RESEARCH PAPER

Seven zinc-finger transcription factors are novel regulators of the stress responsive gene OsDREB1B

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Abstract

Plants have evolved several mechanisms in order to cope with adverse environmental conditions. The transcription factors (TFs) belonging to the DREB1/CBF subfamily have been described as major regulators of the plant responses to different abiotic stresses. This study focused on the rice gene OsDREB1B, initially described as highly and specifically induced by cold. However, here it is shown that OsDREB1B is not only induced by low temperatures, but also by drought and mechanical stress. In order to identify novel TFs that bind to its promoter, a yeast one-hybrid system was used to screen a cold-induced cDNA expression library. Thereby seven novel Zn-finger TFs were identified that bind to the promoter of OsDREB1B. Among them, there were four Zn-finger homeodomain (ZF-HD) and three C2H2-type Zn-finger TFs. Gene expression studies showed that these TFs are differentially regulated at transcriptional level by different abiotic stress conditions, which is illustrative of the crosstalk between stress signalling pathways. Protein–protein interaction studies revealed the formation of homo- and heterodimers among the ZF-HD TFs identified, but not for the C2H2-type. Using a transactivation assay in Arabidopsis protoplasts, all the TFs identified repressed the expression of the reporter gene, driven by the promoter of OsDREB1B. This assay also showed that the dimerization observed between the ZF-HD TFs may play a role on their transactivation activity. The results here presented suggest a prominent role of Zn-finger TFs in the regulation of OsDREB1B.

Key words: Abiotic stress, C2H2, cold, DREB1/CBF, drought, mechanical stress, salt, ZF-HD, Zn finger.

Introduction

Given their sessile nature, plants have adapted to cope with environmental stresses, such as extreme temperatures, drought, and salinity. Many of these adaptations take place at the molecular level and are modulated by transcription factors (TFs), mediating the stress responses. The APE-TALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of TFs plays a prominent role in the response to abiotic stress conditions, particularly through the action of the DROUGHT RESPONSE ELEMENT BINDING 1/C-REPEAT BINDING FACTOR (DREB1/CBF) subfamily (Gilmour et al., 1998; Liu et al., 1998). Elements of this subfamily were first identified in Arabidopsis, but are now known to be present in many other plant species, such as rice (Dubouzet et al., 2003), maize (Qin et al., 2004), grape (Xiao et al., 2006), and cotton (Shan et al., 2007), which illustrates their relevance in plant stress signalling. Even though several genes have been identified as targets of DREB1/CBFs (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004), there is still much to learn about their upstream regulators. ICE1 from Arabidopsis was the first protein identified as a regulator of DREB1/CBF gene expression, through the binding to a specific cis-motif present in the promoter region of DREB1A (Chinnusamy et al., 2003). Afterwards, MYB15 was also described as interacting with ICE1 and binding to the promoters of Arabidopsis DREB1/CBFs, working to repress their transcription (Agarwal et al., 2006). More recently, CAMTA3 was shown to be a positive regulator of Arabidopsis...
DREB1/CBF2, through its binding to the CM2 cis-motif, present in the promoter of that gene (Doherty et al., 2009), while PIF7 was established as a possible link between the light and cold signalling pathways, since it binds to the promoter of DREB1 and DREB1C (Kidokoro et al., 2009) and is a negative regulator of phytochrome signalling (Leivar et al., 2008). Nevertheless, other than in Arabidopsis, very little is known about the regulation of DREB1/CBFs. The current work focuses on the rice gene encoding OsDREB1B, which was initially described as highly and specifically induced by low temperatures (Dubouzet et al., 2003), but later, it was found to also respond to high salinity, osmotic stress, and salicylic acid (Gutha and Reddy, 2008).

There is much interplay in the stress signalling pathways, namely between different families of TFs. One group of TFs that has been described as interplaying with DREB1/CBFs, and having a major role in abiotic stress signalling, is the Zn-finger TFs. Among the different families of Zn-finger TFs, the C2H2-type were described as involved in several stress signalling pathways (Sakamoto et al., 2004; Mittler et al., 2006; Huang et al., 2007; Xu et al., 2008). These TFs, also referred to as the TFIIIA-type finger, are characterized by two Cys and two His residues that bind to a zinc ion (Pabo et al., 2001). Among the members of this class, ZAT12 was described as a negative regulator of the DREB1/CBF regulon (Vogel et al., 2005), while the ZAT10/STZ gene expression was shown to be dependent on DREB1A/CBF3 (Maruyama et al., 2004). C2H2-type TFs are therefore signalling components that can be located either up- or downstream of the DREB1/CBF genes. Another class of Zn-finger TFs that has been implicated in abiotic stress signalling is the Zn-finger homeodomain proteins (ZF-HD), which are characterized by the presence of Zn-finger-like motifs upstream of a homeodomain (Windhovel et al., 2001). Interestingly, coexpression of the stress-inducible ZFHDI and NAC transcription factors enhances the EARLY RESPONSIVE TO DEHYDRATION1 (ERD1) gene expression in Arabidopsis (Tran et al., 2006). Still, there are no reports on the role of the ZF-HD in the responses to abiotic stress in rice.

