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A homeodomain leucine zipper gene from *Craterostigma plantagineum* regulates abscisic acid responsive gene expression and physiological responses

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Abstract

A subset of homeodomain leucine zipper proteins (HDZip) play a role in regulating adaptation responses including developmental adjustment to environmental cues in plants. Here we report the structural and functional characterisation of a dehydration responsive nuclear-targeted HDZip transcriptional regulator, *CpHB-7*. DNA–protein interaction studies suggest that *CDeT6-19*, a known ABA and dehydration responsive dehydrin gene, is a potential target gene of CpHB-7 in the desiccation-tolerant plant *Craterostigma plantagineum*. Transgenic plants that ectopically express *CpHB-7* display reduced sensitivity towards ABA during seed germination and stomatal closure. Expression analysis reveals that genes with induced or repressed expression in *CpHB-7* ectopic expression lines are either mostly repressed or induced by ABA, drought or salt treatment respectively, thus demonstrating that CpHB-7 modifies ABA-responsive gene expression as a negative regulator. *CpHB-7* gene expression is also linked to early organ development, leading to the suggestion that *CpHB-7* is functionally similar to the *Arabidopsis* transcription factor, *ATHB-6*.

Introduction

In plants dehydration is a widespread environmental condition that damages cellular structures and inhibits normal physiological activities, consequently reducing crop yield and restricting the geographical distribution of plants. Plant cells undergo protoplasmic dehydration not only under dehydration stress, but also as a secondary effect under cold and salt stress (Bartels and

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Sunkar, 2005). Significant efforts have been made to understand the mechanisms that plants have evolved to acquire tolerance. One of the strategies to investigate dehydration responses and protective mechanisms is the study of resurrection plants because such plants possess extreme dehydration tolerance (Phillips *et al*., 2002). Studies of the resurrection plant *Craterostigma plantagineum* have revealed that a program of molecular events accompany the desiccation–rehydration process, notably the gradual accumulation of osmoprotective late embryogenesis abundant (LEA) proteins in drying vegetative issues, which disappear upon re-watering (Schneider *et al*., 1993).

Abscisic acid (ABA) plays a central role in regulating plant responses to adverse environmental cues including dehydration (Leung and Giraudat, 1998). Water loss results in an elevation of ABA levels, which leads to stomatal closure, growth inhibition and differential gene regulation for metabolic and developmental adjustment. Stomatal closure is induced by ABA-mediated osmoregulation of guard cells via ion fluxes through ion channels localised at the vacuolar and plasma membranes (Schroeder *et al*., 2001). The ABA stimulus leads to oscillations of cytosolic Ca^{2+} levels and changes in protein phosphorylation, which trigger stomatal closure (Li *et al*., 2000; Allen *et al*., 2001). ABA also functions as a key regulator of differential gene expression in developmental processes such as seed maturation (Finkelstein *et al*., 2002). Progress in understanding the role of ABA during dehydration and in plant development has been achieved by characterizing orthodox seeds from mutants that differ in responses to ABA. Such mutants do not have reduced endogenous ABA content and their phenotypes cannot be reversed to wild-type by exogenous supply of ABA. Multiple loci have now been characterised that either increase or decrease ABA sensitivity in *Arabidopsis* (Koornneef *et al*., 1984; Finkelstein, 1994; Cutler *et al*., 1996). The identities of the mutated genes and the function of the wild-type gene products are known for some of the mutants. Many of the mutations that affect ABA sensitivity result in loss-of-function mutants and the genes affected are signalling molecules that regulate processes such as phosphorylation and transcription (Finkelstein *et al*., 2002).

Different types of transcriptional regulators have emerged as targets of ABA signalling events and comprise members of the basic leucine zipper (bZIP) class, ABI3 (B3 domain), DREB (AP2), basic helix–loop–helix (bHLH) and homeodomain leucine zipper proteins (HDZip) (Kirch *et al*., 2002). HDZip factors have been found exclusively in the plant kingdom (Ruberti *et al*., 1991; Schena and Davis, 1992). Several *HDZip* genes have been linked to environmental adaptation. For example, *ATHB6* (Söderman *et al*., 1999; Himmelbach *et al*., 2002), *ATHB7* (Söderman *et al*., 1996) and *ATHB12* (Lee *et al*., 2001) from *Arabidopsis* and *CpHB-1*, *-2*, *-6* and *-7* (Frank *et al*., 1998; Deng *et al*., 2002) from *C. plantagineum* are linked to drought responses. *CpHB-2*, *-6* and *-7* are induced by both dehydration and ABA, whereas *CpHB-1* is induced only by dehydration, suggesting that HDZips function in ABA-dependent and -independent dehydration responsive signalling pathways. *In vitro* and *in vivo* binding assays have demonstrated that HDZip proteins from *Arabidopsis*, *C. plantagineum* and rice preferentially bind to two 9-bp pseudopalindromic sequences, CAAT(A/T)ATTG (HDE1) and CAAT(G/C)ATTG (HDE2) (Frank *et al*., 1998; Meijer *et al*., 2000; Johannesson *et al*., 2001; Deng *et al*., 2002). Furthermore, different HDZip factors were shown to act either as repressors or as activators of HDE-containing synthetic reporter genes (Meijer *et al*. 1997; Ohgishi *et al*. 2001). Autoregulation through HDE sites in their own promoters was demonstrated for the *Arabidopsis* HDZips, *ATHB2* and *ATHB6* (Ohgishi *et al*., 2001; Himmelbach *et al*., 2002). However, no other studies have linked *cis*-acting HDE elements localised in target gene promoters with their respective *trans*-acting HDZip factor. Therefore, efforts to identify target genes *in planta* will contribute greatly to our understanding of HDZip function.

The ability of HDZip proteins to homodimerise and heterodimerise between members of the same family has been demonstrated (Deng *et al*., 2002). Altered ratios of HDZips that have transcriptional activator or repressor functions could lead to changes in gene expression that allow adaptation to a changing environment or govern plant development. The leucine zipper motif adjacent to the C-terminal of the homeodomain forms an amphipathic alpha-helix with a series of leucine residues responsible for dimerisation of a pair of target

DNA contacting surfaces, thus a fine control mechanism may be established through titration of the homo/heterodimerisation state. The possibility of heterodimerisation of CpHB-7 with other HDZip proteins *in planta* is supported by yeast two-hybrid analysis of CpHB-7 complex formation with CpHB-4/-5/-6 (Deng *et al*., 2002). The leucine zipper motif, therefore, allows a network of interacting HDZip factors to mediate responses to environmental stimuli of different kinds, and integrates information on environmental conditions to regulate target genes.

In this paper, we report the structural and functional characterisation of the nuclear-targeted HDZip transcriptional regulator *CpHB-7* and the identification of *CDeT6-19*, a known ABA and dehydration responsive group 2 *Lea*/dehydrin (*CDeT6-19*), as being a potential target gene of CpHB-7 in *C. plantagineum*. Our analysis revealed that CpHB-7 physically interacts with the HDE motif (GAATTATTA) present in the *CDeT6-19* promoter ($Pro_{CDeT6\text{-}19}$) and activates transcription both independently and synergistically with ABA. We also show how transgenic plants that ectopically express *CpHB-7* display reduced sensitivity towards ABA during seed germination and stomatal closure. Screening of target genes *in planta* by using a combination of genome mining and an *in vivo* expression assay reveals that genes with induced expression in *CpHB-7* ectopic expression lines are mostly repressed by ABA/drought/salt treatment and those with reduced expression in transgenic plants are broadly induced by ABA/drought/salt, indicating that CpHB-7 modifies ABA-responsive gene expression as a negative regulator.

Finally, we observe that *CpHB-7* promoter activity is correlated with early organ development. This not only provides a link between developmental and environmental transcriptional control, but leads to the suggestion that *CpHB-7* has a function similar to *ATHB-6*.

Materials and methods

Plant material and plant transformation

C. plantagineum (Hochst.) was grown under controlled conditions as described (Bartels *et al*., 1990). *Nicotiana tabacum*. cv. petit Havana SR1 and *Arabidopsis thaliana* (Col-0 ecotype) were used as wild-type for transformations. Tobacco was transformed using the Agrobacteria-mediated leaf-disc method (Horsch *et al*., 1985). *Arabidopsis* plants were transformed according to the method described by Clough and Bent (1998). Tobacco plants were grown in climate chambers at 20–22 °C, 75% humidity and 16 h illumination/day. *Arabidopsis* plants were grown in a greenhouse at 20–22 °C and 8 h illumination/day for the first 2 weeks and 16 h illumination/day for further growth. Molecular analysis of transgenic lines was performed according to the methods described in Furini *et al*. (1994).

