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Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the *ERD1* gene in Arabidopsis

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Summary

The ZFHD recognition sequence (ZFHDRS) and NAC recognition sequence (NACRS) play an important role in the dehydration-inducible expression of the *Arabidopsis thaliana EARLY RESPONSIVE TO DEHYDRATION STRESS 1* (*ERD1*) gene. Using the yeast one-hybrid system, we isolated a cDNA encoding the ZFHD1 transcriptional activator that specifically binds to the 62 bp promoter region of *ERD1*, which contains the ZFHDRS. Both *in vitro* and *in vivo* analyses confirmed specific binding of the ZFHD1 to ZFHDRS, and the expression of *ZFHD1* was induced by drought, high salinity and abscisic acid. The DNA-binding and activation domains of ZFHD1 were localized on the C-terminal homeodomain and N-terminal zinc finger domain, respectively. Microarray analysis of transgenic plants over-expressing *ZFHD1* revealed that several stress-inducible genes were upregulated in the transgenic plants. Transgenic plants exhibited a smaller morphological phenotype and had a significant improvement of drought stress tolerance. Using the yeast two-hybrid system, we detected an interaction between ZFHD1 and NACRS-binding NAC proteins. Moreover, co-over-expression of the *ZFHD1* and *NAC* genes restored the morphological phenotype of the transgenic plants to a near wild-type state and enhanced expression of *ERD1* in both Arabidopsis T87 protoplasts and transgenic Arabidopsis plants.

Keywords: zinc finger homeodomain, NAC, ERD1, transcription activator, drought tolerance, microarray.

Introduction

Potentially adverse environmental conditions affect plant growth and development. In response to stress, plants activate a number of defense mechanisms that function to increase tolerance to the adverse conditions. This inducible adaptation or acclimatization process has evolved throughout the plant kingdom and is critical for the survival of all plants. Identification of signalling pathways operating in stress-affected cells and mutual interactions between these pathways is a major research problem that has garnered a substantial amount of attention. Research focused on this area has resulted in spectacular progress and a dissection of the biochemical and physiological pathways involved in stress responses. The early events of plant adaptation to environmental stresses are perception and subsequent stress-signal transduction, leading to the activation of various physiological and metabolic responses (Bray, 2004; Hazen *et al.*, 2003; Kacperska, 2004; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999; Zhang *et al.*, 2004; Zhu, 2002). Furthermore, a large array of genes is activated by the stress conditions, so that a number of proteins are

produced to join the pathways that lead to synergistic enhancement of stress tolerance (Seki *et al.*, 2003; Wang *et al.*, 2003). Currently, a fundamental goal for plant biology is to understand the molecular and cellular mechanisms underlying stress acclimatization and to use such mechanisms to achieve improvements in crop production.

Plant stress responses are regulated by multiple signalling pathways that activate gene transcription and its downstream machinery. In Arabidopsis, *cis*-elements and corresponding binding proteins have been implicated in plant stress responses because their expression is induced or repressed under various stress conditions. Typically, these proteins contain a distinct type of DNA-binding domain such as AP2/ERF, bZIP/HD-ZIP, MYB, MYC, NAC, WRKY and several classes of zinc finger domains (Abe *et al.*, 2003; Liu *et al.*, 1998; Pastori and Foyer, 2002; Tran *et al.*, 2004; Uno *et al.*, 2000; Yamaguchi-Shinozaki and Shinozaki, 2005). Individual members of the same family often respond differently to various stress stimuli; on the other hand, some stress-responsive genes may share the same transcription factors (TFs). This conclusion was based upon the significant overlap of the gene expression profiles that are induced in response to various stresses (Bohnert *et al.*, 2001; Chen *et al.*, 2002; Fowler and Thomashow, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2001).

Analyses of drought-induced genes have indicated the existence of ABA-independent signal transduction cascades occurring between the initial signal of water deficit and the expression of specific genes (Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki and Shinozaki, 2005). Several stress-inducible genes, such as *RD19* and *RD21* that encode different cysteine proteases (Koizumi *et al.*, 1993), and *ERD1* that encodes a putative protein similar to the Clp protease regulatory subunit, are induced through the ABA-independent pathway (Kiyosue *et al.*, 1993, 1994; Nakashima *et al.*, 1997). Molecular analysis of the *ERD1* promoter demonstrated that *ERD1* expression during dehydration mainly depends upon the integrity of both the 14 bp CACTAAATTGTCAC and the CATGTG MYC-like recognition sites (Simpson *et al.*, 2003). Homology searches for putative *cis*-acting motifs within the 14 bp region revealed that this sequence has a reverse complementary homology to an 11 bp region of the site 1 motif AATTGTTACCATGA from the promoter region of spinach *RPS1* gene. Furthermore, the putative core sequence of this *cis*-motif was previously shown to include the 5 bp sequence GTTAC, which is also found within the 11 bp region of homology with the *ERD1* promoter (Simpson *et al.*, 2003; Villain *et al.*, 1994; Zhou *et al.*, 1992).

In a previous study, we isolated three cDNAs encoding the ANAC019, ANAC055 and ANAC072 TFs that bind to the 63 bp promoter region of the *ERD1* gene, which contains the CATGTG MYC-like motif (Tran *et al.*, 2004). Using the yeast one-hybrid system, we determined the complete NAC recognition sequence (NACRS), ANNNNNTCNNNNNNNA-CACGCATGT, containing CATGT and harbouring CACG as the core DNA-binding site (Tran *et al.*, 2004). Plant-specific NAC family TFs have a highly conserved NAC domain at the N-termini of the encoded proteins (Olsen *et al.*, 2005) and have been implicated not only in abiotic and biotic stress responses (Collinge and Boller, 2001; Fujita *et al.*, 2004; Hegedus *et al.*, 2003; Oh *et al.*, 2005; Tran *et al.*, 2004), but also in plant development and senescence (Aida *et al.*, 1997; John *et al.*, 1997; Sablowski and Meyerowitz, 1998; Souer *et al.*, 1996; Vroemen *et al.*, 2003; Xie *et al.*, 2000). In the Arabidopsis genome, about 100 putative NAC genes have been identified (Ooka *et al.*, 2003; Riechmann *et al.*, 2000). Several NAC proteins have been shown to bind to the 35S −90 promoter fragment (Duval *et al.*, 2002; Ernst *et al.*, 2004; Xie *et al.*, 2000). Over-expression of either *ANAC019*, *ANAC055* or *ANAC072* altered the expression of many stress-inducible genes in the transgenic plants, and provided plants with a constitutive increase in drought tolerance (Tran *et al.*, 2004). However, it is important to note that *ERD1* was not upregulated in the transgenic plants. This observation suggests that a coupling TF, which probably binds to the CACTAAATTGTCAC *RPS1* site 1-like sequence, is also required for the induction of *ERD1*.

Here, we describe the cloning and characterization of a novel zinc finger homeodomain (ZFHD) TF, ZFHD1, that specifically binds to the 14 bp *RPS1* site 1-like sequence; which was accordingly renamed to ZFHDRS (zinc finger homeodomain recognition sequence). We analysed the co-activity of both the newly identified proteins and NAC proteins functioning as *trans*-acting factors by using both Arabidopsis T87 protoplasts and Arabidopsis plants over-producing both ZFHD1 and NAC TFs. We used the yeast two-hybrid system as a method to identify the interaction between the ZFHD1 and NAC TFs. Furthermore, to analyse the functions of ZFHD1 TF alone, we studied the gene expression profile of Arabidopsis plants ectopically expressing *ZFHD1* using a 22 K oligo microarray. As a result of this genomic approach, we have been able to identify a number of stress-inducible target genes of the ZFHD1 TF. Lastly, transgenic plants were produced that over-expressed *ZFHD1* either alone or together with ZFHD1 and NAC TFs. The resultant transgenic plants showed significantly increased drought tolerance.

Results

Isolation of cDNAs encoding DNA-binding proteins that bind to the CACTAAATTGTCAC motif in the 62 bp DNA fragment of the ERD1 *promoter*

We utilized the yeast one-hybrid library screening method as a means to clone cDNAs encoding DNA-binding proteins that interact with the 62 bp fragment containing the CAC-

TAAATTGTCAC motif. First, we constructed a YZFHL6 yeast strain, which carried integrated copies of *HIS3* and *lacZ* that served as dual reporter genes. The reporter genes were fused to a six-times tandemly repeated 62 bp DNA fragment of the *ERD1* promoter (−620 to −559), which covered the CACTAAATTGTCAC motif. The resulting yeast strain was tested for *HIS3* and *lacZ* background expression using control assays based on the fact that (i) YZFHL6 transcribes the *HIS3* gene at basal levels, grows on media lacking histidine, but does not grow in the presence of 10 mM 3-aminotriazole (3-AT) and (ii) it forms white colonies on filter papers that have been pre-wetted with X-Gal. In order to screen for cDNAs encoding DNA-binding proteins of interest, the target reporter YZFHL6 strain was separately transformed with two pAD-GAL4 cDNA libraries. These libraries were constructed from cDNA fragments of mRNAs that were prepared from Arabidopsis rosette plants that were unstressed or had been dehydrated for 3 h. In total, we identified 20 clones that were resistant to 10 mM 3-AT, corresponding to four cDNAs from the library prepared from dehydrated Arabidopsis plants: *P1* (16 clones), *P2* (two clones), *P3* (one clone) and *P4* (one clone). All of these clones induced *lacZ* activity and formed blue colonies on filter paper containing X-Gal (Figure 1a).

To determine whether the isolated clones function as transcriptional activators, the cDNA inserts encoding P1, P2, P3 and P4 were cloned into the YepGAP yeast expression vector (Liu *et al.*, 1998), and the resulting plasmids were transformed into the YZFHL6 yeast reporter strain. Yeast cells carrying the plasmid containing the *P1* cDNA grew on the medium lacking histidine in the presence of 10 mM 3-AT and induced *lacZ* activity. However, those carrying the plasmid containing cDNA from either *P2*, *P3* or *P4* did not induce expression of both the *HIS3* and *lacZ* genes (Figure 1b).