In this work, using a yeast one-hybrid (Y1H) system, seven Zn-finger TFs, three C2H2-type, and four ZF-HD, were identified as binding to the promoter of the rice gene OsDREB1B. The genes coding for these TFs are differentially regulated by several abiotic stress conditions. Moreover, all these TFs function as transcription repressors and only the ZF-HD can form homo- and/or heterodimers. This study reports for the first time the direct regulation of a DREB1/CBF gene by Zn-finger TFs.

Materials and methods
Plant materials and construction of the cold-induced cDNA expression library
Eight-day-old rice seedlings (Oryza sativa L. cv. Nipponbare) grown at 28 °C and 12/12 light/dark photoperiod, were subjected to an 8 °C treatment. Whole seedlings were sampled after 2, 5, and 24 h of cold treatment. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Total RNA samples from the three time points were pooled in equal amounts for mRNA purification using the PolyATtract mRNA Isolation System IV (Promega). An unidirectional cDNA expression library was prepared in λACTII using the HybrizAP-2.1 XR cDNA Synthesis Kit and the HybrizAP-2.1 XR Library Construction Kit (Stratagene), according to the manufacturer’s instructions. Three μg mRNA were used to perform the synthesis of first strand cDNA. In vivo mass excision of pACTII phagemid from λACTII was performed as described (Ouwerkerk and Meijer, 2001).

Yeast one-hybrid screening
The promoter of OsDREB1B was divided into four overlapping fragments to be used as baits for the Y1H screening. Fragment lengths and primer pairs used can be found in Supplementary Table S1 (available at JXB online). The fragments were cloned in the pHS3/pINT1 vector system (Ouwerkerk and Meijer, 2001) and integrated into the Y187 yeast strain (Clontech). The bait strains were transformed with the cDNA expression library. For each promoter fragment, over one million yeast colonies were screened in CM-His medium supplemented with 5 mM 3-amino-1,2,4-triazole, as described (Ouwerkerk and Meijer, 2001). The plasmids from the yeast clones that actively grew on selective medium were extracted and the cDNA insert sequenced. These sequences were used to search for homology in the rice genome, using the BLAST algorithm. Plasmids containing genes encoding transcription factors were re-transformed into the respective bait strain, to confirm activation of the reporter HIS gene.

Abiotic stress treatments
Rice seedlings were grown hydroponically in rice growth medium (Yoshida et al., 1976) at 28 °C, 700 μmol photons m−2 s−1, 70% humidity, and 12/12 light/dark photoperiod for 14 days. The seedlings were then transferred to stress conditions 4 h after dawn. At the same time, control seedlings were transferred to fresh growth medium (mock control). Cold treatments were performed by transferring the seedlings to growth chambers at either 5 °C or 10 °C in pre-cooled medium. For salt and abscisic acid (ABA) treatments, seedlings were transferred to growth media supplemented with 200 mM NaCl or 100 μM ABA, respectively. Drought treatment was performed by maintaining the seedlings over dry absorbent paper. The mechanical stress assay was carried out by damaging the seedlings before they were transferred to new growth medium. All other conditions were maintained throughout the assays. Ten plants were sampled for each time point, and roots and shoots were harvested separately. All assays were repeated at least twice.

Semi-quantitative reverse-transcription PCR
Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). First strand cDNA was synthesized from 1 μg total RNA, using an oligo-dT primer (Invitrogen) and the SuperscriptII reverse transcriptase (Invitrogen), following the manufacturer’s instructions. The gene-specific primers, as well as the number of PCR cycles and annealing temperature used for each pair, can be found in Supplementary Table S2. The amplification was performed using GoTaq polymerase (Promega) with 2.5 mM MgCl2, 12.5 μM of each primer, and 0.2 mM dNTPs. In the cases of OsZHD4 and of OsZHD8, 5% DMSO was added to the final mix. ACTIN1 (Os03g50885) was used as an internal control for all experiments, except for shoots in the drought assay and roots at 10 °C, where EUKARYOTIC ELONGATION FACTOR 1α (Os03g08060) and UBQUITIN-CONJUGATING ENZYME E2 (Os02g42314) were used, respectively. The results shown are representative of at least two biological replicates.
Transactivation assay

The reporter plasmids were built using the pCAMBIA1391z promoter-cloning vector backbone, where the kanamycin plant resistance gene, downstream of the full CaMV35S promoter, was removed using XhoI and replaced by the Luciferase gene, excised from plasmid pGL3-basic (Promega) with XhoI and SacI. The minimal CaMV35S promoter (~90 to +8 bp) was cloned upstream of the GUS gene as a Smal–EcoRI fragment. This plasmid was confirmed by restriction analysis and sequencing, and named pLUCm35GUS. The OsDREB1B promoter fragments used in the Y1H screening were cloned in the pLUCm35GUS reporter vector, using the restriction sites SacI and PstI. Effector plasmids were constructed by cloning the coding region of the TFs in pDONR221 (Invitrogen), following the manufacturer’s instructions, to obtain the pENTR-TF. The sequences were then recombined into plasmid pH7WG2 (VIB, Gent), to be under the control of the full CaMV35S promoter.