Isolation of genomic clones of CpHB-7

A phage EMBL 4 genomic library containing partially digested *Sau*3A fragments of *C. plantagineum* genomic DNA (Michel *et al*. 1993) was screened using α-^{32}P dCTP-labelled probes generated from the 150 bp *Bam*HI–*Nco*I fragment of *CpHB-7* cDNA for *CpHB-7* genomic clones. Genomic DNA fragments were subcloned into the pUC19 plasmid vector for DNA sequencing.

Plasmid constructs

The $Pro_{CDeT6\text{-}19}$ (X74067; 889 bp) construct was described by Michel *et al*. (1993). The $Pro_{CpHB\text{-}7}$ (GenBank Locus DQ191407; 10–1112 bp):GUS translational fusion was generated in the pBI101.2 vector (Clontech, Palo Alto, CA). The HDE-deleted $Pro_{CDeT6\text{-}19}$ was obtained using the Altered Sites II Systems (Promega). The $Pro_{CDeT6\text{-}19}$ and HDE-deleted $Pro_{CDeT6\text{-}19}$*:GUS* expression cassettes were individually subcloned into pBluescript KS(+) (Stratagene, La Jolla, CA) for transient transformation assays.

The *CpHB-7* open reading frame (GenBank Locus AF443623: 140–1095 bp) was amplified and inserted into the pRT105 vector (Töpfer *et al*., 1993) under the transcriptional control of Pro_{35S}. The Pro_{35S}*:CpHB-7* expression cassette was subcloned into pBin19 and used for plant transformation. The *CpHB-7* open reading frame was also fused in frame with *GFP* under the control of Pro_{35S}.

Reporter gene assays and microscopy

Transient expression assays and GUS staining was performed according to Michel *et al*. (1993).

Nicotiana tabacum. cv. petit Havana SR1 leaf protoplasts were transfected with 5 μg reporter plasmid and 10 μg effector plasmid were as well as 5 μg of internal control plasmid (*Pro*$_{35S}$: luciferase). Protein concentration was measured according to Bradford (1976). LUC activity was used as internal standard in transient assays. The GUS activity is expressed as pmol 4-methylumbelliferone (MU) mg^{-1} min^{-1} for transgenic plants and as pmol MU$\times$10 000 RLU^{-1} (relative LUC activity) for transient assays. GFP fusions were transformed into tobacco protoplasts and analysed after a 4-h incubation in the dark using an Aristophan fluorescence microscope (Leitz, Wetzlar, Germany) with filter blocks A and I3 (Leitz, Wetzlar, Germany) and filter set 41014 (Chroma Technology, Brattleboro, USA).

Epidermal strips were obtained from the abaxial side of young leaves of 8–10-weeks-old tobacco plants and directly observed under a light microscope (Nikon Eclipse E600). Eight leaves from four plants in each line were examined. Six epidermis strips were obtained from different parts of each leaf (top, middle and base on both sides of the middle rib). Pictures were recorded using a Hamamatsu digital camera C4742-95 and Lucia Image Software on MV-1500 (Version 4.60, Laboratory Imaging). The stomata were counted and the aperture was measured. The same procedure was performed for leaves treated with ABA.

Seed germination test

T2 seeds of transgenic and wild-type tobacco were sterilised using 7% (v/v) NaOCl and 1% (w/v) SDS, thoroughly rinsed with sterile water and sown on MS containing 0–3 μM ABA. For salt stress, seeds were sown on filter paper soaked in 0–200 mM NaCl. Seeds were transferred to 25 °C after 2 days at 4 °C. The germination rate was calculated by dividing the number of germinated seeds by the maximal number of germinated seeds on MS control plates.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed with recombinant CpHB-1 protein produced using the ThioFusion expression system (Invitrogen) as described (Frank *et al.*, 1998) and the following oligonucleotides: HDE-CDeT6-19 (5′-AATTCAGATCT**GAATTATTA**AGAGGATCC-3′ and 5′-GTCTAGA**CTTAATAATT**CTCCTAGGAATT-3′), HDE-cons (5′-AATTCAGATCT**CAATTATTG**AGAGGATCC-3′ and 5′-GTCTAGA**GTTAATAAC**TCTCCTAGGAATT-3′) and HDE-reverse (5′-AATTCAGATCT**GTTAATAAC**AGAGGATCC-3′ and 5′-GTCTAGA**CAATTATTG**TCTCCTAGGAATT-3′). The dyad symmetric sequence of the HDE$_{6\text{-}19}$ core motif is shown in bold. The probes were labeled by filling in the 5′ protruding ends. Binding reactions were performed in 20 μl containing 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol, 4% glycerol, 1 μg polydeoxyinosinic–deoxycytidilic acid (pdIdC), 10 000 cpm-labeled probe (0.1 ng) and 30 ng of purified recombinant *CpHB-1* protein. Competition assays were conducted by adding an excess (10×–100×) of unlabelled probe. After 20-min incubation at room temperature, DNA–*CpHB-1* complexes were separated in a 4% (w/v) acrylamide gel in 0.2× TAE (Tris–Acetate–EDTA) buffer at 4 °C. Dried gels were exposed to autoradiography at −80 °C using KODAK RX film and Trimax X intensifying screens or subjected to PhosphorImager (Molecular Dynamics) analysis using Image Quant Version 1 software. The densitometric units used to quantify the signal were obtained using the volume integration tool, which describes the intensity of the photon emissions released from the phosphor screen during scanning.

Chromosomal immunoprecipitation

Leaves were collected from plants generated by crossing *Pro*$_{35S}$*:CpHB-7* and *CDeT6-19:GUS* transgenic plants and subjected to cross-linking by fixation in 2% formaldehyde (Wang *et al.*, 2002). Nuclei were isolated, sonication-fragmented and immunoprecipitated following the protocol provided by Sharyn Perry (Wang *et al.*, 2002). Antisera directed against the HDZip fragment of *CpHB-1* were used as well as a pre-immune serum as control. PCR amplification of a 259-bp region of *Pro*$_{CDeT6\text{-}19}$ (X74067; −397 to −139 bp) was performed using the following oligonucleotides: 5′-CATCTTTCGCTTGGTCCGATA-3′ and 5′-GCCACGTGCTCTTATGTATG-3′. Three independent experiments were carried out using both transgenic tobacco and *Arabidopsis* systems and similar results were observed.

Data mining, macroarray filters, hybridisation and data analysis

ORFs of putative HDZip target genes were identified from the *Arabidopsis* genomic sequence on the webpage http://www.pedant.gsf.de, by performing pattern searches in each of the five chromosomes with the four possible variants of the HDE sequence: CAATAATTG, CAATTATTG, CAATCATTG and CAATGATTG. From the resulting ORFs, 238 candidates were selected, which contained one or more HDE motifs within 700 bp upstream to the putative start codon (Supplementary Table 1). The coding sequences were amplified from BAC clones or cDNA clones using specific primers (Supplementary Table 1). In addition, fragments of the *Arabidopsis* HDZip class I and II genes and 27 well-established control genes for different stress treatments were amplified with gene-specific primers (Supplementary Table 2). About 0.7 μl PCR products were spotted onto nylon membrane using a Biogridder robot (BioGrid/MicroGrid with cooling, BioRobotics). Several control sequences including actin and ubiquitin were also spotted. GUS, desmin and neblin (Bellin *et al*. 2002) gene fragments were spotted in different amounts and their transcripts generated by *in vitro* transcription were included to prepare RNA probes, as spiking controls (Bellin et al, 2002; Smith et al, 2003). For the wild-type (Col-0) and three *CpHB-7* expressing lines, leaf tissue was harvested from 10 plants and pooled prior to total RNA extraction. Poly $(A)^+$ RNA was subsequently purified and reverse transcribed in the presence of α-^{33}P dCTP. Hybridisations were carried out in Church buffer (7% SDS, 1% bovine serum albumin, 1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2) at 65 °C for 18 h. The filters were washed twice with 40 mM Na-Phosphate, pH 7.2, 0.1% (w/v) SDS at 65 °C, and exposed to a PhosphorImager screen (Molecular Dynamics) for signal detection. Images were read using a Storm PhosphorImager (Molecular Dynamics) imported into the ArrayVision program (version 6.0; Imaging Research, St. Catharines, Ontario, Canada), in which data were normalised with reference to the average intensity of spiking controls on each array experiment. After import of the log-transformed expression data into the ArrayStat program (version 1.0; Imaging Research), common error was determined, and outliers were removed. The values were then normalised by the mean across conditions by an iterative process, and a false-positive error correction is achieved by application of the false-discovery rate method. The *z* test for two independent conditions using the false-discovery rate method (nominal α set to 0.05) was performed to identify statistically significant differential expression genes between each transgenic line and wild-type and to calculate corresponding *P*-values. Global normalisation was adopted for normalizing the difference of signal intensity of each nylon filter: the intensity was calculated as the ratio of each signal to the average intensity of spiking controls. For each line or wild-type control, at least four experiments with different filters and independent cDNA probes from pools of plants were performed for each condition, thus minimizing variation between individual plants, filter and probes. Twelve genes with significant altered expression in transgenic plants were confirmed by RNA blots.

