Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth[1][w]

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Rice (*Oryza sativa*), a monocotyledonous plant that does not cold acclimate, has evolved differently from Arabidopsis (*Arabidopsis thaliana*), which cold acclimates. To understand the stress response of rice in comparison with that of Arabidopsis, we developed transgenic rice plants that constitutively expressed *CBF3/DREB1A* (*CBF3*) and *ABF3*, Arabidopsis genes that function in abscisic acid-independent and abscisic acid-dependent stress-response pathways, respectively. CBF3 in transgenic rice elevated tolerance to drought and high salinity, and produced relatively low levels of tolerance to low-temperature exposure. These data were in direct contrast to CBF3 in Arabidopsis, which is known to function primarily to enhance freezing tolerance. ABF3 in transgenic rice increased tolerance to drought stress alone. By using the 60 K Rice Whole Genome Microarray and RNA gel-blot analyses, we identified 12 and 7 target genes that were activated in transgenic rice plants by CBF3 and ABF3, respectively, which appear to render the corresponding plants acclimated for stress conditions. The target genes together with 13 and 27 additional genes are induced further upon exposure to drought stress, consequently making the transgenic plants more tolerant to stress conditions. Interestingly, our transgenic plants exhibited neither growth inhibition nor visible phenotypic alterations despite constitutive expression of the *CBF3* or *ABF3*, unlike the results previously obtained from Arabidopsis where transgenic plants were stunted.

Drought, high salinity, and low temperature are three important abiotic stresses that are commonly encountered by plants growing in their native environments. Upon exposure to the stresses, many genes are induced and their products are thought to function as cellular protectants of stress-induced damage (Bray, 1997; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Studies on the expression of stress-regulated genes in Arabidopsis (*Arabidopsis thaliana*) have demonstrated the presence of several stress-response pathways involving genes that act either in an abscisic acid (ABA)-dependent or an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 1997). Many transcription factors were characterized and found to function in one of the pathways in Arabidopsis. CBF3/DREB1A (CBF3; Gilmour et al., 1998; Shinwari et al., 1998) and ABF3 (Kang et al., 2002) represent 2 of the characterized transcription factors that are related to the ABA-independent and ABA-dependent pathways, respectively. Promoter regions of many stress-regulated genes in Arabidopsis contain two cis-acting elements, C-repeat (CRT; Baker et al., 1994)/dehydration-responsive element (DRE; Yamaguchi Shinozaki and Shinozaki, 1994) and ABA-responsive element (ABRE; Giraudat et al., 1994) that interact with CBFs and ABFs (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Kang et al., 2002), respectively. When grown under normal conditions, CBF3 in transgenic Arabidopsis enhances expression of its target genes including *cor15a*, *rd29A*, *kin1*, *cor6.6*, and *cor47/rd17* (Liu et al., 1998; Kasuga et al., 1999; Maruyama et al., 2004); on the other hand, ABF3 induces ABA-related genes that encode Lea (*rd29B* and *rab18*) and protein phosphatase 2C (*ABI1* and *ABI2*; Kang et al., 2002). As a result, it has been concluded that CBF3 enhances tolerance to freezing, drought, and high salinity, whereas ABF3 increases tolerance to drought stress alone.

The discovery of CBF3- and ABF3-related pathways in Arabidopsis provides us with strategies to improve the stress tolerance of crop plants. Transcripts of *CBF*-like genes were found to accumulate in response to low temperature in canola (*Brassica napus*), wheat (*Triticum*

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aestivum), rye (*Secale cereale*), and tomato (*Lycopersicon esculentum*) (Jaglo et al., 2001). In addition, overexpression of the Arabidopsis *CBF3* in transgenic canola (Jaglo et al., 2001) and tobacco (*Nicotiana tabacum*; Kasuga et al., 2004) increases tolerance to freezing and drought/low-temperature exposure, respectively. Despite the fact that *CBF* genes and/or related responses are conserved in some plants, various plant species vary greatly in their abilities to survive adverse effects from exposure to these environmental constraints. Obviously, plants that are capable of cold acclimation have evolved differently from those that are unable to undergo cold acclimation (Jaglo et al., 2001). A typical

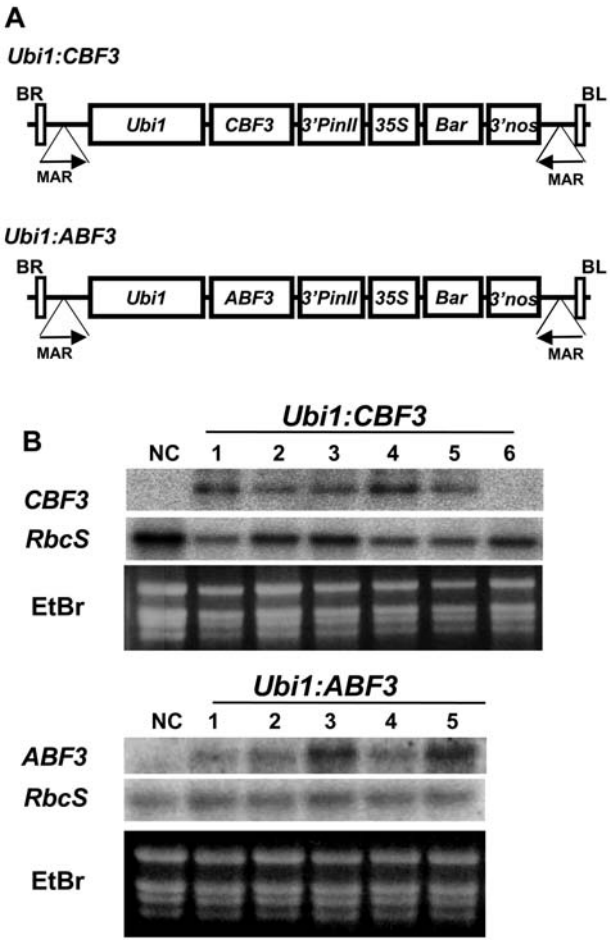

Figure 1. Production of *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants. A, *Ubi1*:*CBF3* and *Ubi1*:*ABF3* consist of the maize ubiquitin promoter (*Ubi1*) linked to the *CBF3* and *ABF3* coding regions, respectively, and the 3′-region of the potato proteinase inhibitor II gene (*3′pinII*), and a gene expression 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 Strätling, 1996). B, RNA gel-blot analysis was performed using total RNAs from young leaves of 6 *Ubi1*:*CBF3* (top) and 5 *Ubi1*:*ABF3* (bottom) lines and from NC plants. The blots were hybridized with the *CBF3* and *ABF3* probes (described in Supplemental Fig. 1) and reprobed with the rice *RbcS* gene (Kyozuka et al., 1993). Ethidium bromide (EtBr) staining of total RNA was for equal loading of RNAs.