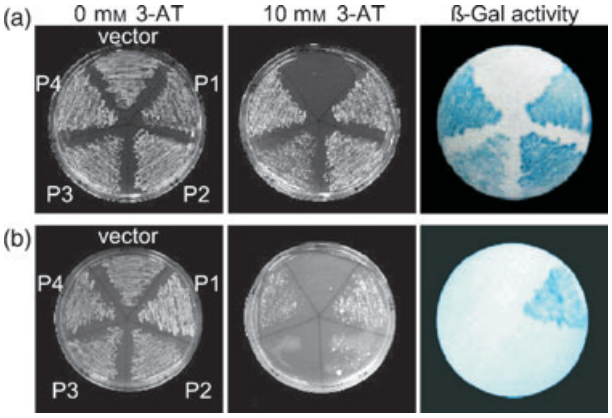

Figure 1. Isolation of cDNAs encoding CACTAAATTGTCAC motif-binding proteins using a yeast one-hybrid system.
(a) Four isolated clones, P1, P2, P3 and P4, displayed 10 mM 3-AT resistance and induced *lacZ* activity.
(b) The cDNA fragments encoding P1, P2, P3 and P4 were cloned into the constitutively expressed YepGAP vector and the resulting plasmids were introduced into the YZFHL6 yeast strain. The transformants were examined for growth in the presence of 10 mM 3-AT and *lacZ* activity. Only P1 was able to transactivate the expression of both the *HIS3* and *lacZ* reporter genes.

Zinc finger homeodomain proteins that bind to the ERD1 *promoter of Arabidopsis*

Based upon sequence database searches with the BLAST algorithm using the deduced amino acid sequences, the four prey proteins were assigned to the ZFHD TF family (Figure 2a). These proteins share three domains with a high degree of sequence similarity: the putative DNA-binding homeodomain which is located at the C-terminus, and the

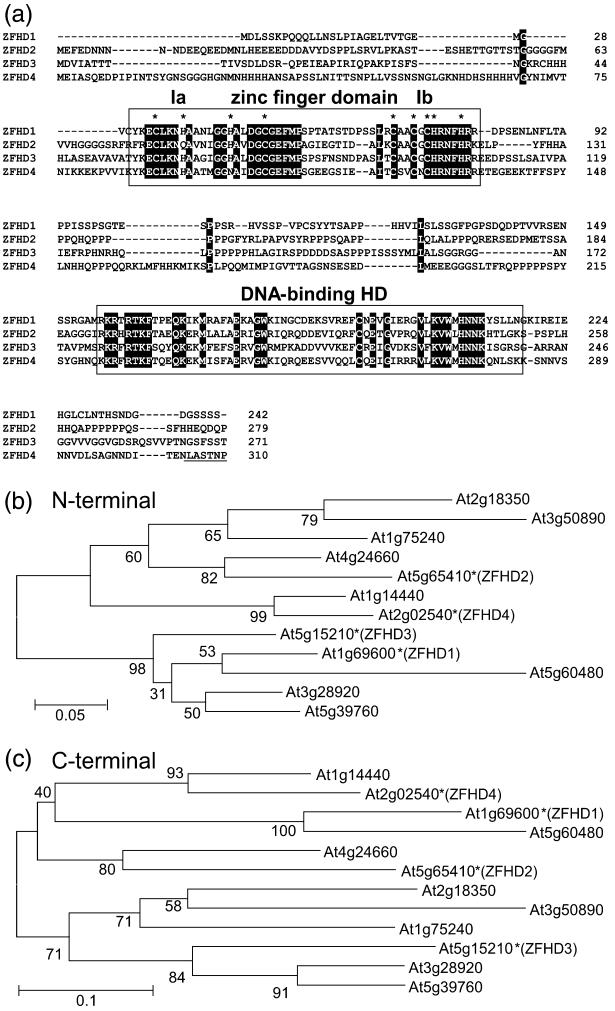

Figure 2. Structural analysis of isolated proteins.
(a) Comparison of the amino acid sequences of ZFHD1, ZFHD2, ZFHD3 and ZFHD4. Identical amino acids are indicated by white letters on a black background. The highly conserved zinc finger domain containing putative zinc-binding amino acids (marked by asterisks) and the putative DNA-binding homeodomain are boxed. The C-terminal serine-rich region is underlined.
(b and c) The evolutionary relationship of the Arabidopsis homeodomain proteins. Either zinc finger domains (b) or DNA-binding homeodomains (c) were used to construct the phylogenetic trees. The bar indicates the relative divergence of the sequences examined. The percentage of bootstrap values (1000 replicates) is shown above the branch nodes.

N-terminal zinc finger domain which consists of two segments (Ia and Ib; Figure 2a). This N-terminal zinc finger domain contains five conserved cysteine residues and at least three conserved histidine residues. It is important to note that the observed spacing pattern of the cysteine and histidines closely resembles zinc-binding domains that are involved in the dimerization of TFs (Mackay and Crossley, 1998). Although the two segments contain at least eight potential zinc-binding amino acids, the unique spacing of the conserved cysteine and histidine residues within segment Ib suggests that both segments form one rather than two zinc finger structures. Some of these putative zinc-binding residues are separated by only one amino acid, a feature that makes it less likely that they contribute to the formation of a second zinc finger structure.

In order to examine the evolutionary relationship between the ZFHD proteins described within our study and other ZFHD proteins found in Arabidopsis, phylogenetic trees were generated with the CLUSTAL X program using zinc finger or DNA-binding homeodomains (Figure 2b,c). The four isolated proteins, named ZFHD1, ZFHD2, ZFHD3 and ZFHD4, belong to four distinct clades. The respective DNA-binding site for these four proteins was subsequently renamed ZFHDRS. At the present time, the biological functions of these isolated Arabidopsis ZFHD TFs remain unknown. Additional database searches revealed that the ZFHD class of homeodomain proteins is present in a broad range of plants such as: *Lactuca sativa* (lettuce), *Solanum tuberosum* (potato), *Oryza sativa* (rice), Glycine max (soybean), *Solanum lycopersicum* (tomato) and C4 species of the genus *Flaveria*. However, there is little information describing the function of this class of proteins, although Windhövel *et al.* (2001) reported the isolation of four *Flaveria* ZFHD TFs that are thought to be involved in regulating the expression of the mesophyll-specific C4 phosphoenolpyruvate carboxylase gene.

Expression of the ZFHD *genes*

To identify the function of the *ZFHD* genes under abiotic stresses, we initially analysed the expression patterns of *ZFHD* genes under various stresses and hormone treatments by RNA gel blot hybridization (Figure 3). Among the four isolated *ZFHD* genes, only the expression of *ZFHD1* was induced by dehydration, high salinity and ABA treatment. The induction of *ZFHD1* was detected within 1 h after dehydration and exogenous ABA treatment. During salt stress, the *ZFHD1* transcription was more strongly induced, and accumulation of the *ZFHD1* mRNA could be observed after 30 min of high salinity stress. Expression of the *ZFHD* genes was not induced by cold treatment, and neither was *ERD1*. The accumulation of *ERD1* mRNA could be observed within 1 h after dehydration and exogenous ABA treatment, and 2 h after high salinity treatment. Furthermore, ZFHD1 was able to activate reporter genes driven by ZFHDRS and minimal promoters, an activity that was not observed with ZFHD2, ZFHD3 and ZFHD4. We therefore focused our efforts on the functional analysis of ZFHD1.

The ZFHD1 protein specifically binds to ZFHDRS

ZFHD1 binds to the 62 bp fragment containing ZFHDRS . We were interested to determine whether ZFHD1 specifically bound to the ZFHDRS *in vivo*. To investigate this, we transformed the pAD-GAL4–ZFHD1 fusion plasmid into a yeast strain carrying the two reporter genes fused to two tandemly

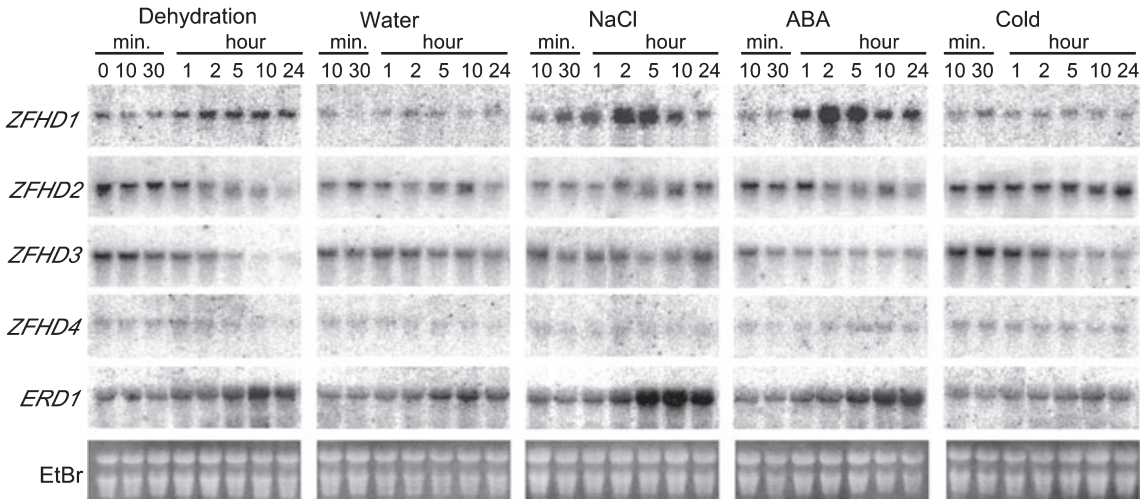

Figure 3. Expression of *ZFHD1*, *ZFHD2*, *ZFHD3* and *ZFHD4*.
Each lane was loaded with 20 μg of total RNA from 3-week-old plate-grown Arabidopsis plants that had been dehydrated (Dehydration), or transferred for hydroponic growth in distilled water (Water), 250 mM NaCl (NaCl), 100 μM ABA (ABA) or at 4°C (Cold). Numbers above each lane indicate the number of minutes or hours after the initiation of treatment before isolation of RNA. mRNA transcription was analysed by RNA gel hybridization. EtBr, ethidium bromide.