Results

Structure of the CpHB-7 gene and intracellular localisation of the gene product

CpHB-7 encodes a 309-amino acid protein and belongs to the HDZip protein family. A highly conserved homeodomain is located between amino acids 89 and 149, and is immediately adjacent to a leucine zipper motif (Deng *et al*., 2002). The *CpHB-7* gene was also previously shown to be rapidly induced by dehydration and ABA in plants and undifferentiated tissues (Deng *et al*., 2002). To further investigate the function of the *CpHB-7* gene, the genomic structure was determined.

The corresponding *CpHB-7* genomic clone was isolated from a λEMBL4 library and its sequence is deposited in GenBank (DQ191407). The *CpHB-7* genomic sequence contains two introns: the first intron (95 bp) is located 47 amino acids upstream of the homeobox and the second (65 bp) is between repeats five and six within the leucine zipper motif. The intron positions in relation to the HDZip domains were similar to class I type *Arabidopsis* HDZip genes *ATHB1*, *ATHB3*, *ATHB5*, *ATHB6* and *ATHB7* (Himmelbach *et al*., 2002). This observation is in agreement with the presence of another class I motif, namely six heptad repeats

within the amphipathic dimerisation domain (Sessa *et al*., 1993). Sequence comparison with functionally characterised HDZips also shows that CpHB-7 belongs to class I, being most similar to ATHB1 (66% similarity/48% identity) and ATHB6 (61% similarity/46% identity).

In silico prediction using the PSORT program (Nakai and Horton, 1999) suggested the presence of a monopartite nuclear localisation signal (NLS) (KKRR) positioned between amino acids 92 and 96 within the homeodomain. Transient expression analysis using a GFP fusion protein was performed to validate this hypothesis. The CpHB-7 protein fused to GFP was restricted to the nucleus of all transfected cells that were visualised (Figure 1), which supports that CpHB-7 functions in the nuclear compartment.

CpHB-7 *is capable of activating a Lea gene via interaction with a HDE element*

Yeast one-hybrid data established that the CpHB-7 protein is capable of binding to consensus HDE elements (Deng *et al*., 2002). An *in silico* search for HDE sequences was conducted on functionally characterised dehydration responsive promoter sequences from *C. plantagineum*. This resulted in the identification of a putative *cis*-element (GAATTATTA, named as $HDE_{6\text{-}19}$), which encompasses the core HDE motif 257 bp upstream of the transcriptional starting point (Figure 2A) in the promoter of the *CDeT6-19 LEA* gene (Michel *et al*. 1994). $HDE_{6\text{-}19}$ was located in a region that was rich in ABA and dehydration responsive *cis*-acting elements, including four ABRE core sequences and two DRE elements (Figure 2A). $HDE_{6\text{-}19}$ is an HDE1 type element since it contains an A/T pair in the middle of the pseudopalindromic sequence and is nearly identical to the functional HDE *cis*-element found in the *ATHB6* promoter (CAATTATTA) (Himmelbach *et al*., 2002). The $HDE_{6\text{-}19}$ motif was specifically recognised as an HDE in competitive electromobility shift assays using a related dehydration responsive HDZip protein, CpHB-1 (Frank *et al*., 1998) (Figure 2B). A reversed HDE fragment failed to compete with the

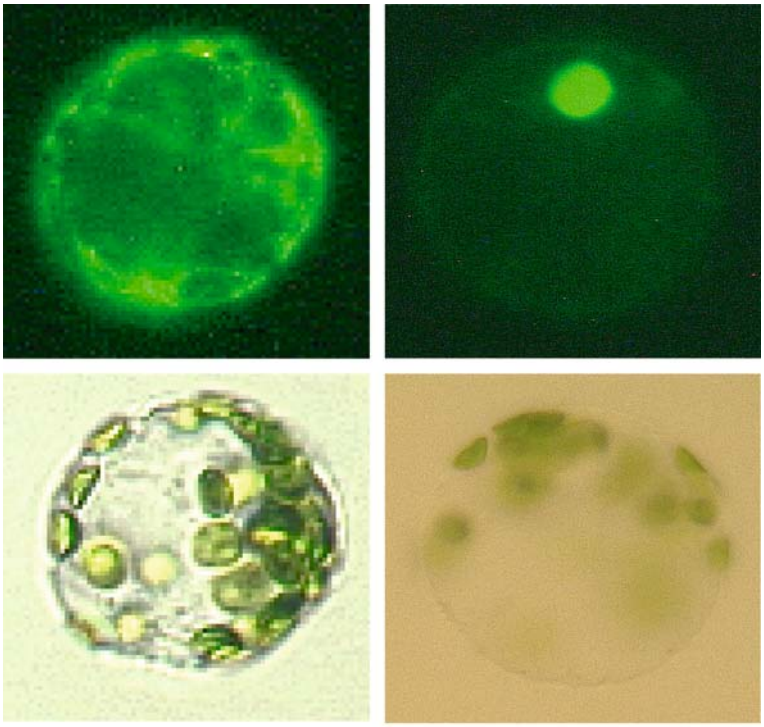

Figure 1. Nuclear localisation of CpHB-7. (A) CpHB-7-GFP gene fusion was transiently expressed in tobacco leaf protoplasts. GFP fluorescence was visualised using fluorescence microscopy and compared to the GFP control.