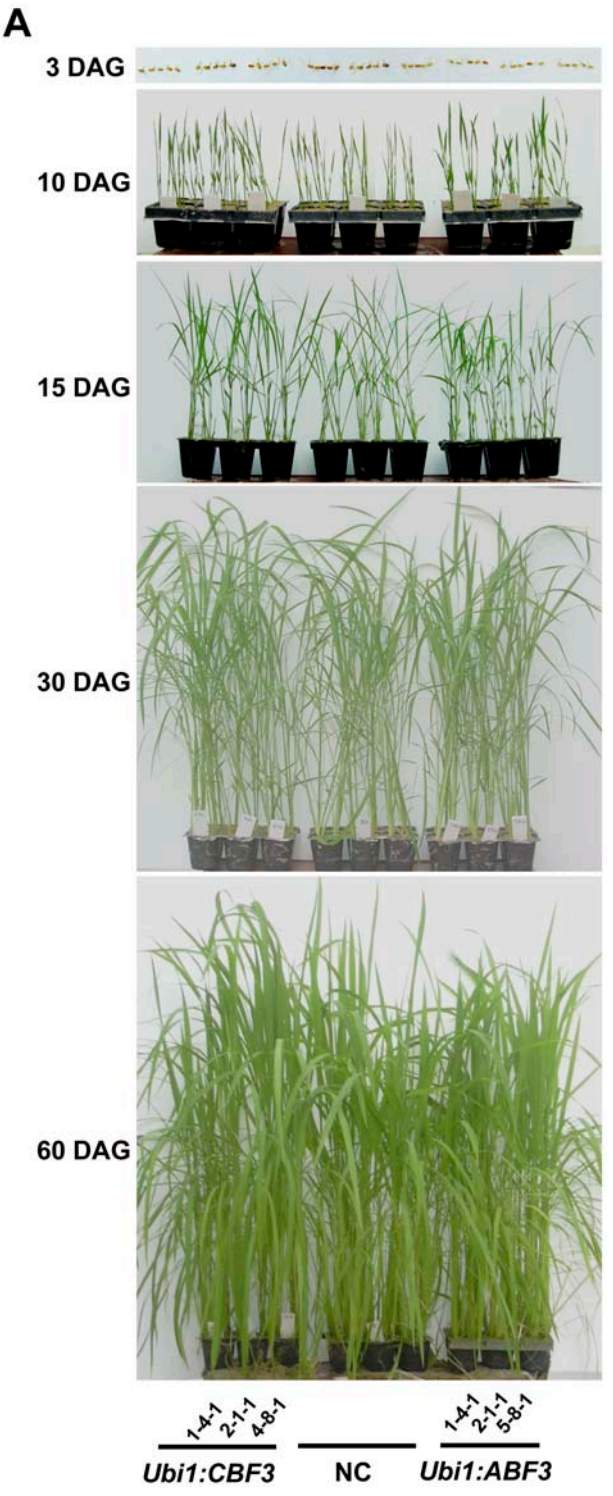

Figure 2. Growth characteristics of *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants. A, Growth phenotypes of 3 independent T_4 homozygous lines for *Ubi1*:*CBF3*, *Ubi1*:*ABF3*, and NC plants at indicated days after germination (DAG). B, Dry weight and fresh weight accumulation of *Ubi1*:*CBF3*, *Ubi1*:*ABF3*, and NC plants. Plants grown in the greenhouse during the same time course shown in A were harvested and fresh and dry weight/10 plants measured. Each data point represents the mean ± SD of triplicate experiments with three different transgenic lines.

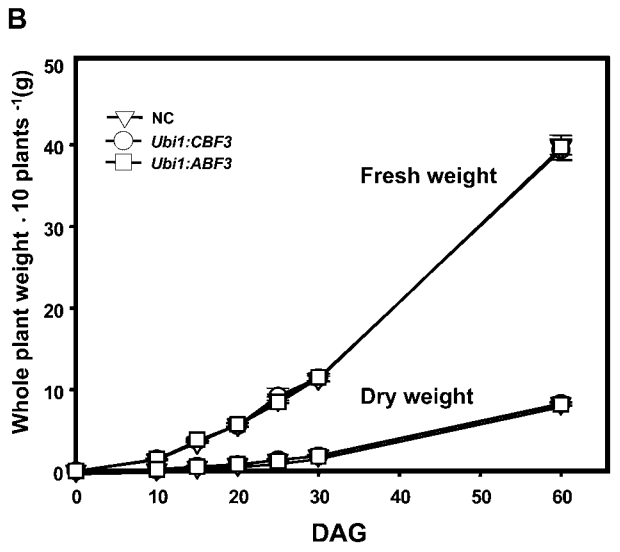

Figure 2. (*Continued.*)

plant from the latter is rice (*Oryza sativa*). In comparison to Arabidopsis and other cereals like wheat and barley (*Hordeum vulgare*) that cold acclimate (Wen et al., 2002), rice is much more sensitive to low temperature exposure. Interestingly, expression of *OsDREB1A*, a rice ortholog of *CBF3*, is different from that of Arabidopsis *CBF3* in that it is induced not only by low temperature but also by exposure to high salinity and wounding (Dubouzet et al., 2003). In Arabidopsis, *CBF3* is induced by low temperature, but it is not affected by drought or high-salinity stress (Shinwari et al., 1998; Gilmour et al., 2000). Microarray analysis of rice stress-regulated genes demonstrated the existence of 22 genes and cis-acting elements that have not been previously reported in Arabidopsis (Rabbani et al., 2003). In addition, 2 signaling pathways for low temperature stress were proposed to exist in rice, one that is responsive to a 12°C stimulus and the other that is induced by exposure to 4°C (Wen et al., 2002). These observations prompted us to examine the stress response of rice in comparison to that of Arabidopsis. We generated transgenic rice plants constitutively expressing *CBF3* and *ABF3* and their ectopic overexpression enhanced stress tolerance in unique ways by activating specific groups of stress-regulated genes. We also discussed the similarities and differences between rice and Arabidopsis in functional aspects of *CBF3* and *ABF3*.

RESULTS

Production of Transgenic Rice Plants That Express Arabidopsis *CBF3* and *ABF3*

To examine the role of *CBF3/DREB1A* (*CBF3*) and *ABF3* in transgenic rice plants, we constructed plasmids for rice transformation, *Ubi1*:*CBF3* and *Ubi1*:*ABF3* (Fig. 1A), in which the genes are under the control of the maize (*Zea mays*) ubiquitin1 promoter including its first intron (*Ubi1*; Christensen and Quail, 1996). Fifteen and 20 independent transgenic lines for *Ubi1*:*CBF3* and *Ubi1*:*ABF3*, respectively, were obtained using the Agrobacterium-mediated transformation method (Hiei et al., 1994). Over 80% of the plants were fertile and T_1, T_2, T_3, and T_4 seeds were collected. Copy numbers and integration events of the