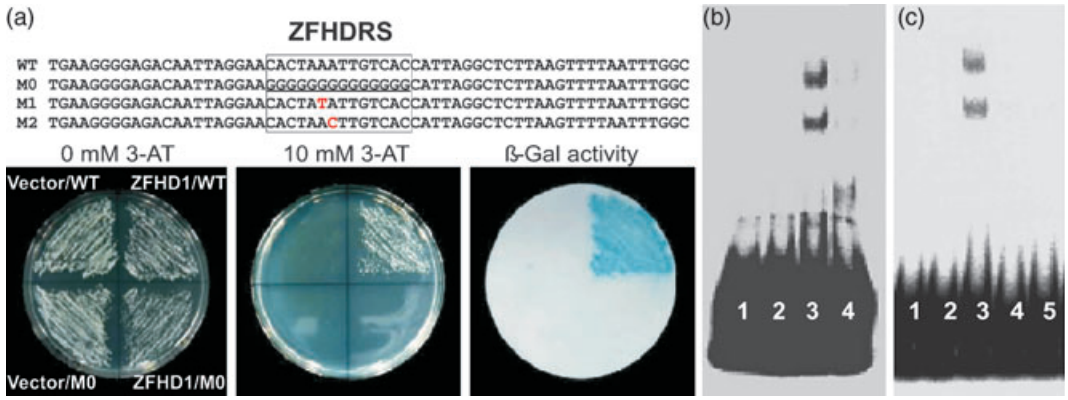

Figure 4. ZFHD1 protein binds specifically to ZFHDRS.
(a) ZFHD1 protein specifically binds to ZFHDRS *in vivo*. A pAD-GAL4 plasmid derivative carrying a cDNA clone encoding the *ZFHD1* gene and the control pAD-WT plasmid were transformed into yeast strains carrying the reporter genes under the control of two tandemly repeated 62 bp promoter fragments containing either wild-type ZFHDRS or mutated motifs M0. The transformants were examined for growth in the presence of 10 mM 3-AT and for β-galactosidase activity, thus demonstrating the binding specificity of ZFHD1 *in vivo*.
(b) *In vitro* DNA-binding reactions were performed with the wild-type 62 bp fragment containing the ZFHDRS (lanes 1 and 3) and the base-substituted 62 bp fragment in which the CACTAAATTGTCAC motif had been replaced by the GGGGGGGGGGGGG sequence (lanes 2 and 4). The radioactive wild-type and base-substituted fragments were incubated with the GST protein (lanes 1 and 2) or the *ZFHD1*–GST fusion protein (lanes 3 and 4).
(c) Mutations of representative canonical nucleotides of ZFHDRS inhibit the binding activity of ZFHD1 *in vitro*. The labelled 62 bp wild-type (lane 3) and base-substituted M1 (lane 4) or M2 (lane 5) fragments were incubated with *ZFHD1*–GST fusion protein. As controls, the labelled 62 bp wild-type fragment was incubated with water (lane 1) or with the GST protein (lane 2).

repeated copies of the mutated 62 bp fragment in which the ZFHDRS was replaced with the sequence GGGGGGGGG-GGGGG (M0) (Figure 4a). The recombinant yeast strains neither grew on medium lacking histidine in the presence of 3-AT nor induced *lacZ* activity. These *in vivo* data indicate that the ZFHD1 protein specifically interacts with the ZFHDRS motif, and not with either the polyG stretch, or the 5′- or 3′-ends of ZFHDRS on the 62 bp fragment (Figure 4a).

The specific binding of ZFHD1 to ZFHDRS was tested *in vitro* using the recombinant ZFHD1 protein. A recombinant ZFHD1–glutathione-*S*-transferase (GST) fusion protein was expressed in *Escherichia coli* and purified for Electrophoretic Mobility Shift assay (EMSA). As shown in Figure 4(c), the recombinant ZFHD1 protein bound the wild-type 62 bp fragment (WT), but did not interact with the base-substituted 62 bp M0 fragment, confirming that ZFHD1 can recognize only the ZFHDRS sequence, but not the sequences located on either the 5′- or 3′-sides of the ZFHDRS (Figure 4b). Within the 14 bp ZFHDRS (CACTAAA-TTGTCAC), there is a sequence (TAAATT) that closely resembles the binding sequence TGTAATT of the α2-homeodomain (Wolberger *et al.*, 1991). Furthermore, Tan and Irish (2006) have recently shown that the Arabidopsis zinc finger homeodomanin At1g75240/ATHB33 bound to the 20 bp segment AGTGTCTTGTAATTAAAA that contains the NNATTA consensus sequence, which is the canonical binding site for most homeodomains with the specificity for the first two bases (Conolly *et al.*, 1999; Fraenkel and Pabo, 1998; Tan and Irish, 2006). The ZFHDRS appears to contain this consensus, which is underlined in the 14 bp ZFHDRS, except that the last nucleotide, which is absent in the α2-homeodomain TGTAATT binding sequence, is G instead of A. This may indicate that it is not indispensable. Two singly mutated 62 bp fragments harbouring a mutation either in the position important for the specificity (M1) or in the consensus nucleotide (M2) (Figure 4a) lost the ability to bind ZFHD1 (Figure 4c). In a separate experiment (Figure 5c) in which a trimer of the 14 bp ZFHDRS was used as a probe, complexes formed through interaction between ZFHD1–GST and labelled DNA fragment were observed, suggesting that the binding sequence for ZFHD1 is well located within the 14 bp ZFHDRS. Together, these results demonstrate that the ZFHD1 protein specifically binds to the 14 bp ZFHDRS sequence containing the consensus NNATTN.

Localization of the DNA-binding and transcription activation domains of ZFHD1

Secondary structure analyses of plant ZFHD proteins predict a helix-turn-helix structure that is typical of the homeodomain; a feature that is probably involved in DNA–protein interactions (Bürglin, 1997). To support this hypothesis, we examined the binding activity of the putative DNA-binding homeodomain of ZFHD1 using an *in vivo* yeast system. A series of fusions of the GAL4 activation domain and different portions of the ZFHD1 homeodomain were designed and assayed for their ability to bind ZFHDRS and activate the *HIS3* and *lacZ* reporter genes in the YZFHL6 yeast strain. As indicated in Figure 5(b), the region of amino acids 156–218 was still able to promote growth of yeast transformants on selective medium lacking histidine in the presence of 5 mM

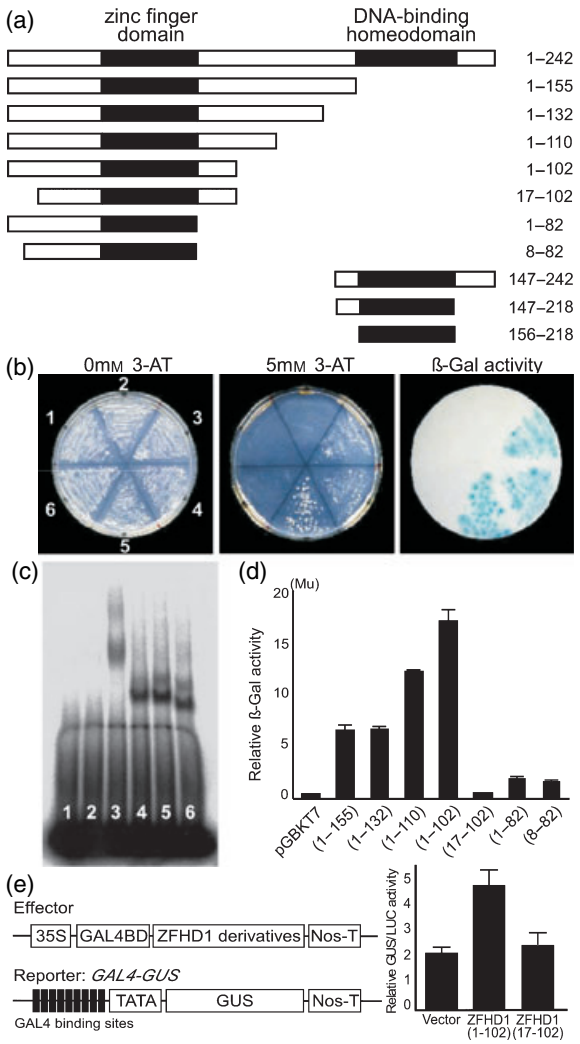

Figure 5. Localization of DNA-binding and activation domains of ZFHD1.
(a) Scheme of fragments used in localization experiments.
(b) The ZFHD1 homeodomain binds to ZFHDRS *in vivo*. The fragments encoding amino acids 1–102 (2), 147–242 (3), 147–218 (4) and 156–218 (5), respectively, were fused to the GAL4 activation domain in the pGADT7 vector. These constructs and the pGADT7 control plasmid (1, 6) were then transformed into a yeast strain carrying the *HIS3* and *lacZ* reporter genes under the control of six tandemly repeated 62 bp *ERD1* promoter fragments containing the ZFHDRS. The transformants were examined for growth in the presence of 5 mM 3-AT and for β-galactosidase activity. The homeodomain was able to interact with ZFHDRS and activate the *HIS* and *lacZ* reporter genes.
(c) The ZFHD1 homeodomains bind to the 14 bp ZFHDRS *in vitro*. The fragments encoding amino acids 1–242 (3), 147–242 (4), 147–218 (5) and 156–218 (6), respectively, were fused with the GST protein, and the fusion proteins were incubated with the labelled trimer of 14 bp ZFHDRS. For control, the labelled trimer of 14 bp ZFHDRS was incubated with water (1) or the GST protein (2).
(d) The N-terminal part of ZFHD1 has activation activity in yeast. The fragments encoding amino acids 1–155, 1–132, 1–110, 1–102, 17–102, 1–82 and 8–82, respectively, were fused to the GAL4 binding domain in the pGBKT7 vector. These constructs were transformed into the Y187 yeast reporter strain, and the β-galactosidase activity of each transformant was determined to analyse the activation activity of the fusion proteins.
(e) The 1–102 N-terminal fragment of ZFHD1 confers activation activity in Arabidopsis protoplasts. The effector plasmid that was driven by a CaMV 35S promoter carries a fusion gene that consisted of the GAL4 DNA-binding domain and either the 1–102 or the 17–102 fragment of ZFHD1. The GUS reporter construct, GAL4–GUS, containing GAL4 binding sites, is fused to the minimal promoter of CaMV 35S. Arabidopsis protoplasts were co-transfected with the GAL4–GUS reporter and the effector construct or the vector DNA. To normalize for transformation efficiency, the CaMV 35S–*LUC* plasmid was also co-transfected in all cases. Bars indicate the standard errors of three replicates.

3-AT and displayed β-galactosidase activity. These observations suggest that the region of amino acids 156–218 covering the homeodomain functions as a potential DNA-binding domain. In order to verify the genetic data, we fused the 147–242, 147–218 and 156–218 C-terminal fragments (Figure 5a), respectively, to the GST protein for EMSA. The three ZFHD1BD–GST fusion proteins were able to form complexes with a labelled DNA construct consisting of a trimer of the 14 bp ZFHDRS, confirming that the homeodomain was able to act as a DNA-binding domain (Figure 5c).