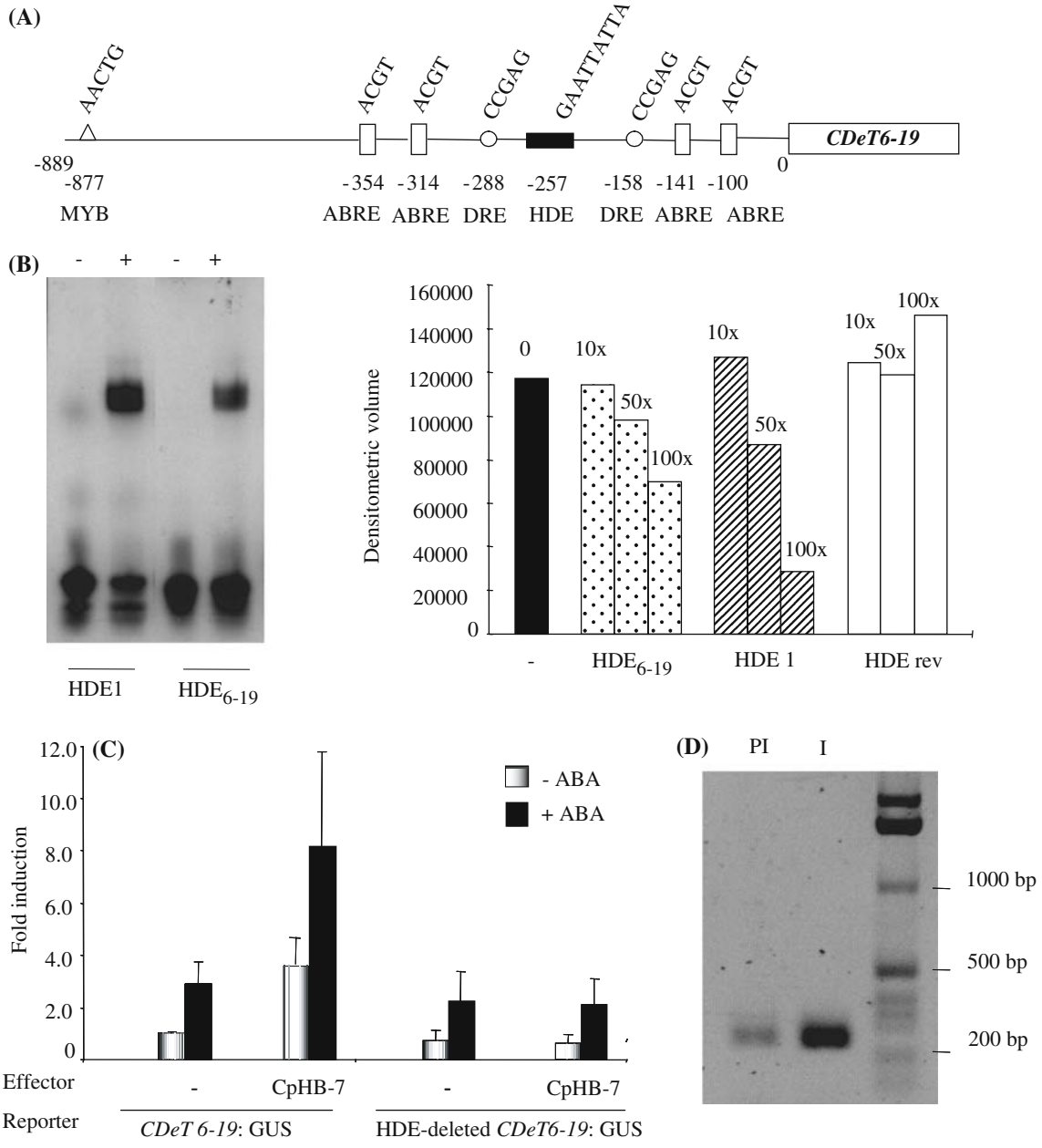

Figure 2. $Pro_{CDeT6\text{-}19}$ is activated via an HDE/protein interaction by CpHB–7. (A) Schematic drawing showing the dehydration and ABA-responsive *cis*-elements found in the $Pro_{CDeT6\text{-}19}$. The upper line summarises the ABRE homologous sequences (squares) identified by Michel *et al.* (1994), DRE box motifs (circles) and a putative MYB binding site (triangle). $HDE_{6\text{-}19}$ (filled rectangle) is located in the centre of the ABRE/DRE box cluster. The positions relative to the transcription start site and the *CDeT6-19* start codon are also shown. (B) Electromobility shift assay with the consensus HDE1 element (5′-CAATTATTG-3′) and $HDE_{6\text{-}19}$-like element (5′-GAATTATTA-3′) and the CpHB-1 protein (left). The interactions between both HDE recognition sites and CpHB-1 were tested in the presence of 10×, 50× and 100× unlabelled specific competitor DNA. No competition was observed when a reversed HDE sequence (5′-GTTAATAAC-3′) was used (right). (C) $Pro_{CDeT6\text{-}19}$, but not HDE-deleted $Pro_{CDeT6\text{-}19}$, was transactivated by CpHB-7 in transient assays using tobacco protoplasts in the presence or absence of 10 μM ABA. Vertical bars indicate the standard deviation; (D) $Pro_{CDeT6\text{-}19}$ fragment was bound by CpHB-7 protein in crosses of tobacco ($Pro_{CDeT6\text{-}19}$:*GUS*×Pro_{35S}:*CpHB-7*) indicated by the enrichment of $Pro_{CDeT6\text{-}19}$ fragment in the DNA pool via a chromatin immunoprecipitation assay using an antibody recognizing the HDZip domain. Independent experiments with *Arabidopsis* crosses ($Pro_{CDeT6\text{-}19}$:*GUS*×Pro_{35S}:*CpHB-7*) were carried out and similar results were observed. PI, pre-immune serum; I, anti-CpHB-1 HD-Zip antiserum.

labeled probe, which suggests that the interaction between the *CDeT6-19 Lea* gene promoter and HDZip proteins is specific.

To address the question whether the *CDeT6-19* gene is transcriptionally regulated by CpHB-7, transient expression assays were performed. The $Pro_{CDeT6\text{-}19}$:*GUS* reporter gene construct was co-transfected with an effector plasmid containing Pro_{35S}:*CpHB-7* into tobacco protoplasts in the presence or absence of ABA. In comparison with a 3-fold enhancement over basal promoter activity caused by exogenous ABA alone, CpHB-7 leads to a 3.5-fold activation of reporter gene expression in the absence of ABA, which was elevated to approximately 10-fold in the presence of ABA (Figure 2C). When the HDE was deleted from $Pro_{CDeT6\text{-}19}$, CpHB-7 failed to activate the GUS reporter gene (Figure 2C). This data indicated that CpHB-7 functions as a transcription activator and modulates ABA-responsive transcription of the *CDeT6-19* gene. Although the effect of ABA on the expression of *CDeT6-19* seemed also weakened by the deletion of HDE, the reduction was not significant. This level of transcriptional activity in combination with exogenously applied ABA was similarly reported in ATHB6-dependent activation experiments using synthetic HDE *cis*-element constructs (Himmelbach *et al.*, 2002).

Evidence for the regulation of *CDeT6-19* transcription by CpHB-7 came also from chromosomal immunoprecipitation (ChIP) experiments. ChIP allows *in vivo* formed complexes of DNA binding protein(s) and associated DNA (Wang *et al.*, 2002). Leaves from stably transformed plants expressing *CpHB-7* in the presence of the *CDeT6-19*:*GUS* reporter gene construct were cross-linked and nuclei were isolated and fragmented by sonication. This was followed by immunoprecipitation using a polyclonal antibody that was raised against the CpHB-1 protein (Frank *et al.*, 1998). Given that the HD-Zip region of CpHB-1 was used as an antigen, the antiserum is likely to cross-react with the related CpHB-7 protein. Using specific primers a fragment of $Pro_{CDeT6\text{-}19}$ that included the $HDE_{6\text{-}19}$ element was preferentially amplified from the immunoprecipitated DNA pool, indicating that the $Pro_{CDeT6\text{-}19}$ fragment was complexed with CpHB-7 protein (Figure 2D). Independent ChIP experiments were carried out using both transgenic tobacco and *Arabidopsis* systems, in both cases similar results were observed. All these data point to the likelihood that *Lea* gene, *CDeT6-19*, is regulated by an HDZip transcription factor, most probably CpHB-7.

Ectopic expression of CpHB-7 promotes seed germination and stomatal closure

To study the physiological function of *CpHB-7*, two transgenic tobacco plant lines (Lines 3 and 5) that express the gene ectopically were analysed. The level of *CpHB-7* expression was higher in Line 5 relative to that observed in Line 3 (data not shown). The transgenic plants appeared phenotypically similar to wild-type plants when grown under normal conditions, except that the transgenic seeds germinated earlier and the seedlings developed more rapidly. Five days after sowing, approximately 90% of the transgenic seeds had germinated and 40–60% of the seedlings had emerged cotyledons and the average root length was more than 6 mm (Figure 3A–C). By comparison, 40% of the wild-type seeds germinated, no seeds/seedlings had emerged cotyledons and the average root length was 4 mm. It was noted that the difference in root length between transgenic and wild-type seedlings was probably due to the difference in germination rate (Figure 3C). Germination of transgenic seeds was also less inhibited by the addition of 0.1–3 μM ABA, when compared to wild-type controls (Figure 3D).