Arabidopsis protoplasts were prepared as previously described (Anthony et al., 2004). For each independent transformation, 5 µg of reporter plasmid and 10 µg of effector plasmid were used. Each transformation was performed in triplicate. Cells were incubated for 24 h at 22 °C in the dark and then collected at 450 g for 1 min in a swing-out rotor. Cell lysis was performed by resuspension in 150 mM K2PO4 (from a 1 M pH 7.8 stock solution), 1 mM EDTA, and pH7WG2 (VIB, Gent), to be under the control of the full CaMV35S promoter.

For bimolecular fluorescence complementation (BiFC), the coding sequences were also recombined from pENTR-TF into vector pH7WG2 (VIB, Gent), to be in a translational fusion with GFP and under the control of the full CaMV35S promoter. Cloning was done according to the Gateway technology (Invitrogen). The empty pH7WG2 vector was used as a negative control for the cellular localization.

Transformation of Arabidopsis protoplasts was performed as described for the transactivation assay. For each transformation, 3 µg of each plasmid were used. The protoplasts were incubated overnight in the dark at 22 °C and observed with a fluorescence microscope (Leica DMR2A). Nuclear staining was performed by incubating the protoplasts in a 600 ng µL⁻¹ DAPI solution for 2 h.

Results

OsDREB1B transcript level is regulated by several abiotic stress conditions

The gene expression of OsDREB1B (Os09g35010) was analysed in plants subjected to several abiotic stress conditions, using semi-quantitative reverse-transcription (RT) PCR. For this, 2-week-old rice seedlings were subjected to cold (5 and 10 °C), salt (200 mM NaCl), drought, and ABA (100 µM) treatments for up to 24 h (Fig. 1A and Supplementary Fig. S1). The results confirmed that OsDREB1B is highly regulated by cold, as previously described (Dubouzet et al., 2003). In addition, it was observed that this regulation is temperature dependent and shows a similar pattern in both shoots and roots (Fig. 1A). When rice seedlings were subjected to 10 °C, the OsDREB1B transcript level was rapidly induced (10 min), reached a peak at 1–2 h, and then started to decrease, returning to basal levels afterwards. At 5 °C, however, the induction of OsDREB1B only started after 40 min of cold and remained high until the end of the assay. Rice seedlings treated with ABA or subjected to high salinity showed a similar gene expression pattern for OsDREB1B in both shoots and roots. The transcript level of OsDREB1B was rapidly (10 min) upregulated after the onset of stress and followed by a downregulation after 20–40 min. This pattern was also observed in shoots under drought stress, whereas in roots the transcript level of OsDREB1B was kept high at least during 24 h after drought treatment. This suggests that OsDREB1B may play an important role in the plant response to drought, particularly at root level. In the case of NaCl and ABA treatments, there was also a transient upregulation of OsDREB1B after 5–10 h of NaCl treatment in shoots and 1–2 h of ABA treatment in roots. However, given that these changes also appear in the mock control, they are likely to be not specific to NaCl and ABA treatments.

Under mock treatment, a circadian regulation of OsDREB1B could be observed, with the transcript level reaching a peak at 2–5 h after the start of the assay (6–9 h after dawn), decreasing afterwards. In addition, under the same treatment, OsDREB1B showed a transient increase of gene expression at 10 and 20 min. Since, in this case, the
only change in conditions was the transfer of the plants to new growth medium, it was hypothesized if this upregulation could be due to a response to mechanical stress. Therefore, another assay was performed, in the same conditions as above, but where the plants were damaged and transferred to new medium. In this case, the OsDREB1B transcript level was highly induced in response to mechanical damage for at least 20 minutes, returning to basal levels afterwards (Fig. 1B). This may also explain the transient upregulation that was observed in the salt, drought, and ABA treatments around 10 and 20 min after the start of stress (Fig. 1A and Supplementary Fig. S1). Nevertheless, the transient induction observed in the salt and drought treatments seems to be more significant than the one seen in the mock treatment.