Thus, it is reasonable to consider that the transcriptional activation domain of ZFHD1 resides within the N-terminal domain of ZFHD1. We tested this hypothesis, first using yeast as an assay system. Various fragments comprising the highly homologous zinc finger domain fused with the GAL4 DNA-binding domain were assayed for their ability to activate transcription of the *lacZ* reporter gene in Y187 from the GAL4 upstream activation sequence. Although the region of amino acids 1–155 was the longest tested N-terminal part, covering the region up to the DNA-binding homeodomain, it showed a moderate level of activation activity compared with the region comprising amino acids 1–102 (Figure 5d). Further deletion analysis of the 1–102 fragment showed that the smallest region that still retained an induction of β-galactosidase activity, approximately twice that of the control, spanned amino acids 8–82 (Figure 5d). The region of amino acids 1–102 induced the highest β-galactosidase activity, thereby indicating that this region covers the potential activation domain. This region of amino acids 1–102 was then used to test the ability of the ZFHD1 to activate transcription in plant cells. We constructed an effector plasmid that was driven by the 35S promoter, carrying a fusion gene that consisted of the GAL4 DNA-binding domain and the 1–102 fragment (Figure 5e). This plasmid was co-transfected into Arabidopsis protoplasts with a reporter plasmid, GAL4–GUS, that contains nine copies of a GAL4 binding site fused to the minimal 35S promoter and the *uidA* gene (GUS). The effector plasmid transactivated the reporter gene, demonstrating that the N-terminal region of ZFHD1 functions as a transcriptional activation domain *in planta*, even in combination with a non-native DNA-binding domain (Figure 5e). Consistent with the yeast experiment, the 17–102 fragment did not exhibit significant activation activity in Arabidopsis protoplasts either (Figure 5e).

ZFHD1 and NAC proteins act in concert to transactivate expression of a GUS reporter gene driven by the ERD1 *promoter and both ZFHDRS and NACRS in Arabidopsis T87 protoplasts*

Simpson *et al.* (2003) showed that induction of the *ERD1* gene required coordinated activity of two *cis*-elements, ZFHDRS and NACRS. Thus, as a means to quickly determine whether the ZFHD1 and NAC proteins are capable of transactivating ZFHDRS/NACRS-dependent transcription in plant cells, transactivation assays were performed using Arabidopsis T87 protoplasts. Protoplasts were co-transfected with a GUS reporter plasmid, which consisted of two tandem copies of the 187 bp fragment of the *ERD1* promoter (from −620 to −434) containing ZFHDRS and NACRS, fused to the minimal *ERD1* promoter and the *uidA* gene (GUS), and one or two effector plasmids. The effector plasmids consisted of the CaMV 35S promoter fused to either *ZFHD1*, stress-inducible NACRS-binding *ANAC019*, *ANAC055* or *ANAC072* cDNAs (Tran *et al.*, 2004). Expression of each of these constructs in T87 protoplasts only transactivated slight expression of the GUS reporter gene. Co-expression of both *ZFHD1* and each *NAC* gene in all possible combinations transactivated significant expression of the GUS reporter gene (Figure 6a). These results suggest that expression of both stress-inducible *ZFHD1* and *NAC* genes is required for the drought-dependent expression of the *ERD1* gene in Arabidopsis.

The ZFHD1 and NAC proteins may cooperatively transactivate the expression of the GUS reporter gene that is driven by the *ERD1* promoter in Arabidopsis T87 protoplasts. We used a yeast two-hybrid system to test whether there is an interaction between the ZFHD1 and NAC proteins. The constructs GAL4BD–ANAC019BD, GAL4BD–ANAC055BD and GAL4BD–ANAC072BD were created as described in Experimental procedures, and were used as baits. The full-length ZFHD1 protein was inserted into the pAD-GAL4 vector in fusion with the GAL4 activation domain (GAL4AD–ZFHD1). These constructs and the empty vectors were transformed into the AH109 yeast strain in all possible combinations. When solely present, none of the fusion proteins induced growth on a minimal medium lacking adenine and histidine that was used to analyse the interaction of the corresponding proteins. In contrast, co-existence of the GAL4AD–ZFHD1 and any GAL4BD–ANACBD fusion proteins induced growth on either minimal medium lacking adenine and histidine, or medium that was lacking histidine in presence of 5 mM 3-AT, demonstrating that there is an interaction between the ZFHD1 protein and each NAC binding domains (Figure 6b). This result might indicate the necessity for the concomitant interaction of two DNA-binding proteins before induction of dehydration-responsive expression of *ERD1*.

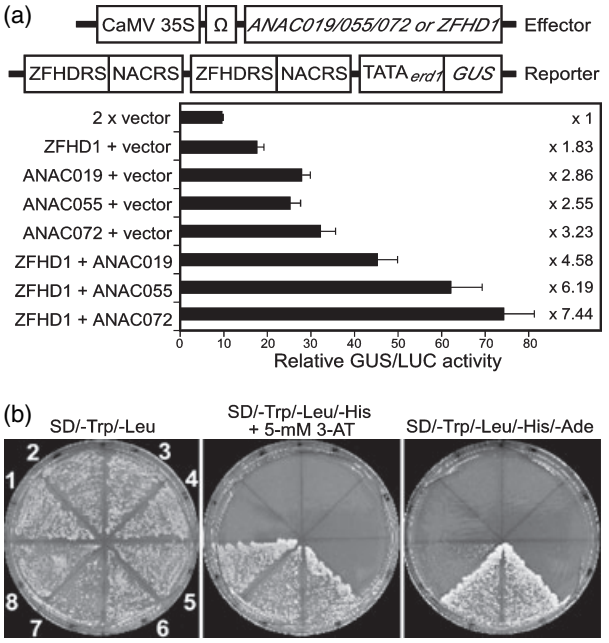

Figure 6. ZFHD1 and NAC proteins act in concert to transactivate expression of a GUS reporter gene in Arabidopsis T87 protoplasts.
(a) ZFHD1 and NAC transcription factors transactivate the *ERD1* promoter–GUS fusion, which is under the control of both ZFHDRS and NACRS. cDNA fragments encoding ZFHD1, ANAC019, ANAC055 and ANAC072 were cloned into plant expression vector pBI35SΩ and co-transformed into T87 protoplasts with the reporter gene plasmids (Reporter) in all possible combinations. To normalize for transformation efficiency, the CaMV 35S–*LUC* plasmid was also co-transfected in all cases. Bars indicate the standard errors of three replicates. Multiplication values refer to the ratio of expression relative to the value obtained with the pBI35SΩvector.
(b) Interaction between ZFHD1 and NAC proteins. The three constructs GAL4BD–ANAC019BD, GAL4BD–ANAC055BD and GAL4BD–ANAC072BD represent fusions of the GAL4 binding domain and NAC binding domains in pGAPGB. The full-length ZFHD1 protein was inserted into the pAD-GAL4 vector in fusion with the GAL4 activation domain, resulting in a GAL4AD–ZFHD1 fusion construct. These constructs and control vectors pGAPGB (GAL4BD) and pGADT7 (GAL4AD) were transformed into AH109 yeast reporter strain in all possible combinations. The transformants were examined for growth on minimal medium lacking histidine in the presence of 5 mM 3-AT, or on minimal medium lacking adenine and histidine, and were used to analyse the interaction of the corresponding proteins. 1, GAL4AD + GAL4BD; 2, GAL4AD + GAL4BD–ANAC055BD; 3, GAL4AD + GAL4BD–ANAC019BD; 4, GAL4AD + GAL4BD–ANAC072BD; 5, GAL4AD–ZFHD1 + GAL4BD; 6, GAL4AD–ZFHD1 + GAL4BD–ANAC055BD; 7, GAL4AD–ZFHD1 + GAL4BD–ANAC019BD; 8, GAL4AD–ZFHD1 + GAL4BD–ANAC072BD.

Functional analysis of the ZFHD1 transcription factor in Arabidopsis: ZFHD1-over-expressing plants display enhanced tolerance to drought

To analyse the biological function of ZFHD1, we generated transgenic plants (35S:ZFHD1) in which the *ZFHD1* gene was constitutively over-expressed under the control of the 35S promoter of cauliflower mosaic virus and the Ω sequence of tobacco mosaic virus. Twenty-four 35S:ZFHD1 lines were subjected to RNA gel blot analysis in order to examine *ZFHD1* gene expression levels. All of the plant lines exhib-

ited a strong over-expression of the transgene, and the RNA blot results for five representative lines are shown in Figure 8. Over-expression of the *ZFHD1* gene reduced the size of rosette plants and caused yellowing in some of the rosette leaves (Figure 7a). At 3 weeks, the maximum rosette radius of the 35S:ZFHD1 plants averaged two-thirds that of the vector control plant (Figure 7d). After 3 weeks of growth on selective plates, plants were transferred to soil pots and grown under continuous light. We observed that the 35S:ZFHD1 plants bolted earlier than vector controls, with five or six rosette leaves for the 35S:ZFHD1 plants and eight leaves for vector control plants ($n = 10$ for each of three independent 35S:ZFHD1 line tested and vector control). At the 5-week stage, the inflorescences of the 35S:ZFHD1 plants were weaker and rounder in comparison with those of vector control plants (Figure 7b). The inflorescences of 7-week-old 35S:ZFHD1 plants were shorter, reaching only 75% of the average length of the vector control inflorescences (Figure 7e,f).

To identify the target genes of ZFHD1 TF, we used the Agilent Arabidopsis 2 oligo microarray that covers over 21 000 genes. The expression profiles in two independent lines of 3-week-old 35S:ZFHD1 under unstressed conditions were compared with those of the vector control plants using Agilent's propagated error method. Genes whose expression levels were significantly upregulated are summarized in Table 1. The complete microarray data set is provided in Table S1 or can be accessed at http://www.ebi.ac.uk/arrayexpress/ with accession number E-MEXP-366. Several genes were randomly chosen and used to confirm the reliability of the microarray data using RNA blot hybridization. *ERD1*, a gene that was not upregulated by over-expression of the *ZFHD1* gene, was included as a negative control. The results shown in Figure 8 support the reliability of the microarray data. *In silico* analysis of the 1000 bp promoter regions upstream of the putative transcription sites of the upregulated genes listed in Table 1 revealed that all of them, except those of *At5g41020* and *At5g26280*, have the consensus TT/AAATT motif within the 14 bp ZFHDRS, closely resembling the TGTAATT motif that plays an indispensable role in binding of the α2-homeodomain and the ZFHD of At1g75240/ATHB33 (Tan and Irish, 2006; Wolberger *et al.*, 1991).