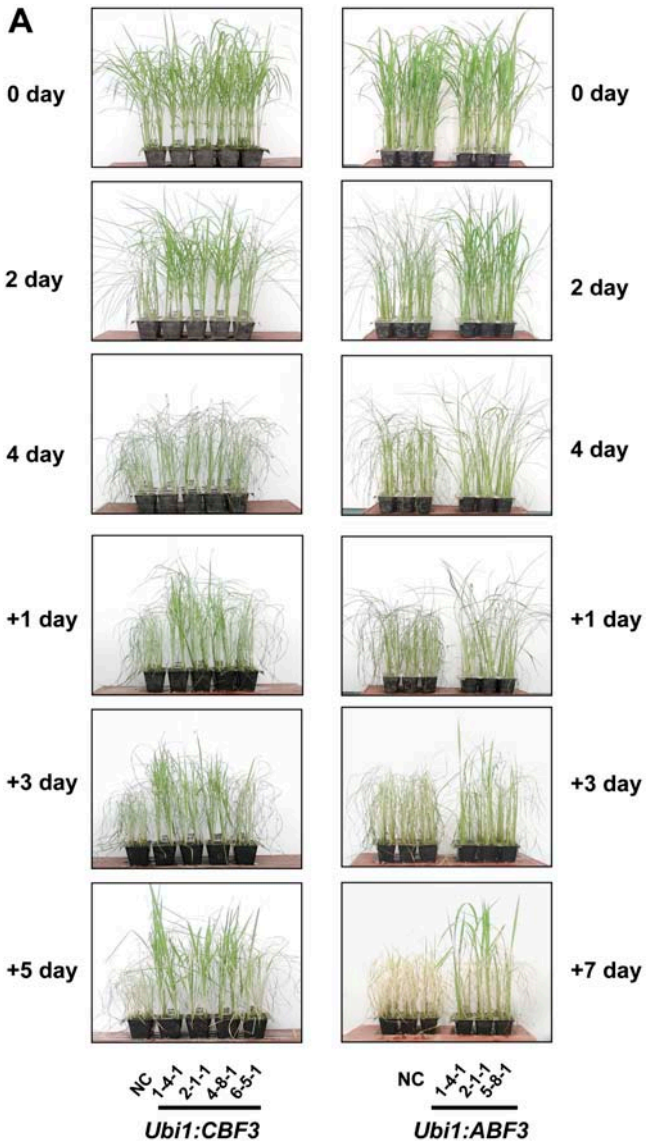

Figure 3. Appearance of plants and changes in chlorophyll fluorescence during drought stress. A, Four and three independent T_4 homozygous lines for *Ubi1*:*CBF3* and *Ubi1*:*ABF3*, respectively, and NC seedlings were grown in the greenhouse for 4 weeks and then subjected to 4 d of drought stress followed by 5 to 7 d of watering. Eighteen plants per each line were tested. Photos were taken at 1- or 2-d intervals; + followed by number denotes days of watering. B, F_v/F_m of the transgenic and NC plants in the same time course of drought stress shown in A was measured using a pulse modulation fluorometer (mini-PAM). F_v/F_m is a measure of accumulated photooxidative damage to PSII. Each data point represents the mean ± SE of triplicate experiments ($n = 6$).

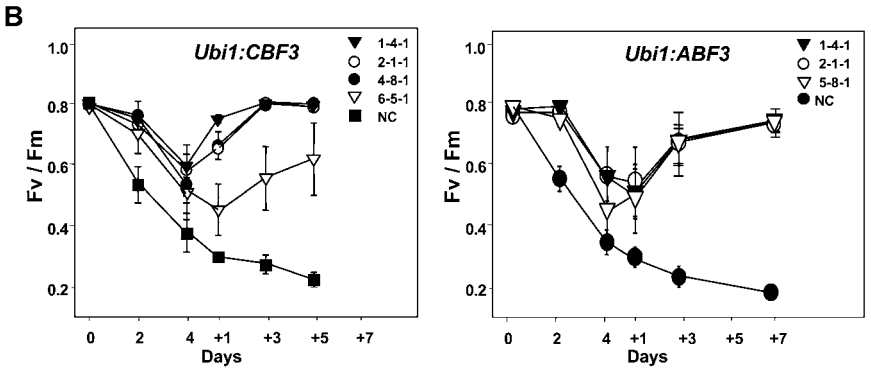

Figure 3. (*Continued.*)

transgene in *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants were determined by genomic Southern blots (Supplemental Fig. 1), which revealed that all the lines are independent and that the copy numbers of corresponding transgenes are either one or two. Expression levels of the transgenes in *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants were examined by RNA-blot analysis using total RNAs from leaf tissues. As shown in Figure 1B, *CBF3* or *ABF3* transcripts were readily detectable in all the lines tested. An exception occurred for line 6 of *Ubi1*:*CBF3* whose hybridization signal was weak but still detectable with longer exposure times. Three or four homozygous T_4 lines that contained single copy insertions of the transgene for the *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants, respectively, were chosen for further analysis.

Unlike the severe stunting observed in Arabidopsis plants that overexpressed *CBF3* or *ABF3* (Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Kang et al., 2002), our *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants displayed normal growth and seed setting. Transgenic and nontransgenic seeds were germinated and their fresh and dry weights determined in the time course after germination. The transgenic plants showed normal vegetative phenotype and fertility as compared to nontransgenic control plants (Fig. 2A), without notable difference in fresh and dry weights (Fig. 2B).

Stress Tolerance in *Ubi1*:*CBF3* and *Ubi1*:*ABF3* Plants

To investigate whether expression of *CBF3* or *ABF3* was correlated with stress tolerance in transgenic plants, 4-week-old nontransgenic control (NC) and T_4 transgenic seedlings were subjected to 4 d of drought stress. After the drought treatments, plants of each line showed wilting and drought-induced rolling of young leaves with a concomitant loss of chlorophyll (Fig. 3A). In contrast to transgenic lines, NC plants exhibited leaf rolling within 2 d of the stress and exhibited considerably more visual symptoms of drought stress. After 4 d of drought stress and subsequent watering for 5 d, the growth of transgenic lines was almost identical to nonstressed control plants. In contrast, the growth of drought-stressed NC plants was severely inhibited, and these plants never recovered and finally died. With the exception of line 6-5-1, after 4 d of drought stress followed by 5 d of watering, almost all of the *Ubi1*:*CBF3* survived, whereas 50% to 67% of the *Ubi1*:*ABF3* plants survived (Table I). Expression level of *CBF3* in the line 6-5-1 was very low (Fig. 1B). These results indicate that overexpression of Arabidopsis *CBF3* or *ABF3* in rice confers increased tolerance to drought stress and that the effect is greater in plants overexpressing *CBF3*. The enhanced drought tolerance of the transgenic plants was further verified by measuring changes in chlorophyll fluorescence. Reductions in the maximum photochemical efficiency of PSII in the dark-adapted state (F_v/F_m) were considerably larger in NC plants than in either the *Ubi1*:*CBF3* or the *Ubi1*:*ABF3* plants throughout the time course (Fig. 3B), thereby validating the increased tolerance to drought stress. Similarly, F_v/F_m was measured in 14-d-old transgenic and NC seedlings including one nullizygous plant after exposure to high salinity and low temperature in addition to drought stress. Levels of F_v/F_m were approximately 30% higher in *Ubi1*:*CBF3* plants than in NC plants

Table I. *Survival of Ubi1:CBF3 and Ubi1:ABF3 plants under drought stress*

Plants[a]	Total[b]	Survival[c]	Survival Rate[d]
Ubi1:*CBF3*			
NC	36	0	0%
1-4-1	36	34	94%
2-1-1	36	36	100%
4-8-1	36	36	100%
6-5-1	36	14	39%
Ubi1:*ABF3*			
NC	36	3	8%
1-4-1	36	21	58%
2-1-1	36	24	67%
5-8-1	36	18	50%

[a]Four-week-old soil-grown plants withheld water for 4 d followed by watering for 7 d and results scored. Plants were considered dead if there was no regrowth 7 d of rewatering. Water loss during the drought periods was similar for all pots. [b]Total number of plants used in each assay. [c]Number of survival plants. [d]Percent of survived plants (survival/total × 100).

under drought and high salinity and 10% higher under low-temperature stress (Fig. 4). F_v/F_m levels in *Ubi1*:*ABF3* plants were higher by 27% as compared to NC plants under drought, but were similar or even lower than NC plants under high-salinity and low-temperature treatments. In summary, CBF3 increased tolerance to drought, high salinity, and low temperature, while ABF3 increased tolerance only to drought in transgenic rice plants.