Seven novel Zn-finger TFs identified as binding to the promoter of OsDREB1B

In order to identify TFs that bind to the promoter of OsDREB1B, a Y1H screening was performed, using that promoter as bait. The 2000 bp upstream of the OsDREB1B ATG start codon were considered to be the promoter region. A bioinformatics prediction of TF binding sites and other cis-regulatory elements in this sequence can be found in Supplementary Table S3. For the Y1H assay, this 2000 bp sequence was divided into four overlapping fragments of around 500 bp, numbered from 1 to 4 (Supplementary Table S1), 1 being the fragment further away from the start codon and 4 the one closest to it (Fig. 2A). The four yeast bait strains (each fragment corresponds to one bait strain) were used to screen a rice cold-induced cDNA expression library, and at least one million yeast colonies were screened for each fragment. This allowed the identification of seven Zn-finger TFs and of a bHLH TF (Figueiredo et al., unpublished results) as binding to the OsDREB1B promoter. Fig. 2A shows the relative position of the Zn-finger TFs identified along the promoter of OsDREB1B. Four ZF-HD TFs were found as interacting with the DNA sequence between –1527 to –961 bp upstream of the OsDREB1B start codon, one C2H2-type Zn-finger TF was found between –1028 to –388 bp, and two other C2H2-type Zn-finger TFs were found between –488 bp and –3 bp. No TFs were found interacting with the promoter fragment ranging from –1945 to –1447 bp upstream of the start codon. In order to confirm the protein–DNA interactions, the yeast bait strains were then re-transformed with the plasmids containing the TF coding sequences. The identified TFs were named according to previous studies (Agarwal et al., 2007; Hu et al., 2008). Table 1 shows the name, gene locus, and number of times each TF was identified in the screening. Fig. 2B shows the protein domains identified in each of the TFs under study. The ZF-HD TFs had a protein sequence.
with the ZF-HD upstream of the C-terminal DNA-binding homeodomain, as previously described (Windhovel et al., 2001). OsZHD1 and 2 had very similar domain structures with the homeodomain close to the C-terminus, whereas in OsZHD4 both domains were more central in the protein. In the case of ZHD8, the homeodomain was localized similarly to the one on OsZHD4, but the ZF-HD domain was closer to the N-terminal of the protein, when compared to the other ZF-HD TFs. As for the C2H2-type TFs, ZOS3-12 and ZOS11-10, only had two. ZOS3-12 showed an additional feature at its C-terminus: a DLN-box/EAR-motif, which was described as a transcriptional repressor domain (Ohta et al., 2001). No such motifs were identified in the other TFs under study. Phylogenetic analysis comparing the sequences of rice and Arabidopsis C2H2 and ZF-HD TFs can be found in previous studies (Agarwal et al., 2007; Hu et al., 2008). Supplementary Fig. S2 shows an alignment of the DNA-binding domains of the Zn-finger TFs identified in this study with the homologous proteins previously described in Arabidopsis.

In order to test whether the TFs identified bind specifically to the promoter fragment used as bait in the Y1H Table 1. Zn-finger transcription factors identified as binding to the promoter of OsDREB1B

<table>
<thead>
<tr>
<th>Promoter fragment (bp)</th>
<th>RGAP gene locus</th>
<th>Conserved domains</th>
<th>Name</th>
<th>No. of times identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>From –1527 to –961</td>
<td>Os09g29130</td>
<td>ZF-HD</td>
<td>OsZHD1</td>
<td>1</td>
<td>Hu et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Os08g37400</td>
<td>ZF-HD</td>
<td>OsZHD2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Os11g13930</td>
<td>ZF-HD</td>
<td>OsZHD4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Os04g35500</td>
<td>ZF-HD</td>
<td>OsZHD8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>From –1028 to –388</td>
<td>Os12g38960</td>
<td>C2H2</td>
<td>ZOS12-7</td>
<td>2</td>
<td>Agarwal et al. (2007)</td>
</tr>
<tr>
<td>From –488 to –3</td>
<td>Os03g32230</td>
<td>C2H2</td>
<td>ZOS3-12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Os11g47630</td>
<td>C2H2</td>
<td>ZOS11-10</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
screening, each yeast bait strain was transformed with the seven Zn-finger TFs, independently. Fig. 3 shows that none of the TFs under study activated the expression of the HIS reporter gene when transformed into the bait strain carrying the OsDREB1B promoter fragment ranging from –1975 to –1447 bp. In addition, the ZF-HD TFs bound only to the promoter fragment ranging from –1527 to –961 bp, which correlates to the fact that this promoter region is the one most enriched in ZF-HD binding motifs (Supplementary Fig. S3; Tan and Irish, 2006). Regarding the C2H2-type TFs, ZOS12-7 bound only to the promoter region ranging from –1028 to –388 bp, while ZOS3-12 and ZOS11-10 bound to the one ranging from –488 to –3 bp, as predicted by the Y1H screening results, but also to the fragment –1028 to –388 bp. Altogether, these results showed that the binding of these TFs is specific to the promoter fragments used and not to the backbone vector. The fact that ZOS3-12 and ZOS11-10 bound to both promoter fragments (–1028 to –388 bp and –488 to –3 bp), correlates with the fact that both fragments have binding sites for these two TFs. There are several reasons why these TFs were initially not identified through the Y1H screening as binding to –1028 to –388 bp (Fig. 2). Among others, they may have higher affinity to fragment –488 to –3 bp or they may have higher transcriptional activity when bound to this fragment as compared to fragment –1028 to –388 bp. Interestingly, the promoter fragment ranging from –1028 to –388 bp shows an enrichment in the A(G/C)T-X3-4-A(G/C)T cis-regulatory element (Supplementary Fig. S3), which had previously been described as a binding site for C2H2-type TFs in Arabidopsis (Sakamoto et al., 2004). The observation that ZOS12-7 only interacts with the promoter fragment ranging from –1028 to –388 bp, suggests that it has a different DNA-binding specificity when compared to ZOS3-12 and ZOS11-10. This is supported by the fact that the DNA-binding domain of ZOS12-7 shares the least similarity when compared to ZOS3-12, ZOS11-10 and the Arabidopsis TFs described by Sakamoto et al. (2004) (Supplementary Fig. S2).