Microarray analysis indicated that several stress-inducible genes are upregulated in the 35S:ZFHD1 plants. In order to evaluate the effect of ZFHD1 over-production on stress tolerance, with a particular emphasis on drought tolerance, we performed assays with three independent 35S:ZFHD1 transgenic lines, all of which behaved in a similar manner. All three *ZFHD1*-over-expressing plants displayed an increased tolerance to drought. More than 90% of the *ZFHD1*-over-expressing plants survived, a rate that was substantially higher than the 6.25% survival rate observed in control plants (Figure 7c,g). To assess whether altered transpiration rates contribute to the better survival of the 35S:ZFHD1 plants, we measured water loss rates and standardized water contents of either 3-week-old whole plate-grown plants or the detached aerial parts of 4-week-old plants that had been transferred to soil for the fourth week. No remarkable difference was found either between the water loss rates or between the standardized water contents of the 35S:ZFHD1 and vector control plants (data not shown). The increased tolerance to drought of the 35S:ZFHD1 plants is likely to be a consequence of their high levels of ZFHD1-regulated expression of downstream genes, for example, LEA class proteins and/or cytochrome P450-dependent proteins.

Co-over-expression of the ZFHD1 *and* NAC *genes enhances expression of the* ERD1 *gene*

Previously, results of studies with Arabidopsis T87 protoplasts indicated that induction of *ERD1* required over-production of both ZFHD1 and NAC TFs (Figure 6a). In order to achieve *in vivo* confirmation in Arabidopsis and to analyse the effects of over-expression of both TFs on the expression of *ERD1*, we generated 35S:ZFHD1/35S:ANAC019, 35S:ZFHD1/35S:ANAC55 and 35S:ZFHD1/35S:ANAC072 transgenic plants over-expressing NAC and ZFHD1. Of 11 lines of 35S:ZFHD1/35S:ANAC019, seven lines of 35S:ZFHD1/35S:ANAC55 and six lines of 35S:ZFHD1/35S:ANAC072, four, three and two stable transformant lines, respectively, and two vector control lines were used to analyse the expression level of *ERD1* using RNA gel blot hybridization. RNAs were purified from 3-week-old plate-grown plants (16 h light/8 h dark cycle), and subjected to

Figure 7. Phenotype of the 35S:ZFHD1, 35S:ZFHD1/35S:ANAC019, 35S:ZFHD1/35S:ANAC055 and 35S:ZFHD1/35S:ANAC072 plants.
The effect of the over-expression of *ZFHD1* and *NAC* genes on plant morphology was studied as follows.
(a) 35S:ZFHD1, 35S:ZFHD1/35S:ANAC019, 35S:ZFHD1/35S:ANAC055, 35S:ZFHD1/35S:ANAC072 and vector control plants were germinated on selective GM medium and grown for 3 weeks.
(b) Three-week-old plants were transferred to pots and grown for two additional weeks.
(c) The drought tolerance phenotype of the 35S:ZFHD1 transgenic plants was investigated as described in Experimental procedures. Control, 4-week-old plants growing under normal conditions; Drought, water withheld from plants for 10 days, then rewatering for 3 days.
(d) Maximum rosette radius (i.e. length of the longest rosette leaf) of 3-week-old plate-grown transgenic plants. Bars indicate standard deviation ($n = 10$).
(e) Three-week-old plants were transferred to pots and grown for four additional weeks for comparison of the length of inflorescences.
(f) Comparison of the length of inflorescence of 7-week-old transgenic plants. Bars indicate standard deviation ($n = 10$).
(g) Comparison of survival rates of transgenic plants under the conditions described in (c). Survival rates and standard deviations (bars) were calculated from results of three independent experiments ($n > 20$ for each experiment).

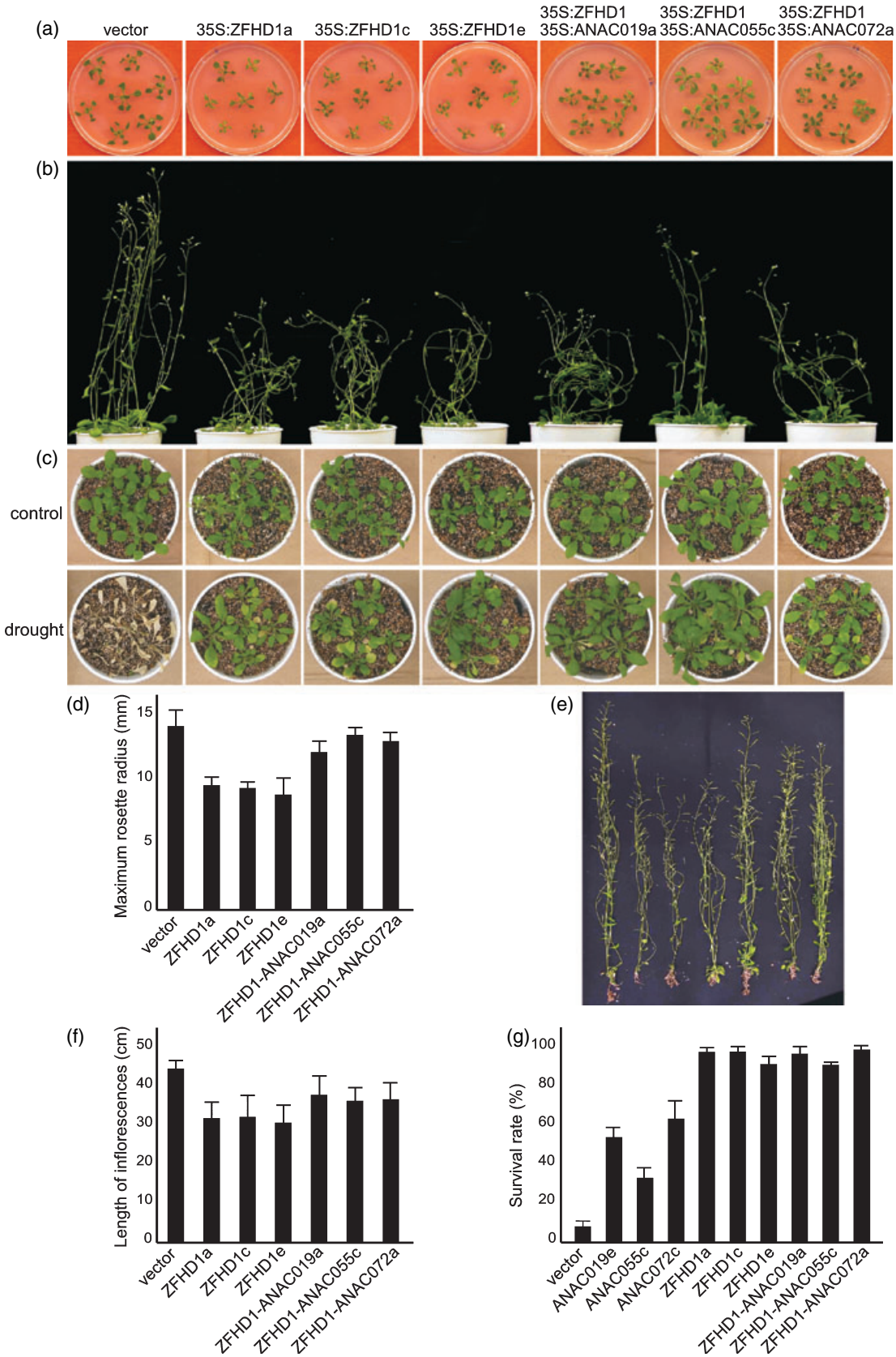

Table 1 ZFHD1-dependent genes detected through microarray analysis

Gene name[a]	MIPS ID[b]	Ratio[c]		Description[d]	Stress response[e]		Number of consensus sequences[f]	
		Line a	Line c		22 K	7 K	TAAATT	TTAATT
RAFL02-05-I05	At4g37990	15.3	12.5	Cinnamyl-alcohol dehydrogenase	A, S	A, D, S	3	1
RAFL15-06-K01	At4g36700	6.5	6.4	Globulin-like protein family	N	–	0	1
	At2g41240	7.3	5.5	bHLH transcription factor	N	–	2	1
	At3g56970	6.9	5.6	bHLH transcription factor	N	–	1	1
RAFL09-81-P03	At1g56430	6.3	5.5	Nicotianamine synthase	N	–	3	0
	At3g56980	6	5.5	bHLH transcription factor	N	–	1	1
RAFL04-16-P21	At4g37370	4.5	6.5	Cytochrome P450	A, D, S, C	A, D, S	0	1
RAFL09-69-I19	At5g64120	6.1	3.9	Peroxidase	N	–	3	1
RAFL11-03-L10	At5g37260	3.6	6.3	Unknown protein	N	N	2	1
	At2g44460	4.9	4.6	Glycosyl hydrolase family 1 protein	N	–	3	1
RAFL04-17-I16	At4g02380	4.5	4.9	LEA3 family protein	D	D	1	3
RAFL05-18-K08	At2g43510	6	3.3	Trypsin inhibitor	N	A	1	1
RAFL07-16-B09	At3g48360	3.5	5.7	Unknown protein	C	S	3	0
	At1g10585	3.8	5	Unknown protein	N	–	1	1
	At5g16730	4.6	4.1	Unknown protein	N	–	2	2
	At4g14760	5.4	3	Centromere protein homologue	N	–	1	1
RAFL21-20-P21	At4g04840	3.6	4.6	Unknown protein	N	–	3	1
RAFL04-18-F22	At5g42760	4.2	3.7	Unknown protein	N	N	1	2
RAFL07-16-N22	At5g41020	4.5	3.3	myb family transcription factor	N	N	0	0
RAFL05-11-H09	At1g05680	3.2	4.5	UDP-glucosyl transferase family protein	A, S	A, D, S	2	1
RAFL21-16-A12	At5g51440	3.5	4.2	Heat shock 22 kDa protein	S	–	1	0
RAFL16-19-D08	At4g30270	4	3.6	Endoxyloglucan transferase	N	–	1	1
RAFL16-34-L18	At1g52100	4.1	3.3	Jasmonate inducible protein, putative	N	–	7	3
RAFL05-13-C23	At5g53450	4.1	2.7	Unknown protein	N	A	0	1
RAFL09-31-F09	At5g26280	4.6	2.1	Unknown protein	N	–	0	0
RAFL14-88-C18	At5g41790	4.3	2.3	Myosin heavy chain-like protein	N	–	2	0
RAFL09-12-B03	At3g60140	3.4	3.1	Glycosyl hydrolase family 1 protein	A	A, D, S	2	1
RAFL11-07-D13	At1g72680	3.5	3	Cinnamyl-alcohol dehydrogenase	S	A, D, S	0	1
RAFL21-17-K19	At1g07400	3	3.4	17.5 kDa class I heat shock protein	S	–	1	0

[a]According to Seki *et al.* (2002c).
[b]Munich Information Center For Protein Sequences (MIPS) entry codes for the cDNAs used in this study.
[c]The ratio is the median of fold changes of two repeats. Fold change was defined as fluorescence intensity (FI) of each cDNA of 35S:ZFHD1 divided by the FI of each cDNA of the vector control line. The first 29 genes with higher expression levels in 35S:ZFHD1 than in control plants are shown. A complete data set is available in Table S1 or at http://www.ebi.ac.uk/arrayexpress/ with accession number E-MEXP-366.
[d]Description as given by the MIPS database.
[e]7 K full-length cDNA and Agilent 22 K oligo DNA microarray results according to Seki *et al.* (2002a,b) and K. Maruyama *et al.*, Biological Resources Division, Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan (unpublished results). Genes with an expression ratio (stressed to unstressed) >3 were considered as stress-inducible genes. A, ABA treatment; D, dehydration; S, high salinity; C, cold.
[f]Number of ZFHDRS consensus sequences in 1000 bp of promoter region upstream of the putative transcription site.
N, no response; –, no microarray data.