CBF3 and ABF3 Activate Different Groups of Stress-Related Genes in Rice

Stress-responsive genes are activated by CBFs and ABFs in Arabidopsis. To identify genes that are up-regulated by CBF3 or ABF3 in rice, global expression profiling was performed on the *Ubi1*:*CBF3* or *Ubi1*:*ABF3* plants in comparison with untransformed plants that were grown under normal growth conditions. The underlying assumption of this approach is that the constitutive expression of *CBF3* or *ABF3* in transgenic plants activates target genes whose expression levels would remain relatively low in nontransgenic plants under normal growth conditions. Profiling was conducted with the 60 K Rice Whole Genome Microarray (GreenGene Biotech, Yongin, Korea). This microarray contains 70-mer oligonucleotide probes with sequences corresponding to 58,417 known or predicted open reading frames that cover the entire rice genome. RNA samples from leaf tissues of 14-d-old transgenic and nontransgenic seedlings were used to generate Cy5- and Cy3-labeled cDNA probes, respectively, which were then hybridized to the microarray. Expression analyses with 3 replicates identified 16 different genes with 1.6-fold greater induction in transgenic plants than in nontransgenic

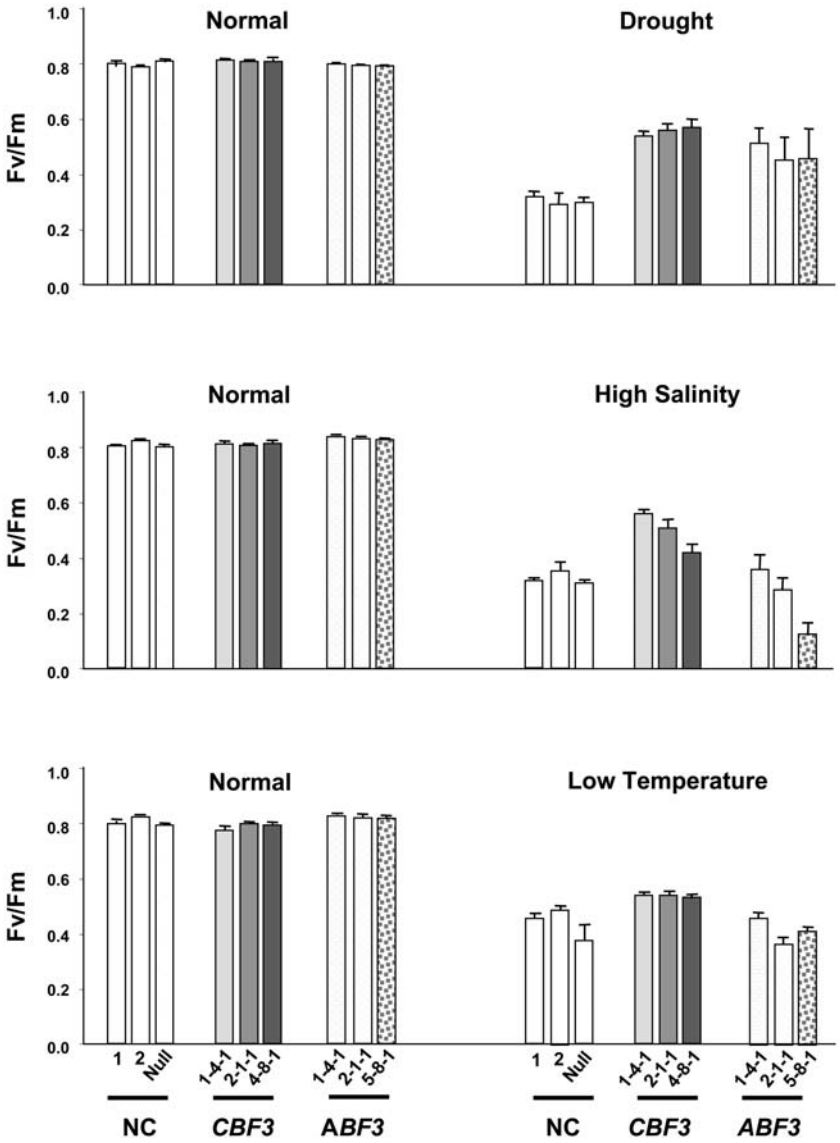

Figure 4. Changes in chlorophyll fluorescence during drought, high-salinity, and low-temperature stresses. Three independent T_4 homozygous lines for *Ubi1*:*CBF3*, *Ubi1*:*ABF3*, and NC seedlings grown in the greenhouse for 14 d were subjected to various stress conditions as described: for drought stress, the seedlings were air-dried for 2 h at 28°C and for high-salinity stress seedlings were exposed to 400 mM NaCl for 2 h at 28°C. For low-temperature stress, they were exposed to 4°C for 6 h. All of the experiments were carried out under continuous light of 150 μmol m^{-2} s^{-1}. Each data point represents the mean ± SE of triplicate experiments ($n = 6$).

Table II. *List of genes that are up-regulated in Ubi1:CBF3 and Ubi1:ABF3 plants under normal and drought stress*

Numbers appearing in bold are the ones that are induced 1.6-fold or more in *Ubi1*:*CBF3* or *Ubi1*:*ABF3* plants.