The genes encoding the Zn-finger TFs identified are differentially regulated under abiotic stress

To understand whether the seven Zn-fingers identified have a role in the plant response to abiotic stress conditions, their gene expression was analysed by semi-quantitative RT-PCR in rice seedlings subjected to the conditions previously tested for OsDREB1B (Fig. 4). The most relevant gene expression profiles were quantified and normalized to the internal controls (Supplementary Fig. S4). All genes under analysis showed a constitutive basal expression in both roots and shoots, and the mock control revealed that some genes undergo a circadian rhythm regulation. This is most striking for genes OsZHD2 and ZOS12-12 in roots and ZOS11-10 in roots and shoots.

Regarding the four ZF-HD genes, their expression in shoots was not significantly or consistently altered.

**Fig. 3.** Analysis of the DNA-binding specificity of the Zn-finger transcription factors (TFs) identified. All the four yeast bait strains used in the Y1H screening were transformed with the seven different TFs fused to the GAL4 activation domain. The left column (–HIS–LEU) shows the selection plates, indicating positive interactions between the TFs and the respective promoter fragments. The right column (+HIS–LEU) shows the positive controls (yeast bait strains grown on histidine-supplemented medium). The bottom diagram indicates the TFs position on each plate.
Fig. 4. Transcriptional profile of the transcription factors (TFs) identified as binding to the promoter of OsDREB1B. The transcriptional profile was obtained by semi-quantitative RT-PCR for plants subjected to cold (5 and 10 °C), ABA (100 μM), NaCl (200 mM), drought, and mock control. All treatments started (time 0) at 4 h after dawn. Genes used as internal controls for each assay are described in the Materials and methods section.
throughout most treatments. There were however some exceptions, like the induction of both OsZHD4 at 5°C and of OsZHD8 by high salinity and drought. We also observed a repression of OsZHD2 and OsZHD4 2 h after drought and of OsZHD8 10 min after ABA treatment. Nevertheless, for most stress assays, the changes in ZF-HD gene expression in shoots were not significant. The same does not apply however to root transcription profiles. The transcript level of OsZHD1 in roots seemed to be transiently induced by cold (only at 5°C) and by drought. The expression of gene OsZHD2, on the contrary, was induced by all stress conditions, even if only for a short period, followed by a return to basal levels. OsZHD4 was mainly induced by ABA, NaCl and drought, but not by cold. OsZHD8 showed an increase in expression under several stress conditions – cold, ABA and drought, with a return to basal levels at the later stages of the treatments.

Concerning the C2H2-type Zn-finger TFs, the three genes showed very distinct expression patterns. The ZOS3-12 transcript level was rapidly and highly upregulated in response to all the stress conditions tested, both in roots and in shoots (except for ABA in roots). The transient induction of this transcript after 10 min in the mock control suggests a response to mechanical stress, as observed for OsDREB1B (Fig. 1). Therefore, the rapid induction (10 – 20 min) upon 10°C and ABA might be biased. Interestingly, and similarly to OsDREB1B, the ZOS3-12 transcript level is more rapidly induced at 10°C than at 5°C. The ZOS11-10 gene expression seemed to be particularly induced under cold in both shoots and roots. The induction is later in the roots, but the transcripts reached higher levels. ZOS11-10 was slightly induced under salt (roots) and drought (roots and shoots), but this regulation might be biased by the circadian rhythm observed in the mock control. In roots, this gene was downregulated in response to ABA treatment. Regarding ZOS12-7, it was late (5 – 10 h) and transiently induced in shoots by ABA and in roots it was induced at 10°C (after 10 h treatment).

The Zn-finger-HD, but not the C2H2-type, TFs form homo- and heterodimers

To test whether the TFs under study would form homo- or heterodimers, and also if they would interact with one another, a direct yeast two-hybrid (Y2H) assay was performed. Each TF was tested for interactions with itself and with the other six TFs under study. As shown in Fig. 5, only interactions for the ZF-HD TFs could be detected, not for any of the C2H2-type TFs under study. The only TFs that were shown to homodimerize in this assay were OsZHD1 and OsZHD4. These two proteins also interacted in yeast with all the other ZF-HD TFs under study, whereas OsZHD2 and OsZHD8 only interacted with OsZHD1 and with OsZHD4, but not with each other.

In order to confirm these interactions, a BiFC assay was performed with the TFs that yielded positive results for the Y2H. Using this system all the interactions previously observed could be validated (Fig. 6). In all cases, the
fluorescence signal was clearly localized, most likely in the nucleus of the cell. This is supported by the nuclear localization of all the individual TFs under study (Supplementary Fig. S5). For some of the interactions several spots of fluorescence could be seen, in what could be nuclear bodies.

The Zn-finger TFs binding to the OsDREB1B promoter have repressor activity

In order to determine whether the TFs identified as binding to the promoter of OsDREB1B were repressors or activators of gene expression, transactivation assays were performed in Arabidopsis protoplasts. Different reporter vectors were used in which the GUS gene is under the control of the minimal CaMV35S promoter plus the respective OsDREB1B promoter fragment that was used in the Y1H screening (Fig. 7A). Using this strategy, this assay also allowed the validation of the interactions of the TFs with the promoter fragments of OsDREB1B.