RNA gel blot analysis. The results shown in Figure 9 demonstrate that, when only one TF was over-expressed, even at elevated levels, it was insufficient for the induction of *ERD1* expression. However, when both TFs were over-expressed simultaneously, regardless of the relative level of either *NAC* or *ZFHD1* transgene over-expression, the *ERD1* mRNA accumulation was markedly increased.

In 3-week-old plate-grown plants under a 16 h light/8 h dark cycle, expression of the *ERD1* gene is detected even under unstressed conditions (Figure 9a). This phenomenon might be due to the absence of light, as the expression of *ERD1* has also been found to be induced by incubation in the dark (Simpson *et al.*, 2003). We therefore used 3-week-old pot-grown transgenic plants, which were grown for 2 weeks on selective plates (16 h light/8 h dark cycle), then transferred to soil and grown for an additional week under continuous light conditions, to confirm the induction of *ERD1* upon co-over-expression of the *NAC* and *ZFHD1* genes observed in Figure 9(a). Under these conditions, *ERD1* expression was hardly detected. Furthermore, we also found a relevant increase in accumulation of the *ERD1* transcript in pot-grown plants over-expressing these two TFs (Figure 9b). Together, these results demonstrate that induction of *ERD1* requires over-expression of both *ZFHD1* and *NAC*; an

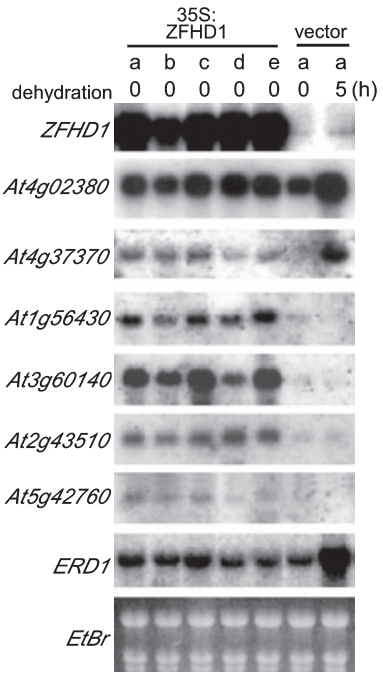

Figure 8. Confirmation of microarray data by RNA gel blot analysis.
Each lane was loaded with 10 μg of total RNA from 3-week-old plate-grown transgenic Arabidopsis plants containing pBI35S:ZFHD1Km (35S:ZFHD1) or pBI35SKm (vector) as a control. Transgenic Arabidopsis plants were untreated or dehydrated as indicated before analysis. EtBr, ethidium bromide.

observation that is consistent with the results of our study using Arabidopsis T87 protoplasts. Moreover, over-expression of both *ZFHD1* and *NAC* genes alleviated the negative effect of an elevated level of ZFHD1 on the size of the resultant transgenic plants (Figure 7a,f), suggesting that the NAC proteins may have a role in balancing the negative effect of ZFHD1 over-production. In addition, transgenic plants over-expressing both *NAC* and *ZFHD1* showed significantly improved drought tolerance compared with transgenic plants in which only *NAC* was over-expressed, due to the presence of the over-expressed *ZFHD1* gene (Figure 7g). The water loss rates and standardized water contents of these transgenic plants were similar to those of control plants (data not shown), suggesting that the enhanced tolerance of the transgenic plants can be attributed to the NAC- and/or ZFHD1-dependent over-expression of the downstream genes.

Discussion

A yeast one-hybrid screening approach using a 62 bp *ERD1* promoter fragment as a bait resulted in the cloning of cDNAs that encode four different members of a novel class of plant ZFHD TFs (Figures 1 and 2). The *ZFHD1* gene, encoding a transcriptional activator, was the most abundant clone out of the four isolated genes, and only the *ZFHD1* gene was upregulated by drought and high salt stresses (Figure 3), suggesting that ZFHD1 may play an important role in abiotic stress responses and be involved in regulation of the drought-inducible expression of the *ERD1* gene. Inspection of the four isolated ZFHD proteins revealed that they share many common features with homeodomain proteins described previously. Specifically, secondary structure analysis predicts a helix-turn-helix structure that is typical of homeodomains, and the four ZFHD proteins contain homeopolymeric amino acid stretches. This is a common feature of many homeodomain proteins (Bürglin, 1994). A sequence motif in the third helix is predicted to recognize and bind to the appropriate DNA sequence, and some amino acids in the DNA-binding helix 3 are thought to be very critical for the DNA-binding specificity of the homeodomain (Bürglin, 1997). Indeed, we showed that the homeodomain of ZFHD1 specifically recognizes and binds to the ZFHDRS (Figures 4 and 5b,c).

In addition to the homeodomains of the zinc finger and other types of homeodomain proteins, such as the HD-BEL (Balasubramanian and Schneitz, 2002), HD-KNOX (Kim *et al.*, 2005), HD-ZIP (Franklin *et al.*, 2003), HD-GL2 (Schrick *et al.*, 2004), PHD finger (Plesch *et al.*, 1997) proteins from plants, sequence comparisons show that the homeodomains of plant zinc finger proteins also share significant sequence similarities with the homeodomains of LIM proteins from animals (Fragkouli *et al.*, 2005). Functional characterization of some members of the homeobox family supports a role for some of these as key regulators of intercellular trafficking (Kim *et al.*, 2005), inflorescence stem growth (Smith *et al.*, 2004), hormone signalling (Himmelbach *et al.*, 2002), adaptive responses to environmental cues (Franklin *et al.*, 2003; Zhu *et al.*, 2004) and pathogen-derived signalling processes (Coego *et al.*, 2005).

The various novel ZFHD proteins reported here have a characteristic feature in that they all contain two highly conserved amino acid motifs in their N-terminal region (Figure 2). These motifs are conserved even in the lettuce, rice, soybean, tomato and *Flaveria* homologues assigned to this class. Thus, the name ZFHD (for ZFHD; Windhövel *et al.*, 2001) has been proposed. The N-terminal domains contain five conserved cysteine and at least two conserved histidine residues. In contrast to the DNA-binding homeodomains, the zinc finger domains of ZFHD members do not display any significant similarities either to the zinc finger domains of LIM homeobox proteins, which consist of two zinc fingers, or to other zinc finger domains. Although the Ia and Ib domains of ZFHD proteins contain at least seven potential zinc-binding amino acids, both domains form one rather than two zinc finger structures. Some of these putative zinc-binding residues are separated by only one amino acid, thus they are unlikely contribute to the formation of a second zinc finger structure.