Gene Name	Accession No.[a]	Normal[b]		Drought[d]	
		Ubi1:*CBF3*[c]	*Ubi1*:*ABF3*[c]	*Ubi1*:*CBF3*[c]	*Ubi1*:*ABF3*[c]
Jacalin1	AK066682	**3.56**	−1.11	**3.04**	**2.92**
Jacalin2	AK101991	**3.32**	−1.17	**2.42**	**2.10**
Dip1	AY587109	**1.66**	1.13	1.10	1.39
Lip5	AB011368	**2.32**	1.29	1.20	−1.10
Lipoxygenase (LOX)	AJ270938	**3.10**	1.43	−1.59	1.11
Glutelin	AK107238	**2.18**	−1.10	−1.22	1.13
Bowman Birk trypsin inhibitor1	AK065846	**1.83**	1.08	−1.19	1.27
Bowman Birk trypsin inhibitor2	AK105455	**1.65**	−1.22	−1.19	1.10
Receptor kinase containing LRR repeats	AK119823	**1.67**	**1.64**	1.37	**1.99**
Unknown protein	AK059202	**1.62**	−2.07	1.12	1.26
Cyt P450	AK069394	1.34	1.11	**5.21**	**3.30**
Seed imbibition protein (Sip1)	AK065100	1.03	−1.03	**2.20**	1.12
Sicrotubule-associated protein MAP65-1a	AK102553	−1.08	1.06	**1.91**	1.22
Unknown protein	NM_188534	−1.07	1.09	**1.93**	1.06
Unknown protein	AK107624	1.10	−1.07	**1.86**	1.17
Unknown protein	AK061456	−1.03	−1.00	**1.68**	1.25
Polygalacturonase (PG2)	AK108477	1.02	1.09	**1.70**	1.47
FtsJ cell division protein	AK070075	1.10	1.10	**1.78**	−1.02
mRNA cleavage factor I subunit	AK061260	1.03	1.13	**1.79**	1.04
Beclin	AK101033	1.19	1.02	**1.73**	1.05
Cyclophilin (CyP)	AK111654	1.04	−1.01	**1.70**	1.21
Phospho sulfolactate synthase (PSLS)	AK072958	−1.01	−1.04	**1.61**	1.02
Gag-pol polyprotein	AK063408	−1.08	−1.18	**1.82**	−1.01
α-Expansin OsEXPA5	AF394546	1.16	1.14	1.41	**1.94**
Hsp70	AK072830	**1.99**	**1.94**	1.39	1.28
Protein phosphatase 2Ca (PP2Ca)	XM_463364	**1.65**	**1.67**	1.21	1.48
Wsi18	D26536	1.00	**2.11**	1.06	**2.27**
RAB21	Y00842	−1.03	**2.60**	1.22	1.54
Phosphate-induced protein 1(phi1)	AK070419	−1.11	**1.67**	1.10	−1.39
Antioxidant protein	AK066452	1.17	**1.68**	1.25	1.15
LEA4	AK107930	−1.01	1.02	1.08	**4.33**
Protein phosphatase 2Cb (PP2Cb)	AK069274	1.12	1.05	1.05	**3.45**
PHD-type zinc finger protein	AK059311	−1.17	1.09	1.07	**3.11**
Unknown protein	AK063747	−1.16	1.09	1.17	**2.83**
Little protein (LP1)	AK063634	1.09	1.09	−1.22	**3.01**
Unknown protein	AK072034	1.08	1.06	−1.11	**2.66**
Unknown protein	AK063680	−1.00	−1.05	−1.08	**2.63**
CCCH-type zinc finger protein	AK106392	1.37	1.05	1.40	**2.56**
26S proteasome AAA-ATPase subunit	AK103936	1.05	1.22	−1.00	**2.26**
Unknown protein	NM_197832	−1.34	1.18	−1.00	**2.24**
Unknown protein	XM_480395	1.01	1.09	1.05	**2.34**
Unknown protein	AK058851	1.03	−1.00	−1.12	**2.38**
Aquaporin (TIP4)	AK121671	1.08	−1.06	1.07	**2.62**
Disease resistance protein (RPH8A)	AK072531	1.13	1.18	−1.07	**2.10**
Unknown protein	AK104155	1.05	1.00	−1.01	**2.20**
1,4-β-D xylan xylanohydrolase	NM_184104	1.09	1.04	1.03	**1.97**
Spore coat protein	AK108917	1.02	−1.02	1.05	**1.93**
Cellulase	XM_469540	−1.06	1.07	1.21	**1.98**
LTI6B-like	AK104060	−1.12	1.09	−1.07	**1.88**
Fusarium resistance protein	AK058343	1.05	1.11	−1.03	**2.20**
ABC transporter	AK105712	−1.08	1.17	−1.19	**1.91**
LEA	AK067556	1.13	1.10	1.22	**1.87**
LEA3	AK102039	−1.02	1.17	−1.17	**2.14**

[a]GenBank accession numbers for full-length cDNA sequences of corresponding genes. [b]Microarrays were hybridized with Cy3- and Cy5-labeled probe pairs of either *Ubi1*:*CBF3* and nontransgenic plants or *Ubi1*:*ABF3* and nontransgenic plants grown in normal growth conditions. [c]The microarray-data sets can be found at the www.http://www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus, GEO). GEO accession number of microarray-data set is GSE2211. [d]Microarrays were hybridized with Cy3- and Cy5-labeled probe pairs of either *Ubi1*:*CBF3* and nontransgenic plants or *Ubi1*:*ABF3* and nontransgenic plants grown in drought-stress conditions.

control plants (Table II; Supplemental Table I). CBF3- or ABF3-induced expression of the candidate genes was subsequently confirmed by RNA gel-blot analysis using the same RNAs for microarray (Fig. 5, left). Our results revealed that CBF3 activates 12 genes including *Lip5*, *Dip1*, *Jacalin1* and *2*, and *LOX*, whereas ABF3 activates 7 genes including *Wsi18* and *Rab21*. Three genes, *Hsp70*, *PP2Ca*, and a receptor kinase gene, are activated by both CBF3 and ABF3. These genes were induced at different levels in nontransgenic rice within 2 h of exposure to stress conditions (Fig. 5, right). The *RbcS* gene whose transcript levels rapidly decrease in response to stress treatments except for low temperature (DeRocher and Bohnert, 1993; Weatherwax et al., 1996) was used to monitor when stress-induced damage initiates. In addition to those genes that are activated by CBF3 or ABF3 under normal growth conditions, we also searched for genes that are further induced by the transcription factors under stress conditions. Expression profiling was performed on the *Ubi1*:*CBF3* or *Ubi1*:*ABF3* plants in comparison with nontransgenic plants that were exposed to drought stress for 2 h. As a result, we identified 15 and 29 genes that are induced further 1.6-fold or more in *Ubi1*:*CBF3* or *Ubi1*:*ABF3* plants, respectively, upon treatment with drought stress (Table II; Supplemental Table I). Some of the genes, *Jacalin1*, *Jacalin2*, *PSLS*, *Wsi18*, *Rab21*, *LEA4*, and *PP2Cb*, were confirmed by RNA gel-blot analysis using RNAs from leaf tissues of 14-d-old transgenic and nontransgenic seedlings that were exposed to drought, high salinity, ABA, and low temperature for 2 h (Fig. 5, right). Interestingly, the genes that are activated under stress conditions in the transgenic plants are different from those that are activated under normal growth conditions, except for *Jacalin1*, *Jacalin2*, *Wsi18*, and *Rab21* that are common to both conditions. The difference may be due either to the activation of rice CBF3 and/or ABF3 homologs under stress conditions or to the increased stress tolerance of transgenic plants that was set by the activated genes under normal growth conditions. Expression of 3 *CBF* homologs, *OsDREB1A*, *OsDREB1B*, and *OsDREB2A* (Dubouzet et al., 2003), and 2 *ABF* homologs, *OsTRAB1* (Hobo et al., 1999) and AK065873, were examined by the microarray analysis described above, which revealed that none of them was increased in expression levels by 2 h of exposure to drought stress (Supplemental Table I). These observations led us to suggest that those genes that are activated under stress conditions are not the direct targets of CBF3 and ABF3 in the transgenic plants. Instead, their increased expression under stress con-