Regarding the C2H2-type TFs, ZOS3-12 had a predicted transcriptional repressor DLN-box/EAR-motif domain (Ohta et al., 2001), but for ZOS11-10 or ZOS12-7 no trans-acting domains could be predicted. Nevertheless, in Arabidopsis, four C2H2-type TFs (STZ, AZF1, AZF2 and AZF3) were shown to repress transcription through a (G/C)T-X₃₋₅-A(G/C)T cis-element (Sakamoto et al., 2004), which is present in both OsDREB1B promoter fragments used in the Y1H screening to identify ZOS11-10 or ZOS12-7 (Supplementary Table S3 and Supplementary Fig. S3). In the study of Agarwal et al. (2007), a phylogenetic tree of C2H2-type TFs showed that the rice proteins ZOS3-12, ZOS11-10, and ZOS12-7 are in the same clade as the Arabidopsis proteins STZ, AZF1, AZF2, and AZF3. These results, together with the alignment of the conserved DNA-binding domains (Supplementary Fig. S2), suggest that these TFs have similar DNA-binding specificities.

As for the ZF-HD TFs, no predicted repressor or activator domains identified were present in any of these proteins. Nevertheless, the results indicated that, in the conditions tested, all seven Zn-finger TFs are repressors of gene expression (Fig. 7B). The repressor activity, measured as a GUS/LUC ratio, ranged from 20% for ZOS3-12 to almost 80% for ZOS11-10, which was the strongest repressor. All the other TFs had intermediate activities, in the range of 40–60% of the initial GUS/LUC ratio. Curiously, the only Zn finger having a canonical repressor domain (ZOS3-12) showed the lowest repressor activity.

In order to test whether the formation of ZF-HD heterodimers (Figs. 5 and 6) had an effect on their transactivation activity, Arabidopsis protoplasts were co-transformed with all possible combinations of the ZF-HD TFs binding to OsDREB1B promoter. Fig. 7C shows that the repression activity observed for OsZHD1, when transformed alone, is no longer observed when this TF is co-transformed with any of the three TFs with which it forms heterodimers. In contrast, the co-transformation of OsZHD2 and OsZHD4 had a clear synergistic effect in repressing transcription. When OsZHD2 and OsZHD8, which do not dimerize with one another (Figs. 5 and 6), were co-transformed, the transactivation activity observed was similar to the ones of the single TFs. The same was observed for the co-transformation of OsZHD4 and OsZHD8, even though these two TFs were observed to
heterodimerize and OsZHD4 was shown to form homodimers (Figs. 5 and 6).

Discussion

The rice gene OsDREB1B was initially reported as specifically and strongly induced by cold (Dubouzet et al., 2003) and shown to be also regulated, to some extent, by salt, osmotic, and oxidative stresses and salicylic acid (Gutha and Reddy, 2008). In the work here presented, it was observed that OsDREB1B gene expression is indeed highly induced by low temperature and that this induction is dependent on the intensity of the stress. Interestingly, the faster induction of OsDREB1 gene expression at 10 °C, as compared to 5 °C, was also observed for ZOS3-12 and ZOS11-10 (Figs. 1 and 4 and Supplementary Figs. S1 and S4). This may be due to the fact that 5 °C is a very severe stress for rice plants. When subjected to such severe conditions, rice plants are most likely biochemically impaired, resulting in a delay in molecular responses. In our research group, this response has been observed for other rice genes (Serra et al., unpublished results). Moreover, it was also observed that OsDREB1B is very responsive to drought conditions, but only in the roots. This highlights the importance of analysing different tissues separately, in order not to mask differences in expression between them. Gutha and Reddy (2008) reported an activation of OsDREB1B under salt stress that was not observed in the gene expression studies here presented. The fact that these authors used a different rice variety may be an explanation for this difference, but also the experimental designs may account for some of the gene expression differences. Interestingly, an early induction of OsDREB1B could be detected in the mock-treated plants, which turned out to be a response to mechanical stress. The fact that this gene responded to the moving of the plants from one flask to another, i.e. responded to touch, indicates that OsDREB1B is highly responsive to mechanosensing. This is in agreement with the findings by Gutha and Reddy (2008), who observed that OsDREB1B transcription is induced in response to salicylic acid and that tobacco plants over-expressing this gene have an enhanced resistance to viral infection. Moreover, Gilmour et al. (1998) had also reported an activation of the Arabidopsis DREB1B/CBF1 in response to mechanical agitation. Therefore, the involvement of this gene in mechanosensing and biotic stress