Another difference was the ratio of open:closed stomata between wild-type and transgenic plants (Figure 3E, Table 1). Under well-watered conditions, 50% of the stomata in wild-type leaves were opened as indicated by their aperture greater than 4 μm, and approximately 10% of the stomata were closed as indicated by their aperture width less than 1 μm in wild-type leaves. In comparison, only 19–24% of the stomata in transgenic leaves showed an aperture width greater than 4 μm, and as much as 32–41% of the stomata were closed as their aperture width less was than 1 μm (Table 1). Concentrations of 1 and 10 μM ABA stimulated stomatal closure in wild-type leaves to 37% and 40%, respectively, but did not affect transgenic plants in the same way, in that the percentage of closed stomata remained at approximately 40% (Figure 3E). Similar phenotypes were also observed in transgenic *Arabidopsis* leaves

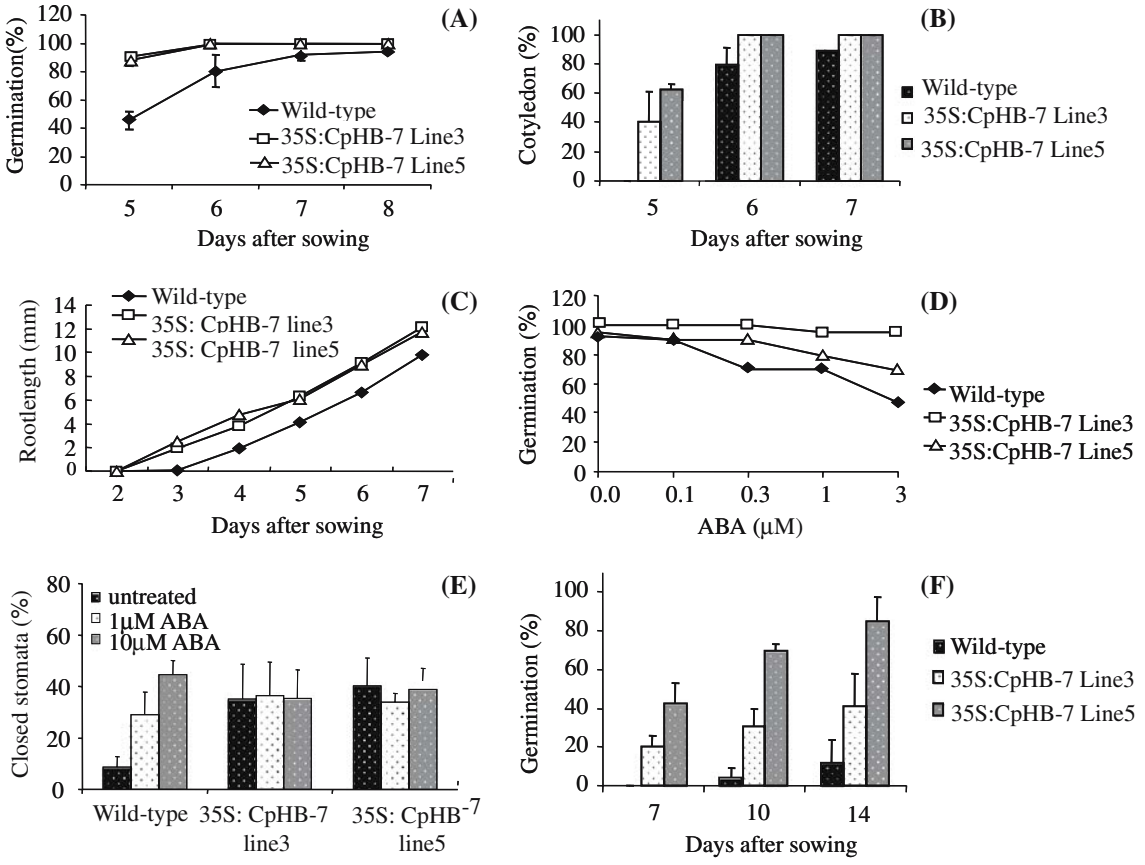

Figure 3. Phenotypic analysis of transgenic tobacco plants that ectopically express *CpHB-7*. (A) Seed germination rates of wild-type (SR1) and transgenic tobacco expressing *CpHB-7* determined from a total of 500 seeds per line from 10 individual experiments; transgenic and wild-type seeds (SR1) were germinated on MS media (unless indicated) after 2 days incubation at 4 °C in the dark; (B) Percentage of seedlings with visible cotyledons at 5th–7th day after sowing, determined from a total of 500 seeds per line from 10 individual experiments; (C) Root lengths of seedlings on the 2nd–7th day after sowing, determined from 200 seeds per line from two individual experiments; (D) Seed germination in the presence of 0–3 μM ABA on the 5th day after sowing, determined from a total of 250 seeds per line from 6 individual experiments; (E) Stomatal response to 1 and 10 μM ABA in three leaves per line of a comparable developing stage and position from 5-week-old plants pretreated with water or ABA, total numbers of stomata are indicated in Table 1; (F) Seed germination on filter paper soaked with 200 mM NaCl determined on the 7th, 10th, and 14th days after sowing, as determined from a total of 150 seeds per line from five individual experiments.

Table 1. Numbers (upper) and percentages (lower) of the stomata with different aperture width in leaves of plants expressing *CpHB-7* in comparison with wild-type plants grown in the same climate chambers.

Line	Stomatal aperture width (μm)						Total number observed
	0–1	1–2	2–3	3–4	4–5	>5	
3	113 (32%)	15 (4%)	53 (15%)	91 (25%)	64 (18%)	23 (6%)	359 (100%)
5	180 (41%)	27 (6%)	62 (14%)	89 (20%)	63 (14%)	21 (5%)	442 (100%)
Wild-type	31 (10%)	3 (1%)	35 (11%)	92 (28%)	106 (33%)	56 (17%)	323 (100%)

(data not shown). These results show that *CpHB-7* expression affects ABA-related phenotypes.

Transgenic plants showed no significant difference when subjected to dehydration or cold stress (data not shown). However transgenic seeds showed increased tolerance to salt stress when germinated in the presence of 100–200 mM NaCl. Even in the presence of 200 mM NaCl, less than

10% of wild-type seeds germinated, however, 40–80% transgenic seeds were still able to germinate and most of the plantlets survived (Figure 3F). The level of transgene expression did not broadly affect the observed phenotypes, except in the germination tests. It was noted that an increase in germination on salt containing media was observed in Line 5 relative to that observed for Line 3.

Tissue specific expression of CpHB-7

Homeobox containing genes are known to regulate developmental pathways in both animals and plants. To examine the relationship between developmental and environmental control of gene expression, we analysed $Pro_{CpHB\text{-}7}$ in transgenic *Arabidopsis* plants and the results are shown in Figure 4. $Pro_{CpHB\text{-}7}$ drives expression in all tissues of germinating seeds, however no GUS staining was detectable in dry seeds. GUS reporter gene activity was observed in roots and leaves of young seedlings, with intense staining in organs that were emerging and developing (Figure 4A–C). Leaf cross sections revealed that staining was essentially present in all cells, with slightly increased intensity in the phloem (data not shown). The staining gradually diminished as the organs matured. In older plants staining was also observed in the pistils during early floral development and seed formation (Figure 4D–L).