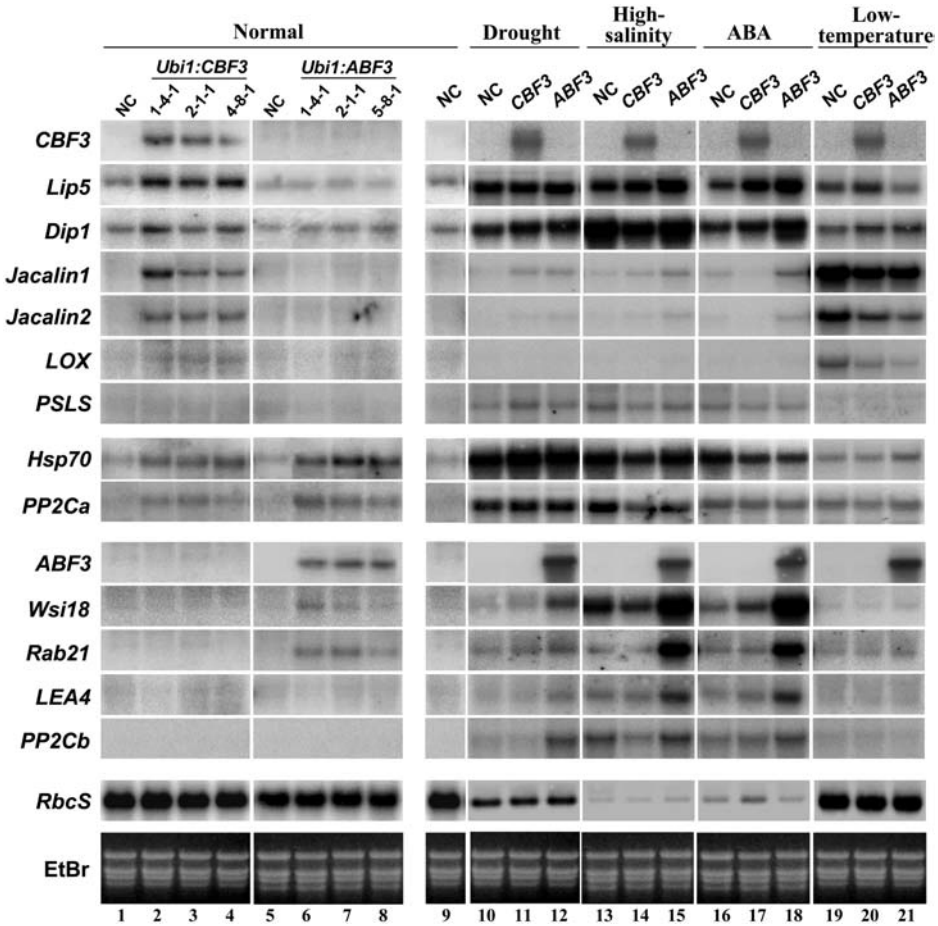

Figure 5. Induction of stress-related genes in *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants. Three independent T_4 homozygous lines for *Ubi1*:*CBF3*, *Ubi1*:*ABF3*, and NC seedlings were grown in the greenhouse for 14 d. Transgenic and NC plants were then treated for 2 h with drought (the seedlings excised before being air-dried for 2 h), high salinity (400 mM NaCl) at the greenhouse, and with low-temperature stress (4°C) at the cold chamber under continuous light of 150 μmol m^{-2} s^{-1}. For ABA treatments, 100 μM ABA was applied to each 14-d-old seedling for 2 h. RNA gel blots of total RNAs from transgenic and NC plants grown either under normal growth conditions (left) and under stress conditions (right) are indicated. RNA gel blots of NC plants grown under normal growth conditions were included on the left-hand side of each section for clarity of comparison. The blots were hybridized with probes for *CBF3*, *ABF3*, *Lip5* (AB011368), *Dip1* (AY587109), *Jacalin1* (AK066682), *Jacalin2* (AK101991), *LOX* (AJ270938), *PSLS* (AK072958), *Hsp70* (CF280418), *PP2Ca* (CF304401), *Wsi18* (D26536), *Rab21* (Y00842), *LEA4* (AK107930), *PP2Cb* (AK069274), and *RbcS* (Kyozuka et al., 1993). EtBr staining of total RNA was used to ensure equal RNA loading.

ditions resulted from secondary effects of enhanced stress tolerance of the transgenic plants that protected those genes from being down-regulated by stress treatments. Overall, Arabidopsis transcription factors, CBF3 and ABF3, activate 12 and 7 target genes in transgenic rice plants, respectively, which appears to render the corresponding plants acclimated for stress conditions. The target genes together with 13 and 27 additional genes are induced further upon stress treatments (Table II; Fig. 5), consequently making the transgenic plants more tolerant to stress conditions.

To determine the functional significance of interaction between CBF3/ABF3 and the promoters of the target genes that they induce, we chose the *Wsi18* promoter as an example. Constructs containing the promoter linked to the β-glucuronidase (GUS) reporter gene and effector constructs containing CBF3 or ABF3 under the control of the *Ubi1* promoter were used for transient transformation by microprojectile bombardment of 7-d-old rice seedlings. The *LUC* gene was used as an internal control to evaluate transformation efficiency. After particle bombardment, the samples were incubated in one-half-strength Murashige and Skoog (MS) medium either with or without ABA for 3 d. As seen in Figure 6, ABF3 activated the *Wsi18* promoter and resulted in an 8-fold increase in GUS activity when compared to the activity of the promoter without the effector. Activity of the *Wsi18* promoter was highly induced when ABA was applied and resulted in an 18-fold increase in relative GUS activity. CBF3, however, did not elevate expression of the *Wsi18* promoter at all under the tested experimental conditions. These results are in agreement with the increased expression of *Wsi18* in *Ubi1*:*ABF3* plants, but not in *Ubi1*:*CBF3* plants (Fig. 5), thus demonstrating that CBF3 and ABF3 in transgenic rice both enhance tolerance to stress by activating different groups of stress-regulated genes.

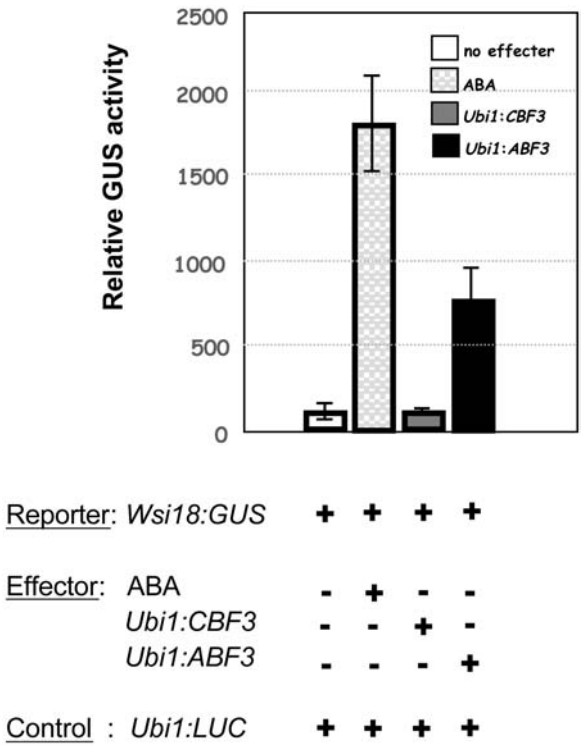

Figure 6. Transactivation of the *Wsi18*:*GUS* fusion by ABF3. The *Wsi18*:*GUS* construct was cotransformed with the effector constructs, either *Ubi1*:*CBF3* or *Ubi1*:*ABF3*, or with the expression vector alone; 4 μg of each construct with 2 μg of *Ubi1*:*LUC* as an internal standard was used in all cases. Each bar represents the mean value of the relative GUS/LUC activities from four independent experiments.

DISCUSSION

We developed transgenic rice plants constitutively expressing *CBF3/DREB1A* (*CBF3*) and *ABF3*. CBF3 overexpression in transgenic rice (*Ubi1*:*CBF3*) substantially elevated tolerance to drought and high salinity but had only limited effect on low-temperature stress tolerance. The minimal enhancement of low temperature tolerance was rather unexpected because CBF3 in Arabidopsis is a key regulatory factor that functions primarily in freezing tolerance by activating a battery of downstream genes (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Fowler and Thomashow, 2002; Maruyama et al., 2004). One explanation includes differences in the composition of CBF3-regulated genes in rice. Using microarray and RNA gel-blot analyses, we identified 12 stress-regulated genes in rice that are up-regulated by CBF3. In contrast, a total of 38 stress-regulated genes were identified in Arabidopsis as the target genes of CBF3 by a similar approach (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004). *OsDREB1A*, a rice ortholog of CBF3, in transgenic Arabidopsis activates 7 CBF3-target genes (Dubouzet et al., 2003). Thus, CBF3 activates a smaller number of target genes in rice than in Arabidopsis, causing relatively lower levels of tolerance to low temperature in *Ubi1*:*CBF3* plants. This may be due to the possibility that the Arabidopsis CBF3 cannot recognize the sequence in the promoter region of certain stress-related rice genes.