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**Fig. 7.** Transactivation activity of the transcription factors (TFs) identified as binding to the OsDREB1B promoter. (A) Constructs used for Arabidopsis protoplast transformation. Effector constructs used correspond to the TF coding region under the control of the full CaMV35S promoter. Reporter constructs contain the GUS gene driven by the minimal CaMV35S promoter (m35S) plus the fragment of the OsDREB1B promoter (promOsDREB1B) used as bait in the Y1H screening. The LUC gene under the control of the full 35S promoter was used to normalize GUS expression levels. (B) Transactivation or repression analysis as a GUS/LUC activity ratio. Each plot refers to a specific OsDREB1B promoter fragment, as used for the Y1H screening, which is highlighted in the schemes on top right corners. Values shown are multiples of the GUS/LUC ratio obtained with the reporter vector without effector. Values are mean ± SD (n = 3). * Differences to the GUS/LUC values of the reporter vector alone are statistically significant (t-test, P < 0.1).
responses seems to be conserved in different species. This is further supported by the fact that the promoter of OsDREB1B has several putative binding sites for WRKY TFs (Supplementary Table S3), which are normally associated with biotic stress signalling. The fact that this study did not identify any WRKY TF binding to the promoter of OsDREB1B is probably due to the fact that it used a cDNA expression library prepared from seedlings subjected to 2, 5, and 24 h of cold. The use of a biotic stress-induced cDNA expression library would certainly result in the identification of additional TFs binding to the promoter of OsDREB1B.

Even though the DREB1/CFB regulon has been the subject of many studies, in several plants, not much is still known about the TFs that control of expression of these genes. Phytochrome interacting factor 7 (PIF7) has been shown to be involved in the regulation of DREB1/CFBs in Arabidopsis (Kidokoro et al., 2009). Also, in Arabidopsis, at least three other TFs were reported to bind to the promoters of DREB1/CFBs and regulating their transcription: ICE1, MYB15, and CAMTA3 (Chinnusamy et al., 2003; Agarwal et al., 2006; Doherty et al., 2009). In rice however, no TFs had been identified as regulators of OsDREBs. In this work, seven Zn-finger TFs, both homeodomain and C2H2-type, were identified as binding to the promoter of OsDREB1B. Zn-finger TFs had already been shown to interplay with DREB1/CFBs in the response to abiotic stresses, both up- and downstream of the DREB1/CFB regulon (Maruyama et al., 2004; Vogel et al., 2005). However, there are no reports on the direct regulation of DREB1/CFBs by Zn-finger TFs. The fact that in Arabidopsis no Zn-finger TFs have been found as binding to the promoters of DREB1/CFBs, may mean that the regulation of these genes is different in rice and Arabidopsis. Only a better understanding of the TFs that regulate DREB1/CFB expression in several plant species will unveil whether these regulatory pathways are species-specific or not. Nevertheless, it must also be taken into account that the TFs identified so far, as regulating DREB1/CFBs, may only be a small fraction of the total TFs involved in the regulation of these genes.

The gene expression of ZF-HD and C2H2-type TFs has been already described as regulated by abiotic stress conditions in Arabidopsis (Sakamoto et al., 2004; Tan and Irish, 2006) and in rice (Agarwal et al., 2007; Jain et al., 2008). In the current study, the gene expression of some TFs was highly altered by all stresses applied (such as ZOS3-12), while for others the transcript level did not show significant variations (like OsZHD1), and others responded specifically to one particular type of stress (such as ZOS12-7, both in shoots and roots). Again, and similarly to what happens with OsDREB1B, for some of the genes, major differences could be observed between expression patterns in roots and shoots, indicating that stress responses are tissue-specific. In Arabidopsis, it has been observed that several genes are differentially regulated in shoots and roots in response to several abiotic stress conditions (Kreps et al., 2002; Kilian et al., 2007). The fact that the expression of genes encoding regulators of OsDREB1B is modulated by these many environmental conditions is indicative of the crosstalk between different stress signalling pathways, where OsDREB1B seems to play an important role.

Microarray data are already available on the expression profiles of rice ZF-HD TFs under abiotic stress conditions (Jain et al., 2008). These authors observed an upregulation of OsZHD1 in drought and of OsZHD4 in cold, which correlates with the data here presented. Regarding the C2H2-type TFs, the expression data in this study for ZOS3-12, indicating an induction by several types of stress, also correlate with previous microarray data that reported its upregulation by cold, salt, and drought (Agarwal et al., 2007). In that study, the authors had also observed an induction of ZOS11-10 by cold, similarly to what is observed here, both in roots and shoots. The fact that a time course up to 24 h was analysed here, instead of single time point, as performed by Jain et al. (2008) and Agarwal et al. (2007), may explain why some gene expression responses described here were not observed in those studies.

It was also interesting to note that, even though the Zn-finger TFs identified were shown to be negative regulators of OsDREB1B transcription, their gene expression pattern under cold is, in some cases, very similar to the one of OsDREB1B. This is particularly striking in the case of ZOS3-12 (Figs. 1 and 3 and Supplementary Figs. S1 and S4). Nevertheless, the increased ZOS3-12 transcript level, caused by either 5 or 10 °C treatment and observed in both roots and shoots, starts always later than the increase observed for OsDREB1B transcript level. The fact that the induction of ZOS3-12 upon 5 °C treatment does not seem to have an immediate negative effect on the transcript levels of OsDREB1B may be due to competition with other TFs that activate the expression of OsDREB1B. In addition, it must be noted that an increase in gene expression does not always correlate directly with an increase in protein levels.