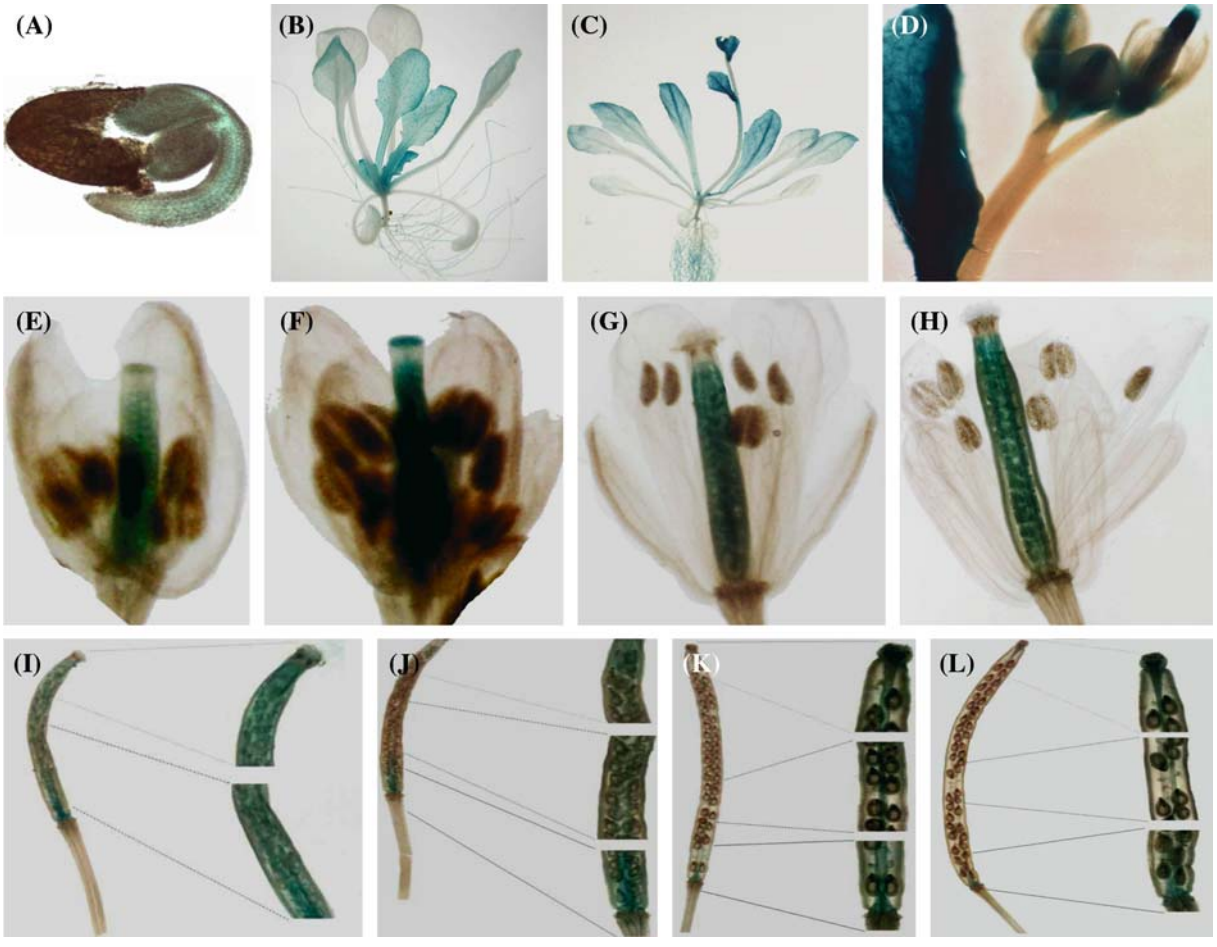

Figure 4. $Pro_{CpHB\text{-}7}$ is developmentally regulated. (A)–(D), $Pro_{CpHB\text{-}7}$ expression in transgenic *Arabidopsis* at consecutive stages from germinating to flowering; (E)–(H), flowers at different stages; (I)–(L), siliques at different stages with magnification of the part indicated.

Ectopic CpHB-7 expression leads to activation and repression of target genes

The function of CpHB-7 as a transcription factor was further investigated by combining a data mining approach and DNA array technology in *Arabidopsis*. In order to identify possible target genes of HD-Zip transcription factors in the *Arabidopsis* genome, the pattern search function of the Protein Extraction, Description and Analysis Tool (http://www.pedant.gsf.de) was used. By pattern searches with the HDE sequences 807 putative ORFs, whose promoter regions contain one or more HDE motifs within 1 kb upstream to each putative start codon, were identified in the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis* genomic sequence database. A selection of 238 ORF fragments (300–500 bp) was successfully amplified using specific primers designed according to the sequence information in the database (Supplementary Table 1). The corresponding PCR products were purified and arrayed together with control probes, including all 26 *Arabidopsis* HD-Zip genes and 27 control genes for different stress treatments (Supplementary Table 2). The resulting targeted cDNA array is, therefore, useful for comparative expression profiling of genes with HDE target sequences.

PolyA$^+$ RNA samples extracted from wild-type *Arabidopsis* and three independent *CpHB-7* ectopic expression lines were labeled and hybridised in four independent experiments. Hybridisation data was normalised and statistic significance was analysed using ArrayStat software (Imaging Research Inc., Canada). The results revealed that nine clones had elevated expression levels (>2-fold increased in at least two independent lines with *P* value <0.01) in lines expressing *CpHB-7* (Table 2) and 16 showed reduced expression levels (>2 fold decreased in at least two independent lines with *P* value <0.01, Table 3). The remaining 213 genes were broadly unaffected, in that expression levels were <2-fold affected in transgenic compared to wild-type plants. The gene products with altered expression in transgenic lines are involved in the following cell processes: photosynthesis, transcription, phosphorylation, carbohydrate metabolism, protein degradation, osmoprotection, cytoskeletal adjustment and ATP synthesis. The functions of five identified genes remain unknown. RNA blots were performed with seven candidates to validate the array data. These RNA blots confirmed the activation of four presumptive target genes and the repression of three other presumptive target genes (Figure 5).

Analysis of the expression data of these clones reported in RARGE database (http://www.rarge.gsc.riken.jp) revealed that those with induced expression in *CpHB-7* ectopic expression lines are mostly repressed by ABA, drought and salt treatment and those with reduced expression in transgenic plants are broadly induced by ABA, drought and salt, indicating that CpHB-7 modifies ABA responsive gene expression as a negative regulator.

Known abiotic and biotic stress-responsive *Arabidopsis* genes including *COR47* (AT1G20440), *KIN1* (AT5G15960), *COR15A* (AT2G42540), *ERD10* (AT1G20450), *GST1* (AT1G02930), *PGDH* (AT1G17745), *RC12A* (AT3G05880), *PDF1.2* (AT5G44420), *PR1* (AT2G19990), *PR2* (AT1G75040), *RD20* (AT2G33380), *RAB18* (AT5G66400) and *RD29A* (AT5G52310) were also shown to be strongly repressed in the *CpHB-7* expressing lines (Table 3). RNA blots were performed for *COR47*, *KIN1, PDF1.2* to verify the array data (Figure 5). With the exception of *PR1*, *PR2* and *PDF1.2*, the identified stress-responsive genes are ABA and dehydration inducible according to RARGE database and previously published by Seki *et al*. (2002). *In silico* analysis of the 1 kb 5′ upstream sequences revealed the presence of HDE-like sequences (AAT A/T/G/C ATT) in 8 out of the 13 genes that were deregulated by the ectopic expression of *CpHB-7* (see Table 3).

Three *HDZip* genes (*ATHB6*, *ATHB12* and *HAT1*) were also found to be repressed in the *CpHB-7* ectopic expression lines (Table 3, Figure 5). *ATHB6* and *ATHB12* are known to be induced by drought, salt and exogenous ABA (Söderman *et al*., 1996; Johannesson *et al*., 2003). Functional characterisation links them to developmental adaptation in response to stress conditions and in all cases, HDE elements are present in the promoter region (Table 3). Pro_{ATHB6} has been analysed in most detail and it was concluded that *ATHB6* is subject to feedback regulation mediated by an HDE/HDZip interaction (Himmelbach *et al*., 2002). Therefore, it cannot be ruled out that *CpHB-7* ectopic expression may modulate transcription of native HDZip genes in *Arabidopsis*.

Table 2. List of genes with increased expression in transgenic *Arabidopsis* lines expressing *CpHB-7*.

ORF identity[a]	Putative function[b]	RAFL No.[c]	Comments from RARGE[c]	Central nucleotide[d]	Distance from ORF (bp)	Fold increase[e]		
						Line 2	Line 7	Line 8
AT4G28660	Photosystem II protein W-like	RAFL09-49-M01	–	T	636	15.5×	13.8×	7.6 ×
AT3G09260	Thioglucosidase 3D precursor	RAFL04-15-P18	Drought, salt and ABA-repressed	T	93	5.4×	6.4×	7.2 ×
AT4G37800	Endo-xyloglucan transferase-like protein	RAFL11-10-A22	Drought and salt-repressed	G, T	362, 85	9.2×	5.5×	5.9×
AT1G75780	Tubulin beta-1 chain	RAFL16-81-K22	–	A	676, 692	2.5×	3×	2.7×
AT5G06290	2-cys peroxiredoxin-like protein (thiol-specific antioxidant protein)	RAFL06-08-E07	Drought, salt and ABA-repressed	T	447	6.6×	2.9×	2.9×
AT2G03450	Putative esterase	RAFL16-06-H08	–	T	138	2.8×	2.8×	2.4×
AT2G43910	Putative methyl chloride transferase	–	–	A	148	2.5×	2.8×	1.9×
AT4G38770	Extensin-like protein (PRP4)	RAFL06-11-A22	Drought, salt and ABA-repressed	A	234	5.4×	2.2×	4.5×
AT4G04640	H^+-transporting ATP synthase (EC 3.6.1. 34) gamma-1 chain precursor	RAFL05-11-F15	–	A	299	2.2×	1.6×	2.9×

[a]MIPS entry codes for the cDNA used in this study.
[b]Description as given by the MIPS database.
[c]According to RARGE database http://www.rarge.gsc.riken.jp/index.html
[d]Central nucleotide in the putative HDE elements found in the 5′-regulatory region of the corresponding genes.
[e]Fold induction is given as the average fold change of four independent hybridisations. Fold change was defined as the intensity of each cDNA hybridised with RNA from plants expressing *CpHB-7* relative to the intensity of each cDNA hybridised with wild-type control RNA. Only clones showing expression ratio > 2-fold and *P*-value < 0.01 in at least two independent lines were considered as significant.