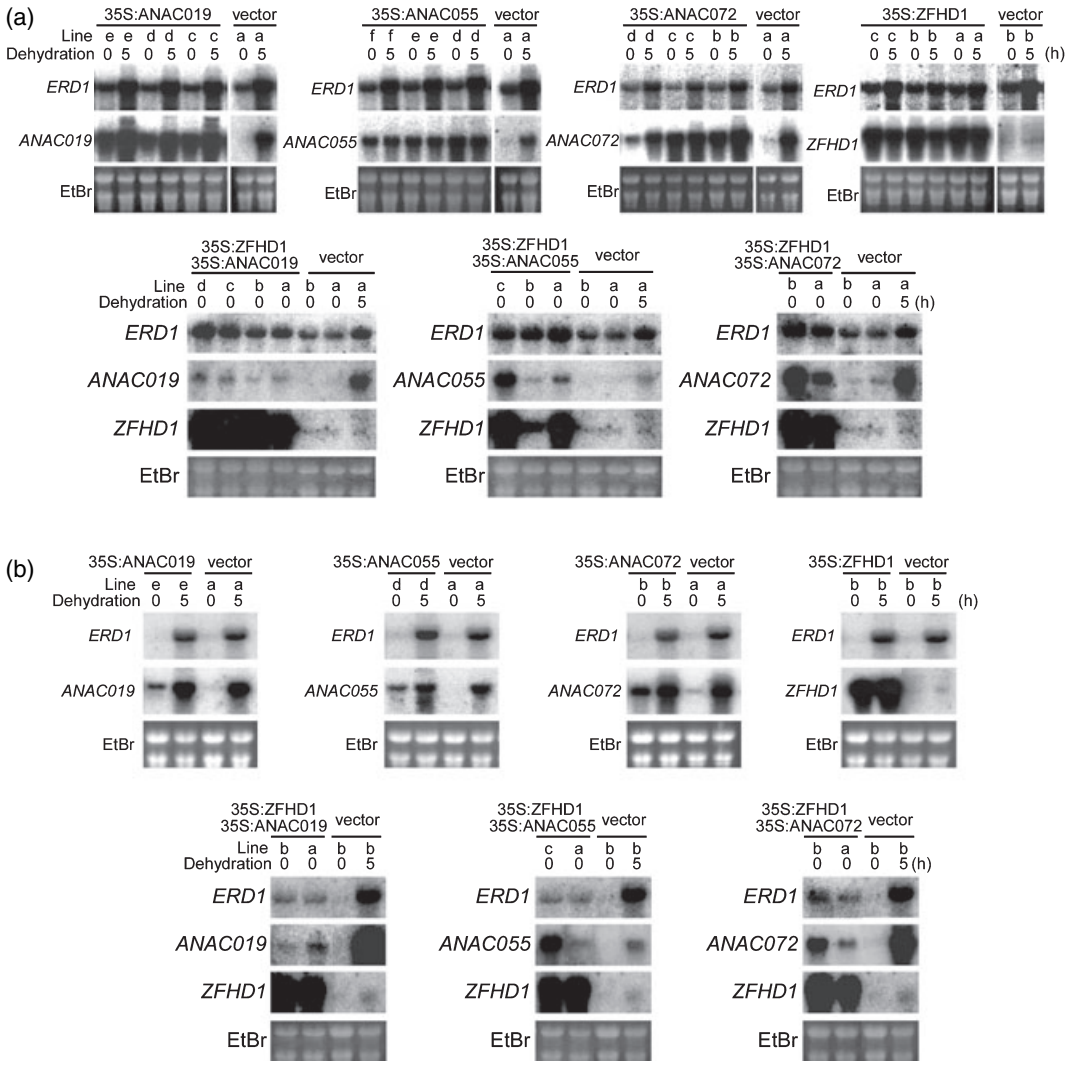

Figure 9. Co-over-expression of *ZFHD1* and *NAC* genes induced expression of the *ERD1* gene in Arabidopsis transgenic plants.
(a) Each lane was loaded with 10 μg of total RNA from 35S:ANAC019, 35S:ANAC055, 35S:ANAC072, 35S:ZFHD1, 35S:ZFHD1/35S:ANAC019, 35S:ZFHD1/35S:ANAC055, 35S:ZFHD1/35S:ANAC072 or vector control Arabidopsis plants. Transgenic Arabidopsis plants were grown for 3 weeks on selective plates (16 h light/8 h dark cycle). Plants untreated or dehydrated as indicated before analysis. EtBr, ethidium bromide.
(b) Each lane was loaded with 10 μg of total RNA from 35S:ANAC019, 35S:ANAC055, 35S:ANAC072, 35S:ZFHD1, 35S:ZFHD1/35S:ANAC019, 35S:ZFHD1/35S:ANAC055, 35S:ZFHD1/35S:ANAC072 or vector control Arabidopsis plants. Transgenic Arabidopsis plants were grown for 2 weeks on selective plates (16 h light/8 h dark cycle), then transferred to soil and grown for an additional week under continuous light conditions. Plants were untreated or dehydrated as indicated before analysis. EtBr, ethidium bromide.

We expected that a TF that functions in the stress-inducible expression of *ERD1* would be a transcriptional activator. Among the proteins identified, only ZFHD1 was shown to function as a transcriptional activator for ZFHDRS-dependent transcription in the yeast system (Figure 1b). Because the regulatory domains of the ZFHD group are unique and have not been described so far, we took the advantage of the yeast one-hybrid system and Arabidopsis protoplasts to study the regulatory domain of ZFHD1. Functional analysis of the N-terminal region covering the zinc finger domain of Arabidopsis ZFHD1 indicated that this domain is a potential activation domain (Figure 5d,e). In addition, the amino acids located at the N- and C-termini of the zinc finger domain of ZFHD1 are also essential for activation activity as indicated by deletion analyses (Figure 5d,e). These amino acids differ in ZFHD1, ZFHD2, ZFHD3 and ZFHD4, and ZFHD2, ZFHD3 and ZFHD4 did not display relevant activation activity (Figure 1b), again suggesting the important role of these amino acids in activation activity. The zinc finger domain also functions as a novel dimerization domain for the formation of homo- and heterodimers, and the highly conserved cysteines are essential for protein/protein interaction (Windhövel *et al.*, 2001; Tan and Irish, 2006). Gel shift analysis showed that the ZFHD1 protein

could bind to ZFHDRS as a dimer (Figure 4b,c). The potential homo- and heterodimerization may potentiate transcriptional activity of the ZFHD proteins, which would have a significant effect on the expression of target genes and may consequently affect stress responses.

Microarray analysis of transgenic plants over-expressing ZFHD1 revealed that several stress-inducible genes were upregulated, and these transgenic plants exhibited a strong drought tolerant phenotype (Table 1, Figure 7c,g). These data further support the hypothesis that ZFHD1 functions in stress signalling. Using microarrays, we identified the 29 genes with the greatest increase in expression (Table 1). However, it may be difficult to determine the downstream genes of ZFHD1 using only array and RNA gel blot analyses (Figure 8), as we used a strong constitutive CaMV 35S promoter to over-express *ZFHD1*. Therefore, we analysed expression of these 29 upregulated genes under stress conditions and searched for the consensus motif in their promoter regions. With the exception of *At5g41020* and *At5g26280*, all genes contain the A/T-rich consensus TA/TAATT in their promoter regions, which closely resembles the TGTAATT motif that has an indispensable role in binding of the α2-homeodomain and the ZFHD At1g75240/ATHB33 and covers the NNATTN consensus sequence (Conolly *et al.*, 1999; Fraenkel and Pabo, 1998; Tan and Irish, 2006; Wolberger *et al.*, 1991; Table 1). Of these, eight genes that are both stress-inducible and contain the consensus motif are excellent candidates as direct target genes of ZFHD1 (Table 1). However, elevation of a single ZFHD1 TF is not sufficient to produce increased *ERD1* transcript (Figure 8); an observation which suggests that coupling factor(s) is/are required. Several reports have demonstrated the involvement of two or more transcriptional activators in the transcriptional regulation of stress-responsive genes. *RD22* gene transcription during dehydration stress was controlled via the coordinated action of AtMYC2 and AtMYB2 factors (Abe *et al.*, 2003). In addition, ABA-responsive expression of the *RD29A* gene was shown to require both DREB- and AREB-interactive transcriptional activators (Narusaka *et al.*, 2003). However, there is no direct evidence for a possible interaction between AtMYC2 and AtMYB2 TFs or between DREB and AREB TFs.

There are two drought-responsive *cis*-elements, ZFHDRS and NACRS, that reside within the *ERD1* promoter. During water stress, *ERD1* gene transcription is controlled by coordinated activity between these two *cis*-acting elements (Simpson *et al.*, 2003), and consequently their respective TFs. Indeed, under unstressed conditions, transgenic plants over-expressing the *NAC* genes did not induce expression of *ERD1* as indicated by microarray and RNA gel blot hybridization analyses, although many other stress-inducible genes were induced (Figure 9; Tran *et al.*, 2004). However, in transgenic Arabidopsis plants over-expressing both ZFHDRS-binding ZFHD1 and NACRS-binding NAC proteins, the *ERD1* transcript accumulated to a higher level than in control plants (Figure 9a,b). This clearly indicated that the *ERD1* gene induction required the concerted action of the ZFHD1 and NAC TFs. In addition, Figure 9(b) shows that co-over-expression of NAC and ZFHD1 does not increase the expression of *ERD1* to the amount found in stressed plants, suggesting that other unknown factors may be involved and/or post-translational modification might be necessary. In Arabidopsis T87 cell studies, we also demonstrated that expression of a GUS reporter gene placed under the control of the minimal *ERD1* promoter and both ZFHDRS and NACRS *cis*-elements was more significantly increased in the presence of both ZFHD1 and NAC TFs than in the presence of a single TF alone (Figure 6a). In the transient activation assay, ZFHD1 alone induced a lower level of expression of GUS driven by ZFHDRS–NACRS and the minimal TATA box of *ERD1* than did any ANAC protein, showing that the ZFHD1 has a weaker influence in promoting the induction of *ERD1* expression than the ANAC proteins. This result is consistent with the results of *cis*-element studies by Simpson *et al.* (2003), in which it was observed that base substitution of ZFHDRS had a smaller impact than base substitution of NACRS. The ZFHD1 and NAC TFs appeared to interact, as shown by a two-hybrid analysis (Figure 6b), and cooperatively transactivated the expression of the *ERD1* gene. In addition, over-expression of the *ZFHD1* gene induced several stress-inducible genes, which were not upregulated by over-expression of the *NAC* genes (Table 1; Tran *et al.*, 2004). These data suggest that, during water stress, the ZFHD1 TF functions as a transcription activator in cooperation with the NAC TFs or alone.

Over-expression of ZFHD1 resulted in an improvement in drought tolerance (Figure 7c,g); however, on average, the plant size was reduced to approximately 75% of that of the control plants (Figure 7a,e,f). The increased survival ratio of the 35S:ZFHD1 plants under drought conditions was not associated with altered transpiration rates, thus this may be the result of the induction of stress-inducible genes, such as *At4g02380* and *At4g37370* encoding LEA3 class and/or cytochrome P450-dependent proteins, respectively, that are controlled by ZFHD1 in the 35S:ZFHD1 plants. It is has been proposed that LEA class proteins play a role in counteracting crystallization of cellular components or the irreversibly damaging effects of increasing ionic strength induced by water deficit (Bartels and Sunkar, 2005; Ingram and Bartels, 1996; Thomashow, 1999; Zhu, 2001). Therefore, the improved resistance of 35S:ZFHD1 plants may derive from the induction of *At4g02380* encoding a LEA3 protein, which has a detoxification role and alleviates such cellular damage rather than suppressing water loss. Alternatively, the cytochrome P450-like protein, encoded by *At4g37370*, may also be involved in increasing the tolerance of 35S:ZFHD1 plants because cytochrome P450-dependent mono-oxygenases represent the primary enzymes respon-

sible for preventing the accumulation of high, toxic concentrations of free fatty acids liberated by phospholipases in the early stress responses in the plant cells (Duan and Schuler, 2005; Kahn and Durst, 2000).

The over-expression of stress-inducible genes is often associated with growth retardation, with the degree of growth retardation being dependent on the expression level of the transgenes (Aharoni *et al.*, 2004; Kasuga *et al.*, 1999). When both *ZFHD1* and *NAC* genes were over-expressed in all possible combinations, the transgenic plants exhibited a higher degree of drought tolerance. This effect was significantly greater than when the *NAC* gene was over-expressed alone (Figure 7g), and it also reduced the negative effect of over-production of *ZFHD1* on the plant size (Figure 7a,e,f). NAC family TFs were also found to play a role in a diverse set of developmental processes (Olsen *et al.*, 2005). Thus, we cannot exclude the possibility that ANAC019, ANAC055 and ANAC072 may play additional roles in other aspects of development, or in aerial parts of the plant. Over-expression of the *NAC1* gene gave the transgenic plants a bigger phenotype with larger leaves and a thicker stem (Xie *et al.*, 2000). Transgenic plants over-expressing *ANAC055* had a similar kind of morphological phenotype to 35S:NAC1 plants, depending on the expression level of the transgene (Tran *et al.*, 2004).

Transgenic plants over-expressing DREB1A showed an increased tolerance to drought, salt and freezing; however, its over-production resulted in a dwarf phenotype (Liu *et al.*, 1998). One strategy to overcome the problem of growth retardation is to use a stress-inducible promoter as reported by Kasuga *et al.* (1999). The results presented here suggest a new strategy for improving plant tolerance to environmental stress without significant growth retardation under normal conditions. NAC and ZFHD TFs are present in a broad range of plants, including crops, and can therefore be used to improve the stress tolerance of agriculturally important crops by gene transfer. Furthermore, this study represents an important insight in to the molecular mechanisms as well as the functions of the ZFHD TFs in the abiotic stress response. Detailed functional analyses of each member of the ZFHD TF family in response to abiotic stresses may provide further information concerning the complex regulation of the stress response in plants.

