant lines analyzed, still showed other differences in gene expression patterns, such as gene activation occurring preferentially in roots rather than in shoots (Fig. 3).

Discussion

In the present work we have generated transgenic rice plants (cv. Nipponbare) ectopically expressing the *HvCBF4* gene from barley (Choi *et al.* 2002) under the influence of a constitutive promoter (maize Ubi1 promoter) (Christensen and Quail 1996) or a stress inducible one (*Arabidopsis* RD29A promoter). Transgenic plants expressing *HvCBF4* have not showed increased tolerance to cold or to high-salt stress. This is different from what Oh *et al.* (2007) have found. These divergent results may be due to the different rice cultivar used (Nipponbare vs. Nakdong) and thus different genomic background, leading to the activation/repression of a different set of genes. Also divergent from the results of Oh *et al.* (2007), in our case, Ubi::*HvCBF4* transgenic plants have shown growth retardation (Table 2) which is a common undesired effect of transformation assays using transcription factors (Kasuga *et al.* 1999, Ito *et al.* 2006). *HvCBF4* may activate different genes in the two cultivars thus inducing growth retardation in one and not in the other. Another possible explanation is possible somaclonal variation occurring during the *in vitro* culture (Oh *et al.* 2007).

Aiming to understand in physiological terms how the transgenic plants respond to drought stress, we also performed chlorophyll fluorescence analyses in these transgenic plants. In this study, we compared transgenic lines *versus* NT plants in terms of maximum photochemical efficiency of PS 2 (F_v/F_m) in normal growth conditions and under drought stress. The transgenic Ubi::*HvCBF4* plants have shown a higher quantum yield when under drought stress (Table 3) as compared to the AtRD29A::*HvCBF4* and NT plants especially at higher photosynthetic active radiation (PAR). This result reveals that, the photochemistry of Ubi::*HvCBF4* plants functions better under drought conditions than that of the AtRD29A::*HvCBF4* and NT plants. In laboratory conditions there is a strong correlation between the quantum yield of PS 2 and the efficiency of carbon fixation (Maxwell and Johnson 2000). However a careful interpretation must be made because under certain stresses a discrepancy between these two parameters may occur (Fryer *et al.* 1998). When analyzing the qP and the qN, Ubi::*HvCBF4* plants showed a higher amount of open reaction centres than AtRD29A::*HvCBF4* and NT plants. These results agree with the data obtained for quantum yield and ETR (Table 3). The AtRD29A::*HvCBF4* and NT plants revealed an increased proportion of closed PS 2 reaction centres. This may be due to photosynthesis saturated by light, although stomatal closure is another possible explanation. Less CO_2 uptake and less carbon fixation in the Calvin cycle can lead to the closure of the reaction centres in the PS 2 site. The changes of qN also agree with this hypothesis since, upon closure of PS 2 reaction centres, the excess energy not used in photosynthesis has to be dissipated as heat. Taken together, these results revealed that, under drought stress, the Ubi::*HvCBF4* plants were photosynthetically more efficient than the other two plant lines. This may be due to some kind of thylakoid protection, better stomatal regulation and/or more efficient water uptake from the soil. However, to test these hypotheses further analyses are required, for instance assessing carbon fixation rates and stomatal conductances. Similar results were obtained by Savitch *et al.* (2005) who overexpressed in *Brassica napus* two *CBF/DREB1*-like genes from the same species, resulting in increased photosynthetic capacity. In these studies, the non-acclimated transgenic plants showed higher photochemical efficiency in photosynthesis, and increased capacity for linear electron transfer (ETR) (Savitch *et al.* 2005).

The RWC results obtained here (Table 3) have shown that Ubi::*HvCBF4* plants maintained a better water content during the whole drought stress period, which may result from either better stomatal regulation of water loss or better water uptake by the roots.

The low-temperature dependent binding of HvCBF4 and the phylogenetically related HvCBF2 (Xue 2003) to the DRE/CRT motif was previously described in barley (Skinner *et al.* 2005). In rice, gene activation occurred even in normal conditions revealing that, at least in this species, low-temperature is not needed for binding and gene activation. In a recent work (Oh *et al.* 2007) identified 15 rice genes as *HvCBF4* targets. However, our results showed that some of the 15 rice genes that Oh *et al.* (2007) found as being activated by the constitutive expression of *HvCBF4* had little or no expression changes when the gene was driven by the AtRD29A promoter (Fig. 3). We could detect more than 500 genes differentially expressed in the AtRD29A::*HvCBF4* plants in control conditions, as well as almost all the genes selected from the top-50 up-regulated genes, had a DRE1 (G/ACCGAC) or DRE2 (G/ATCGAC) (Xue 2003) *cis*-motif in the promoter region (data not shown). In fact, the HvCBF4 transcription factor may act as a master-switch, because several other transcription factors showed up-regulation, namely a *NAM*, an *AP2/ERF* domain TF, a putative *CRT/DRE factor1*, a transcription factor *CBF1*, and a *WRKY*-binding domain TF. Since other *DREB1/CBF* genes were activated, it is not possible to identify which genes are the direct targets of the HvCBF4 protein. Additionally, several other stress-related genes have been activated in our array, like cytochromes P_{450} and an α-amylase (Ito *et al.* 2006, Oh *et al.* 2007), genes encoding proteins such as trehalose-6-phosphate phosphatase (Garg *et al.* 2002) and a dehydrin Rab16b, known to respond to water stress (Ono *et al.* 1996), and a cysteine proteinase precursor.

To validate the *Affymetrix* array data, we further analyzed the expression of several genes by RT-PCR, including some genes detected by other authors when over-expressing DREB1/CBF in rice (Ito *et al.* 2006, Oh *et al.* 2007). Ubi::*HvCBF4* expression was found to activate the expression of genes previously reported by Oh *et al.* (2007), both under control and stress conditions (Fig. 3), but not those found up-regulated by the AtRD29A::*HvCBF4* in *Affymetrix GeneChip*. In contrast, in RT-PCR analyses, AtRD29A::*HvCBF4* led to the activation of the genes we selected from the microarray study, mainly in roots (Fig. 3).

The explanation of the differential gene expression activation depending on the promoter used is still unclear. Apparently, the low expression levels of *HvCBF4* in plants carrying AtRD29A-driven constructs (Fig. 2*B*) was enough to induce expression changes even in stress absence due to a leaky-expression of the promoter (data not shown). This leaky-expression of AtRD29A promoter has also been previously reported (Pino *et al.* 2007). Nevertheless, despite the fact that several stress-related genes were up-regulated, these plants failed to survive under drought, cold or salt stress. This may be due to a better function of the RD29A promoter in roots than in shoots (Ito *et al.* 2006) or to promoter failure during long stress periods. The Ubi::*HvCBF4* plants were able to survive drought stress and the plants showed consistently better chlorophyll fluorescence parameters as compared to the other transgenic line and NT. However, none of the genes that showed up-regulation in the *Affymetrix* rice array could be amplified by RT-PCR for this line (Fig. 3). The Ubi::*HvCBF4* plants could, however, activate the expression of genes described in Oh *et al.* (2007) as targets of HvCBF4 (Fig. 3).

To the best of our knowledge, the identification in a *GeneChip* microarray of a discrepancy in gene expression activation as a result of regulation by different promoters had not been previously reported.

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