ABF3 in transgenic (*Ubi1*:*ABF3*) rice also exhibited increased tolerance to drought, but did not have enhanced tolerance to high salinity or to low-temperature stress (Fig. 4). These data were in agreement to those obtained in Arabidopsis overexpressing *35S*::*ABF3* that possessed enhanced drought tolerance (Kang et al., 2002). We identified 7 ABF3-target genes that are stress regulated in rice. Three of those, *Hsp70*, *PP2Ca*, and a receptor kinase gene, were also activated by CBF3 and these data allow us to suggest that ABA-dependent and ABA-independent pathways converge, at least in part, into these common target genes. Four ABF3-target genes that are stress regulated were identified in Arabidopsis (Kang et al., 2002) and none of them are activated by CBF3.

The core conserved sequences of CRT/DRE and ABRE, G/ACCGAC and ACGTG, were found in

multiple copies in promoter regions of the rice target genes within 1 kb upstream of the ATG start codon (Supplemental Table II). Interestingly, CBF3-target genes carry more DREs than ABREs, while ABF3-target genes contain more ABREs than DREs. For example, *Lip5*, *Dip1*, *Jacalin2*, and *LOX* contain 2 or 3 DREs and 1 ABRE or none, whereas *Rab21*, *Wsi18*, and *PP2Cb* contain 1 DRE and 2 to 5 ABREs, respectively. The presence of multiple CRT/DRE elements is a common characteristic of Arabidopsis genes that are induced by CBF3 (Gilmour et al., 2000; Dubouzet et al., 2003; Maruyama et al., 2004). CBF3 binds equally well to both ACCGAC and GCCGAC, whereas OsDREB1A has higher affinity binding for GCCGAC than for ACCGAC (Sakuma et al., 2002). Similarly, HvCBF1 binds to GCCGAC more efficiently than to ACCGAC in barley (Xue, 2002). Thus, in monocots, GCCGAC appears to be the preferred binding site for CBF3 or its orthologs. CBF3-target genes contain much more GCCGAC than ACCGAC in their promoter regions (Supplemental Table II). Whether CBF3 directly binds to the promoters of the target genes in rice remains to be determined. However, our observation that *CBF3* overexpression increased their transcript levels reflects that the interactions are likely to be involved. ABF3-target genes have 2 to 5 copies of ABRE in their promoters (Supplemental Table II), consistent with the observation that more than 2 copies of ABREs are required to confer ABA responsiveness (Skriver et al., 1991). The Arabidopsis *rd29B* promoter that lacks a DRE, but contains 2 ABREs, is induced by ABA (Uno et al., 2000) and also by ABF3 (Kang et al., 2002). Overexpression of *ABF3* in our *Ubi1*:*ABF3* plants resulted in increased expression of the target genes including *Wsi18*. Moreover, activity of the *Wsi18* promoter was enhanced in rice seedlings by ABF3, but not by CBF3, in our transactivation assays (Fig. 6). Taken together, these results suggest that at least 2 copies of DRE or ABRE are required for CBF3 or ABF3 to activate corresponding target genes in rice.

Overexpression of Arabidopsis genes, *35S*:*CBF3* and the *35S*:*ABF3*, in transgenic Arabidopsis resulted in various levels of growth inhibition under normal growth conditions (Kasuga et al., 1999; Kang et al., 2002). Similarly, overexpression of a rice gene, *35S*:*OsDREB1A*, in transgenic Arabidopsis also caused stunted growth (Dubouzet et al., 2003). Interestingly, our *Ubi1*:*CBF3* and *Ubi1*:*ABF3* plants exhibited neither growth inhibition nor visible phenotypic alterations in rice, despite constitutive expression of the transgenes (Fig. 2A). This may have occurred because lower levels and/or fewer numbers of target genes are activated by CBF3 or ABF3 in rice than in Arabidopsis, and hence, the effects on plant growth might be minimized in rice. In fact, transcript levels of the target genes in our transgenic plants under normal growth conditions were lower than levels in nontransgenic plants that were treated with stress (Fig. 5). Alternatively, rice is evolutionarily more tolerant to the expression of stress-regulated genes than dicots, including Arabidopsis. This is supported by our previous observation that transgenic rice plants producing trehalose were stress tolerant and exhibited normal growth, unlike the results obtained for transgenic dicots, such as potato (*Solanum tuberosum*) and tobacco that were severely stunted (Jang et al., 2003). Overall, our results demonstrated that Arabidopsis genes *CBF3* and *ABF3* function in stress-response pathways in rice, an important agronomic crop, without causing undesirable growth phenotype.

MATERIALS AND METHODS

Plasmid Construction and Transformation of Rice

Expression plasmids, *Ubi1*:*CBF3* and *Ubi1*:*ABF3*, contain the *bar* gene under the control of the cauliflower mosaic virus *35S* promoter for herbicide-based selection and a pair of the matrix attachment region sequence from the chicken lysozyme gene for stable expression of transgene (Phi-Van and Strätling, 1996). The ubiquitin1 promoter, together with its intron (*Ubi1*), was used to drive constitutive expression (Christensen and Quail, 1996). The coding region of *CBF3* was PCR-amplified from Arabidopsis (*Arabidopsis thaliana*) genomic DNA using a pair of primers, 5′-GATGAACTCATTTT-CAGCTTTTC-3′ and 5′-ACAGTGCTCTCTTGTGGGAC-3′, and *ABF3* cDNA was kindly provided by Dr. S.Y. Kim (Choi et al., 2000). The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating and embryogenic calli from mature rice (*Oryza sativa*) cv Nakdong seeds were transformed as previously described by Jang et al. (1999). To make *Wsi18*:*GUS* for transactivation assays, the *Wsi18* promoter region was PCR-amplified from rice genomic DNA using the primers 5′-AAGCTTGAGTCATAGGGAGA-3′ and 5′-AGTGATTCCAGCCAAGTTTGGATCC-3′ (Joshee et al., 1998). The control plasmid, *Ubi1*:*LUC*, containing the firefly luciferase gene driven by the *Ubi1* promoter, was obtained from Dr. Peter Quail at University of California at Berkeley.

Growth Measurements

Transgenic and nontransgenic rice (cv Nakdong) seeds were germinated in a one-half-strength MS solid medium in a growth chamber in the dark at 28°C for 3 d, transplanted into soil pots, and grown in the greenhouse (16-h-light/8-h-dark cycles) at 28°C to 30°C. Each pot (5 × 5 × 6 cm) was filled with nursery soils (Bio-media, Kyeongju, Korea) and planted with 6 seedlings. The fresh weight of plants was determined in the time course by harvesting and weighing the whole plant parts including roots of 10 plants/line. The dry weights were determined after drying the plants at 80°C for 48 h. Each experiment was repeated three times with three independent transgenic lines.