Table 3. List of genes that reduced expression in transgenic *Arabidopsis* lines expressing *CpHB-7*.

ORF identity[a]	Putative function[b]	RAFL No.[c]	Comments from RARGE[c]	Central nucleotide[d]	Distance from ORF (bp)	Fold reduction [e]		
						Line 2	Line 7	Line 8
AT1G02300	Hypothetical protein/cathepsin B-like cysteine proteinase	RAFL05-5-05-A16		C	64	2.1×	3.7×	4.4×
AT3G60880	Dihydrodipicolinate synthase precursor	RAFL08-13-I24	–	A	545	2.4×	6.3×	2.9×
AT3G26740	Putative light regulated protein,	RAFL06-15-H18	Drought, ABA-inducible	T, T	409, 196	4.2×	6.3×	11.1×
AT3G14230	DNA-binding protein	RAFL11-05-O04	Rehydration inducible	A	311	1.8×	2.6×	2.6×
AT3G28710	Adenosine triphosphatase	RAFL05-10-J16	Not responsive	G	33	10×	2×	2.6×
AT3G50500	Protein kinase SnRK2.2, similar to ABA-responsive protein kinase from *Vicia faba*	RAFL09-88-K16	–	T	171	2.4×	3.2×	4.6×
AT3G49000	RNA polymerase III subunit-like protein	RAFL09-65-D16	–	C	447	4.2×	7.7×	25×
AT4G20260	Endomembrane-associated protein	RAFL06-88-G20.	–	T	336	2.4×	3.9×	3×
AT4G01080	Hypothetical protein	RAFL15-13-H2O	–	C	591	1.4×	2.6×	4.8×
AT4G01400	Hypothetical protein	RAFL09-06-F18	–	C	586	1.4×	3×	3.6×
AT4G02380	Similar to *Phaseolus aureus* IAA-induced protein ARG	RAFL04-17-I16	Drought- and NaCl-inducible	G	583	3×	2.2×	5.3×
AT4G36500	Hypothetical protein	RAFL15-25-I11	–	A	315	2.4×	1.8×	5.9×
AT5G23580	Calcium-dependent protein kinase	RAFL16-36-G04	–	G	183	2.9×	5×	8.3×
AT5G39610	NAM/CUC2-like	RAFL08-11-H20	Drought, NaCl and ABA-inducible	A	235	9.1×	14.3×	5×
AT5G63800	Beta-galactosidase	RAFL08-18-L11	ABA-inducible	T	238	1.5×	8.3×	3.5×
AT5G66880	Protein kinase, similar to ABA responsive protein kinase from wheat	RAFL06-89-M02	–	T	175	10×	2×	2.3×
AT2G22430	*ATHB6*, HDZip transcription factor	RAFL05-19-N01	ABA, drought and NaCl-inducible	C*, T*, A*	1396, 322,1178, 817,687	2.2×	2.9×	2.1×
AT3G61890	ATHB12, HDZip transcription factor	RAFL11-01-J18	Drought, NaCl and ABA-inducible	C*, T*, G*, A*	1832, 1091, 792, 752, 58	1.1×	3×	2.2×
AT4G17460	HAT1, HDZip transcription factor	RAFL15-36-J21		A*, C*	850, 285, 615	2.5×	10×	4.6×

Table 3. (Continued)

ORF identity[a]	Putative function[b]	RAFL No.[c]	Comments from RARGE[c]	Central nucleotide[d]	Distance from ORF (bp)	Fold reduction [e]		
						Line 2	Line 7	Line 8
AT1G20440	COR47, dhn/lea/rab-like	RAFL04-20-N09	Cold, drought, NaCl and ABA-inducible	A*	191	3.3×	6.7×	5.6×
AT1G20450	ERD10, Group II LEA protein	RAFL05-08-P17	Cold, drought, NaCl and ABA-inducible	T*	922; 609	1.9×	3.9×	6.3×
AT1G02930	GST1, glutathione S-transferase	RAFL05-16-O07	Cold and NaCl-inducible	G*	786	3.6×	6.7×	9.1×
AT1G17745	PGDH, D-3-phosphoglycerate dehydrogenase	–	–	T*	402	5.9 ×	4.8 ×	7.7×
AT2G42540	COR15A	RAFL05-03-A05	Cold, drought, NaCl and ABA-inducible	T*, G*	947, 809, 780	2.7×	5.9×	7.1×
AT5G66400	RAB18, glycine-rice dehydrin protein	–	ABA, drought, and cold inducible	C*	440	1.8×	5.6×	3.3×
AT5G52310	RD29A	RAFL04-17-F01	Cold, drought, NaCl and ABA-inducible	T*	666	4.4×	5.6×	5.9×
AT2G33380	RD20, putative Ca^{2+}-binding EF-hand protein	RAFL08-16-M12	Drought, NaCl and ABA-inducible	T*	51	2.3×	4.4×	4.8×
AT5G15960	KIN1, cold and ABA-inducible protein kin1	RAFL06-08-N16	Cold, drought, NaCl and ABA-inducible,			7.7×	25×	5.3×
AT3G05880	RC12A	RAFL05-03-J08	Drought and ABA-inducible,			1.5×	2.9×	3.1×
AT5G44420	PDF1.2, antifungal protein-like	–	–			33.3×	50×	50×
AT2G19990	PR1, pathogenesis-related protein 1 (PR-1)	–	–			2.2×	4.6×	4.8×
AT1G75040	PR2, pathogenesis-related protein 5 (PR-5)	RAFL04-13-G17	–			4×	6.3×	7.7×

[a] MIPS entry codes for the cDNA used in this study.
[b] Description as given by the MIPS database.
[c] According to RARGE database http://www.rarge.gsc.riken.jp/index.html
[d] Central nucleotide in the putative HDE elements found in the 5′-regulatory region of the corresponding genes.
[e] Fold reduction is given as the average fold change of four independent hybridisations. Fold change was defined as the intensity of each cDNA hybridised with RNA from plants expressing *CpHB-7* relative to the intensity of each cDNA hybridised with wild-type control RNA. Only clones showing expression ratio <0.5-fold and P-value <0.01 in at least two independent lines were considered as significant.
* Indicates the central nucleotides of near-perfect HDE sequences found in the 5′-regulatory region of the corresponding genes.