Experimental procedures

Plant materials and stress treatments

Wild-type plants (*Arabidopsis thaliana*, ecotype Columbia) were grown on germination medium (GM) agar plates for 3 weeks (16 h light/8 h dark cycle) as previously described (Nakashima *et al.*, 1997). Transgenic plants were grown either on selective plates for 3 weeks (16 h light/8 h dark cycle, referred as plate-grown), or on selective plates for 2 weeks and then transferred to soil pots and grown under continuous light for an additional week (referred as pot-grown). Various treatments were applied according to the methods described by Nakashima *et al.* (1997). T87 cultured cells, derived from Arabidopsis, were maintained, grown and treated as previously described (Axelos *et al.*, 1992).

Yeast one-hybrid screening of Arabidopsis cDNA libraries

Reporter plasmids were constructed and the yeast reporter strain was selected as described by Liu *et al.* (1998). Approximately 2.1×10^6 and 4.5×10^6 yeast transformants were screened according to the manufacturer's protocol (Clontech Matchmaker One-Hybrid System, Palo Alto, CA, USA) using 40 μg of AD-cDNA libraries prepared from unstressed and 3 h-dehydrated Arabidopsis plants as previously described (Liu *et al.*, 1998). The titers of the unstressed and 3 h-dehydrated primary libraries were 5.8×10^6 pfu and 6.2×10^6 pfu, respectively. Twenty positive colonies were obtained from selective plates containing 10 mM 3-AT. When a colony-lift filter assay was performed to verify the DNA–protein interaction, as described in the Yeast Protocols Handbook (Clontech), all 20 colonies conferred β-galactosidase activity. The cDNA isolation, subcloning and sequencing of these 20 clones were performed as described previously (Liu *et al.*, 1998). For initial analysis of the binding specificity of ZFHD1, we used four tandemly repeated copies of a mutated 62 bp fragment in which the CACTAAATTGTCAC motif was replaced with GGGGGGGGGGGGGG for the construction of the reporter plasmids.

RNA gel blot analyses

RNA extraction and gel blot hybridization were performed as previously described (Nakashima and Yamaguchi-Shinozaki, 2002).

Transactivation experiment with T87 protoplasts

cDNA fragments encoding ANAC019, ANAC055, ANAC072 and ZFHD1 were cloned into plant expression vector pBI35SΩ to create effector plasmids (Abe *et al.*, 1997). To construct pBI-ZFHDRS-NACRS-TATA$_{ERD1}$, two tandem copies of the 187 bp fragment of the *ERD1* promoter (from −620 to −434) containing the ZFHDRS and NACRS motifs were ligated to the *Hin*dIII site of pSKTATA, into which the *ERD1* minimal TATA sequence had previously been cloned (Simpson *et al.*, 2003). Then, the CaMV 35S promoter in pBI221 was replaced with ZFHDRS-NACRS-TATA$_{ERD1}$ fused fragments. Reporter and effector plasmids were co-transformed into T87 protoplasts and the transactivation assay was performed according to the method described by Satoh *et al.* (2004).

Liquid culture assay for β-galactosidase activity

β-Galactosidase activity, expressed in Miller units, was measured as described in the Yeast Protocols Handbook using *o*-nitro-phenyl-β-D-galactopyranoside as a substrate.

Determination of interaction of ZFHD1 and NAC TFs by a two-hybrid system

To investigate the possible interaction between ZFHD1 and NAC proteins, the DNAs encoding the respective NAC binding domains, ANAC019BD (1–186 aa), ANAC055BD (1–185 aa) and ANAC072BD (1–185 aa) were fused to the GAL4 binding domain (GAL4BD) in pGAPBD to create the following fusions: GAL4BD–ANAC019BD,

GAL4BD–ANAC055BD and GAL4BD–ANAC072BD, respectively. The full-length ZFHD1 was fused to the GAL4 activation domain (GAL4AD) in pAD-GAL4 to create the GAL4AD–ZFHD1 fusion. The pGAPGB plasmid was constructed by replacing the truncated P_{ADH1} promoter with the strong P_{GAP} promoter from the YepGAP vector (Liu *et al.*, 1998) to increase the expression level of the fusion constructs. The AH109 yeast strain was co-transformed with BD and AD plasmid constructs in all possible combinations, and plated on selective medium lacking leucine, tryptophan, histidine and adenine. pGAPGB (BD) and pGADT7 (AD) were used as a negative control for these experiments.

*Preparation of glutathione-*S*-transferase fusion proteins and gel retardation assay*

A fragment encoding ZFHD1 was PCR-amplified and fused in-frame to the pGEX-4T-2 vector (Amersham Biosciences, Buckinghamshire, UK). The recombinant pGEX-4T-2 plasmid was introduced into the BL21 *E. coli* strain. GST fusion protein was produced and purified as previously described (Urao *et al.*, 1993). Gel electromobility shift assays were conducted according to the methods described by Sakuma *et al.* (2002) and Chinnusamy *et al.* (2003).

Transgenic plants over-expressing ZFHD1*,* ZFHD1 *and* ANAC019*,* ZFHD1 *and* ANAC055*, and* ZFHD1 *and* ANAC072 *cDNAs*

The cDNA encoding ZFHD1 was inserted into the pBI35SΩKm plasmid (Liu *et al.*, 1998), while cDNAs encoding ANAC019, ANAC055 and ANAC072 were inserted into pBI35SΩHyg plasmids (Tran *et al.*, 2004). The resulting plasmid pBI35SΩ:ZFHD1Km was introduced into Arabidopsis either solely or in combination with either pBI35SΩ:ANAC019Hyg, pBI35SΩ:ANAC055Hyg or pBI35SΩ:ANAC072Hyg. These multiple transformations were used to create transgenic plants over-expressing either *ZFHD1* (35S:ZFHD1), *ZFHD1* and *ANAC019* (35S:ZFHD1/35S:ANAC019), *ZFHD1* and *ANAC055* (35S:ZFHD1/35S:ANAC055) or *ZFHD1* and *ANAC072* (35S:ZFHD1/35S:ANAC072). Transgenic vector control plants were created by transforming Arabidopsis with the pBI35SΩHyg or pBI35SΩKm plasmids lacking inserts.

Microarray analysis

Three-week-old 35S:ZFHD1 transgenic plants and pBI35SΩKm vector control plants were grown on GM-kanamycin plates and were used for the extraction of total RNA with Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Two independent transgenic lines were used for each experiment. For each biological replicate, material from 10 plants was pooled to provide a single sample for RNA purification. Total RNA extracts were used to prepare Cy5-labelled and Cy3-labelled cDNA probes for microarray experiments using an Agilent Arabidopsis 2 oligo microarray (Agilent Technologies Inc., Santa Clara, CA, USA). All microarray experiments, including data analysis, were carried out according to the manufacturer's protocols. The reproducibility of the microarray analysis was assessed by incorporating a dye swap into the experimental plan. Under our experimental conditions, genes showing a signal value <1000 in both Cy3 and Cy5 channels of the control plants were not considered for the analysis because expression of these genes was not always detected reproducibly by RNA gel blot analysis. In addition, only genes with *P*-values <0.001 were studied. According to our previously published data, most genes with changes in expression greater than threefold are clearly and reproducibly confirmed by RNA gel blot or real-time quantitative RT-PCR analyses (Fujita *et al.*, 2004; Maruyama *et al.*, 2004; Rabbanni *et al.*, 2003). Feature extraction and IMAGE ANALYSIS software (version A.6.1.1; Agilent Technologies Inc.) was used to locate and delineate every spot in the array and to integrate each spot's intensity, filtering and normalization using the Lowess method. Gene clustering analysis was performed with GENESPRING 6.1 (Silicon Genetics, San Carlos, CA).

Drought stress tolerance of transgenic plants

Plants were grown aseptically in Petri dishes containing GM selective agar medium for 3 weeks and were subsequently transferred to 8 cm pots filled with a 1:1 mixture of perlite and vermiculite. Plants were grown in this soil medium for an additional week prior to exposure to drought stress. Drought stress was imposed by with-holding water for 10 days in growth chamber that was maintained at 22°C, 50–60% relative humidity, and continuous 55 μmol m^{-2} sec^{-1} photon flux density. The drought stress was continued until lethal effects of dehydration were observed on most of the control plants. Plants were removed from the drought stress and received water. Three days subsequent to the re-watering, the numbers of plants that survived and continued to grow were counted and recorded (Tran *et al.*, 2004).

Analysis of plant water relations

Water loss and standardized water content were measured as described previously (Ma *et al.*, 2004; Yoshida *et al.*, 2002), with minor modifications. The transgenic plants were grown either on selective plates for 3 weeks (16 h light/8 h dark cycle, plate-grown), or on selective plates for 3 weeks and then transferred to soil pots and grown under continuous light for an additional week (pot-grown). The fresh weight of whole 3-week-old plate-grown and detached aerial parts of 4-week-old pot-grown plants was determined at various time points. The plants were then dried in an oven at 65°C for 48 h and dry weight was measured.

Phylogenetic analysis

The amino acid sequences of the zinc finger and homeodomains of 12 Arabidopsis ZFHD proteins were aligned using CLUSTAL X (Thompson *et al.*, 1994) with the following parameter set: gap open penalty = 5.00, gap extension penalty = 0.5. The alignment was finally adjusted manually and a phylogenetic tree was constructed by the neighbor-joining method using MEGA software (Saitou and Nei, 1987). The confidence level of monophyletic groups was estimated using a bootstrap analysis of 1000 replicates.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 The complete set of microarray data obtained from microarray analysis of 35S:ZFHD1 plant

[a]According to Seki *et al* (2002c)

[b]Munich Information Center for Protein Sequences (MIPs) entry codes for the cDNAs used in this study.

[c]The ratio is the median of fold changes of two repeats. Fold change was defined as flourescence intensity (FI) of each cDNA of 35S:ZFHD1 divided by the FI of each cDNA of vector control line.

[d]Description as given by the MIPs database.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Accession numbers

The sequences of ZFHD1, ZFHD2, ZFHD3, ZFHD4 genes are available from GenBank with accession numbers: AC021046 (At1g69600), AB011479 (At5g65410), AL353993 (At5g15210), AC004136 (At2g02540), respectively.