This study also tested whether the Zn-finger TFs identified interact with themselves and one another. Interactions were only observed between the ZF-HD TFs. The C2H2-type TFs did not interact with one-another, nor with the ZF-HD TFs, in yeast. Homo- and heterodimers had already been described for ZF-HD TFs in Arabidopsis (Tan and Irish, 2006) and in Flaveria (Windhovel et al., 2001), meaning that this feature is conserved in several species for this type of TFs. The heterodimerization of two homeodomain proteins from the mushroom Coprinus cinereus was shown to be necessary for their function as transcriptional regulators, namely in what concerns their targeting to the cell nucleus (Spit et al., 1998). This is illustrative of the functional significance of TF heterodimerization.

In this transactivation assay in Arabidopsis protoplasts, the TFs identified as binding to the promoter of OsDREB1B all functioned as transcription repressors. Among previously described ZF-HD TFs, the Arabidopsis ZHD11/ATHB29/ZFHD1 was described as having transcriptional activation activity, while all the other ZF-HD TFs in this plant did not show such activity (Tan and Irish,
2006; Tran et al., 2006). It was not determined however if those TFs would work to repress transcription. Interestingly, the members of this family have been described as redundant in Arabidopsis (Tan and Irish, 2006), which may explain the fact that the ZF-HD here identified in rice as regulators of OsDREB1B all act as in the same way, to repress its transcription. Regarding the C2H2-type TFs, different members of this family had already been described as transcriptional repressors under several abiotic stress conditions, including cold, in Arabidopsis (Sakamoto et al., 2004). Moreover, a cis-element present in the OsDREB1B promoter fragments used in the YIH screening was described as a binding site for Arabidopsis C2H2 TFs (STZ, AZF1, AZF2, and AZF3) and to act as a negative regulator of transcription (Sakamoto et al., 2004). The fact that the C2H2-type TFs binding to the OsDREB1B promoter share high sequence similarity with the Arabidopsis STZ, AZF1, AZF2, and AZF3 (Agarwal et al., 2007), indicates that their DNA-binding specificity and transactivation activity must be similar to the one of the Arabidopsis proteins. Nevertheless, other C2H2-type TFs involved in abiotic stress conditions have also been described as transcriptional activators (Huang et al., 2009; Sun et al., 2010).

The identification of seven transcriptional repressors binding to the promoter of OsDREB1 is a gene known to be highly induced by cold (Fig. 1 and Supplementary Fig. S1), leads to the question of why so many repressors were identified as binding OsDREB1B promoter. A possible hypothesis is suggested: when rice seedlings are subjected to 10 °C, the OsDREB1B gene expression is quickly induced, reaches a peak at 2 h, and then starts to decrease (Fig. 1). In addition, the cold-induced cDNA expression library used for the YIH screening was prepared from plants subjected to 8 °C and collected 2, 5, and 24 h after the start of the stress (see Materials and methods). Thus, if at 8 °C the regulation of OsDREB1B gene expression is similar to what happens at 10 °C, it is expected that after 2 h at 8 °C, the cDNA library will be enriched in OsDREB1B repressors rather than activators.

This study showed that formation of heterodimers among the ZF-HD binding to the OsDREB1B promoter affected their transactivation activity and that the dimerization effect depends on the interacting partners. The repressor activity of OsZHD1, which can form homodimers, was lost when it was co-transformed with any of the other ZF-HD TFs, with which it can form heterodimers. This suggests that the heterodimers formed have different DNA-binding specificities, which do not match the cis-elements present in the promoter of OsDREB1B. Similarly, in mammals the heterodimerization of C/EBPβ with E2F proteins was also shown to inhibit the transactivation activity of C/EBPβ alone (Zaragoza et al., 2010). The dimerization of TFs has long been described to regulate their binding to DNA and also their transactivation activity. For instance, the TF heterodimers Myc–Max and Mad–Max were shown to have opposing functions in regulating transcription (Ayer et al., 1993). Moreover, homo- and heterodimers of two homeodomain proteins, Atx4 and Gcs, were described as having different DNA-binding specificities, as well as transactivation activities (Tucker and Wisdom, 1999).

This work allowed the identification of several novel players in the abiotic stress-signalling pathway. These novel TFs bind to the promoter and interplay to repress the expression of OsDREB1B, are involved in the response to different abiotic stresses, and may also play a role in biotic stress. Together with previous reports, these data suggest that Zn-finger TFs may be a pivotal component in the regulation of DREB1/CBF genes in plants.

**Supplementary material**

Supplementary data are available at JXB online.

**Supplementary Fig. S1.** Analysis of the OsDREB1B gene expression under abiotic stress conditions.

**Supplementary Fig. S2.** Alignment of conserved DNA-binding domains of the Zn-finger TFs.

**Supplementary Fig. S3.** Putative Zn-finger binding elements present in the OsDREB1B promoter.

**Supplementary Fig. S4.** Expression analysis of the TF-encoding genes under abiotic stress conditions.

**Supplementary Fig. S5.** Cellular localization of the Zn-finger TFs.

**Supplementary Table S1.** Primers used to isolate the OsDREB1B promoter fragments.

**Supplementary Table S2.** Primers used for semi-quantitative RT-PCR.

**Supplementary Table S3.** cis-Acting elements in the promoter region of OsDREB1B.

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**References**


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