Stress Tolerance of Plants Grown in Soil

Transgenic and nontransgenic rice seeds were germinated in a one-half-strength MS solid medium in a growth chamber in the dark at 28°C for 4 d, transplanted into soil, and grown in the greenhouse (16-h-light/8-h-dark cycles) at 28°C to 30°C. Eighteen seedlings from each transgenic and nontransgenic line were grown in pots (5 × 5 × 6 cm; 6 plants/pot) for 4 weeks before performing the drought-stress experiments. For drought stress, 4-week-old NC and transgenic seedlings were subjected to 4 d of drought followed by 5 to 7 d of watering. F_v/F_m values of transgenic and NC plants were measured in the time course with a pulse modulated fluorometer (mini-PAM, Walz, Germany) as previously described (Jang et al., 2003). The numbers of the plants that survived or continued to grow were scored.

Chlorophyll Fluorescence under Conditions of Drought, High Salinity, and Low Temperature

Transgenic and nontransgenic rice seeds were germinated and grown in a one-half-strength MS solid medium for 14 d in a growth chamber (16-h-light of 150 μmol m^{-2} s^{-1}/8-h-dark cycles at 28°C). Green parts of approximately 10 seedlings were cut by scissors before stress treatments in vitro. For low-

temperature stress, the seedlings were incubated at 4°C water for up to 6 h under continuous light of 150 μmol m^{-2} s^{-1}. For high-salinity stress treatments, they were incubated in 400 mM NaCl for 2 h at 28°C under continuous light of 150 μmol m^{-2} s^{-1} and for drought stress they were air-dried for 2 h at 28°C under continuous light of 150 μmol m^{-2} s^{-1}. F_v/F_m value was measured as previously described (Artus et al., 1996; Jang et al., 2003).

60 K Rice Whole Genome DNA Chip Analysis

Expression profiling was conducted with the 60 K Rice Whole Genome Microarray. Information of the Microarray can be found at www.ggbio.com/rice60kchip.html (GreenGene Biotech). The 60 K Microarray was designed to represent all the genes in rice. In total, 60,727 oligomers were designed from gene-specific regions of both *japonica* and *indica* subsp. These include 58,417 from known and predicted genes and 66 randomized DNA. Among these, 2,310 genes were also designed as antisense oligomers. Oligomer sequences were extracted by Qiagen-Operon (Cologne, Germany) based on rice genome information from Beijing Genomics Institute (Yu et al., 2002). Oligomers were synthesized and purified by Qiagen-Operon and spotted on SuperAmine slide using facilities of the Dr. David Galbraith lab at the University of Arizona (http://ag.arizona.edu/microarray/deconvolution.html). A set of Rice 60 K Microarray is composed of 2 slides and has a total of 64,896 spot addresses. Each slide is formatted with 48 (12 $\times$ 4) blocks with addresses composed of 676 (26 $\times$ 26) spots. Blank spots (4,099) were also included for easy alignment of scanning format. Each oligomer, 70 nucleotides long and average melting temperature of 78°C, was printed in each spot address with a diameter of 100 μm.

Total RNA (100 μg) was prepared from leaf tissues of 14-d-old transgenic and nontransgenic seedlings (5–10 plants each) as reported previously (Jang et al., 2002) and the mRNA was purified from total RNAs using a Qiagen oligotex column (Qiagen, Valencia, CA). For drought stress, 14-d-old seedlings were excised from the seedlings before being air-dried for 2 h in the greenhouse under continuous light of approximately 900 to 1,000 μmol m^{-2} s^{-1}. Preparation of fluorescence labeled probes and microarray hybridization was performed as procedures provided by Genisphere 3DNA Array Detection Array 50 kit (v. 2, Genisphere, Hatfield, PA). The microarray was scanned with Genepix 4000B (Axon Instruments, Union City, CA) and the quality of the chip data was analyzed with statistical R language and sma package in Bioconductor project (http://www.bioconductor.org/) implemented on Linux platform. Noncorrelation of signal and background intensities were confirmed by plotting base 2 log background intensity in x axis and base 2 log intensity subtracted with background intensity in y axis. Prior to normalization, normal distribution of Cy3 and Cy5 intensities were tested by qqplot function in R statistical language. The spatial effects on the chip during the hybridization process were checked with spatial func in sma package. The variance difference between Cy3 and Cy5 intensities within microarray was tested by the Student's t test under the assumptions firstly uniform and then nonuniform variances. The ANOVA difference of signal intensities between microarrays was performed by one-way ANOVA. Block-by-block Lowess normalization (Yang et al., 2002) and multivariate statistics such as clustering, principal component analysis, multidimensional scaling, etc. were analyzed with Acuity 3.1 (Axon Instruments). Spots with flag of 0 and a diameter greater than 51 pixel size were used for the analysis. Cy3 significant spots were determined when its log ratio is less than -0.67 [2**(-0.67) = 1.6-fold decrease] and its background subtracted intensity is higher than 500. On the contrary, Cy5 significant spots was determined when its log ratio is greater than 0.67 [2**0.67 = 1.6-fold increase] and its intensity is higher than 500. A preliminary microarray experiment using nontreated wild-type RNA labeled Cy3 and Cy5, respectively, gave less than 0.5% false positive signals in the analysis. Hybridization of different microarrays with the same mRNA samples indicated a good correlation. To assess the reproducibility of the microarray analysis, we repeated the experiment three times with independently prepared total RNA (Supplemental Table I).

RNA Gel-Blot Analysis

Transgenic and nontransgenic rice seeds were germinated on soil and grown in the greenhouse (16-h-light/8-h-dark cycles). For low-temperature stress treatment, 14-d-old seedlings were exposed to 4°C at a cold chamber for 2 h under continuous light of 150 μmol m^{-2} s^{-1}. For high salinity and ABA treatment, 14-d-old seedlings were grown in a nutrient solution, 0.1% (v/v) Hyponex (Busan, Korea), for 2 d and then transferred to fresh nutrient solution containing 400 mM NaCl or 100 μM ABA for 2 h in the greenhouse under continuous light of approximately 900 to 1,000 μmol m^{-2} s^{-1}. For drought stress, 14-d-old seedlings were excised from the seedlings before being air-dried for 2 h in the greenhouse under continuous light of approximately 900 to 1,000 μmol m^{-2} s^{-1}. Preparation of total RNA and RNA gel-blot analyses were previously reported (Jang et al., 2002). Hybridization signals were captured by using a phosphor imager analyzer (FLA 3000, Fuji, Tokyo).

Transactivation Assay

Rice seeds were germinated and grown in one-half-strength MS solid medium for 7 to 9 d in a growth chamber with cycles of 16-h light/8-h darkness at 28°C. Green parts of approximately 3 to 4 seedlings, including leaves and sheaths, were cut by scissors and spread on a one-half-strength MS solid medium before particle bombardment. The tissues were transformed by particle bombardment with 4 μg of the *Wsi18*:*GUS* plasmid, 2 μg of *Ubi1*:*LUC* as an internal standard, and 4 μg of either *Ubi1*:*CBF3* or *Ubi1*:*ABF3* plasmid DNA, as previously described (Klein et al., 1987). The samples were then incubated in a MS medium, or a MS medium containing 100 μM ABA if necessary, for 72 h in the dark at 25°C before freezing in liquid nitrogen. Luciferase and GUS assays were carried out as previously described (Busk et al., 1997). Relative GUS activity was calculated as the reading of the GUS assay divided by that of the luciferase assay.

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