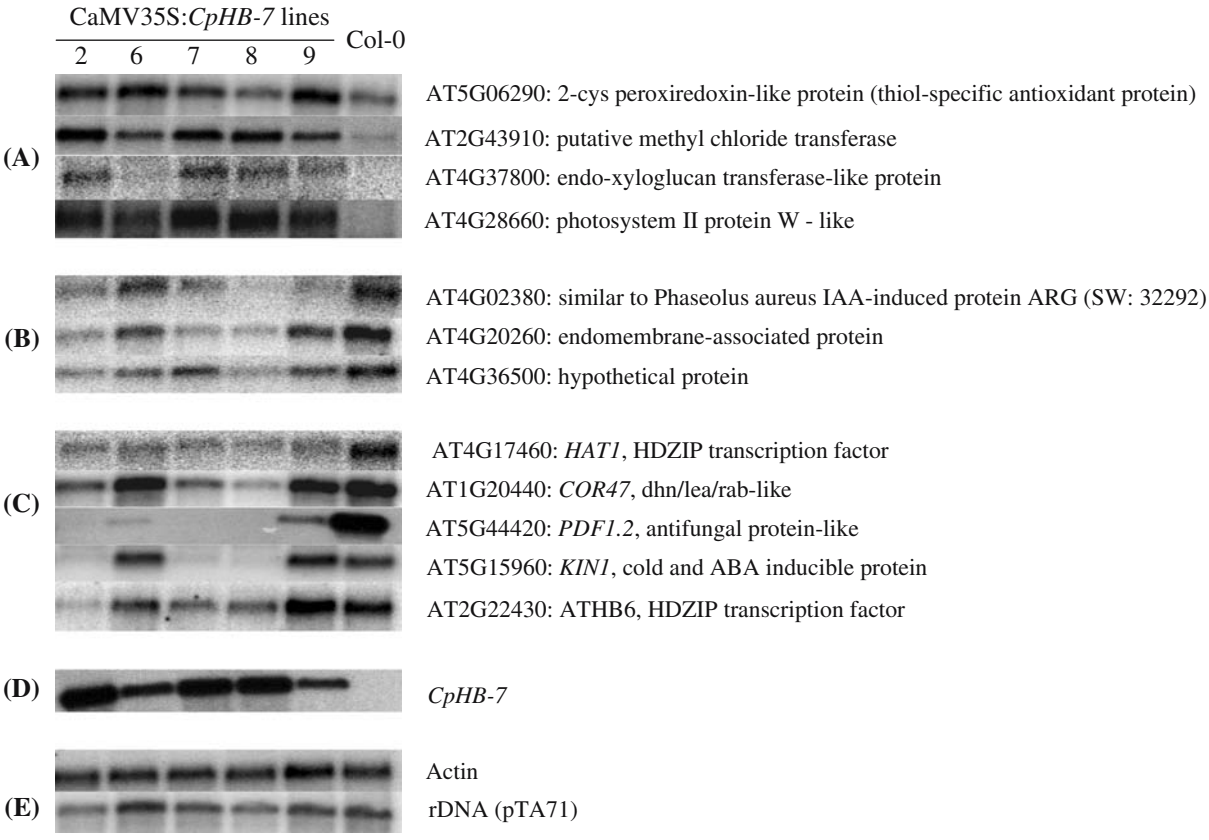

Figure 5. Differential gene expression analysis of transgenic plants that ectopically express *CpHB-7* by Northern hybridisation. About 3 μg poly-A enriched RNA was isolated from five independent transgenic lines and wild-type *Arabidopsis* plants, separated by electrophoresis and hybridised with PCR amplified gene specific probes as indicated. Two groups of genes, whose promoter regions contained one or more HDE motifs within 1 kb upstream to the putative start codon, were activated (A: AT5G06290, AT2G43910, AT4G37800 and AT4G28660) and repressed (B: AT4G02380, AT4G20260 and AT4G36500) in transgenic plants expressing *CpHB-7* via Pro_{35S} (D) compared to wild-type plants (Col-0). (C) Repression of the HD-Zip factors, *HAT-1* (AT4G17460) and ATHB6 (AT2G22430), and three known abiotic stress responsive genes (*COR47*, AT1G20440; *PDF1.2*, AT5G44420; *KIN1*, AT5G15960). (E) Actin and rDNA (pTA71; Gerlach and Bedbrook, 1979) probes were included as loading controls.

Discussion

Homeobox genes are transcription factors characterised by the homeodomain (HD), which is a variation on a helix–turn–helix structure that binds DNA (Gehring *et al*., 1994). These genes play crucial and diverse roles in many aspects of development, including the early development of animal embryos, the specification of cell types in yeast, and the initiation and maintenance of the shoot apical meristem in flowering plants (Burglin, 1997). One class of HD proteins are characterised by a leucine zipper (Zip) motif, which facilitates homo- and heterodimerisation (Ruberti *et al*., 1991). HDZip proteins are thought to have occurred after the divergence of plants and animals, as a result of an exon capture event (Schena and Davis, 1994).

The mode of action of HDZips is poorly understood. *In vitro* site selection assays were used to define potential HDZip binding sites, which led to the discovery of specific consensus pseudopalindromic HDE sequences (Sessa *et al*., 1993). The identification of HDE *cis*-elements in non-HDZip gene promoters that are bound by HDZip factors has yet to be published. Given that it is unlikely that HDZip factors function solely as autoregulatory molecules, we have attempted to identify additional genes that are regulated by HDZip factors through HDE *cis*-elements.

Abscisic acid-responsive gene expression is regulated at the transcription level and an

understanding of the transcription factors and *cis*-elements involved is an important research goal. Most studies to date have concentrated on ABRE, DRE and MYB/MYC type *cis*-elements and the associated DNA binding factors. Our study is based on the observation that (i) dehydration and ABA induces expression of specific Class 1 HDZips from the resurrection plant *C. plantagineum* and (ii) HDE binding activity is induced in *C. plantagineum* during early dehydration (Frank *et al.*, 1998; Deng *et al.*, 2002).

CpHB-7 *is a nuclear localised transcriptional activator*

As demonstrated using a CpHB-7-GFP chimeric protein, the HDZip protein CpHB-7 from *C. plantagineum* is targeted exclusively to the cell nucleus. Nuclear localisation of CpHB-7-GFP was independent of ABA or osmotic adjustment. Therefore, upon induced synthesis, CpHB-7 must be subject to immediate nuclear import. Nuclear import can occur by diffusion or by active transport (Görlich and Mattaj, 1996). Due to its predicted molecular mass of 35 kDa, CpHB-7 is unlikely to diffuse freely into the nucleus but probably requires NLS sequences for active transport. CpHB-7 contains a putative monopartite NLS sequences that fits the consensus of the SV40 large T-antigen-type (K-R/K-X-R/K; Garcia-Bustos *et al.*, 1991) and is probably responsible for the nuclear import.

We show that CpHB-7 has *in vivo* transactivating properties when bound to an HDE *cis*-element (CAATNATTG). Preliminary evidence for a transcriptional activator function was obtained using a yeast expression system (Deng *et al.*, 2002). When fused to a GAL4 DNA binding domain, CpHB-7 activated reporter gene expression via the GAL1 upstream activator sequence. Deletion of a C-terminal region (238–309 amino acids) resulted in a loss of transcriptional activation, however the truncated CpHB-7 polypeptide functioned in protein-protein interaction assays. The deleted region contains two acidic regions (located between 256–261 and 279–285 amino acids), which has been defined as one type of transcriptional activation domain (Triezenberg, 1995).

To date, functional HDE elements have only been reported in HDZip promoters (Ohgishi *et al.* 2001; Himmelbach *et al.* 2002). We have found an HDE-like element (named HDE_{6-19}) in $Pro_{CDeT6\text{-}19}$, a *Lea* gene induced by dehydration/ABA in *C. plantagineum*. This *cis*-element is able to interact with HDZip protein *in vitro*. We also present evidence that CpHB-7 is capable of activating $Pro_{CDeT6\text{-}19}$ synergistically with ABA. These findings suggest that *CDeT6-19* is a target gene of CpHB-7 in *C. plantagineum* and that CpHB-7 functions as a transcriptional activator *in planta*.

CDeT6-19 transcripts are expressed at a low level and are gradually up-regulated in all vegetative tissues by dehydration and ABA, reaching the highest level when *C. plantagineum* is desiccated. In comparison, *CpHB-7* transcripts appear transiently during the early stages of dehydration or after ABA treatment, predominantly in developing organs. It is therefore likely that *CDeT6-19* is regulated by *CpHB-7* when they co-exist *in planta*. Other additional factors must participate in *CDeT6-19* transcriptional regulation and candidates may be mediated by other *cis*-elements clustered around $HDE_{6\text{-}19}$ in $Pro_{CDeT6\text{-}19}$.

Our study was extended to look at the effects on HDE/CpHB-7 mediated gene expression using *Arabidopsis* as a host system. Ectopic expression in heterologous systems has been successful in numerous cases to assign biological function (Zhang, 2003). For example, expression mediated via Pro_{35S} of an *ABI3*-like gene from yellow cedar in transgenic tobacco revealed that the transcription factor has a role in abscisic acid action that is similar to that of ABI3 proteins in angiosperms (Zhang *et al.*, 2003). The Arabidopsis *CBF1* gene also has an equivalent function in transgenic tomato when expressed under the control of Pro_{35S}. A limitation of overexpression strategies is the possible generation of neomorphs, where the introduced transcription factor confers a new function through sequestration of other components of transcriptional machinery, thus resulting in the activation or repression of unrelated genes (Ptashne, 1988). This is particularly important given that it is likely that ectopic expression of *CpHB-7* could titrate out complexes of endogenous *Arabidopsis* HDZips that are involved in ABA responses. HDZip functional analysis must therefore be interpreted with caution, nevertheless the Pro_{35S}*:HDZip* strategy has allowed insight

into the function of ATHB6 (Himmelbach *et al*., 2002), ATHB-8 (Baima *et al*., 2001), ATHB13 (Hanson *et al*., 2001) and Oshox1 (Scarpella *et al*., 2002).

A systematic search of the *Arabidopsis* genome sequence was performed to investigate the role of HDE sequences as *cis*-acting elements. Genes were selected to generate a customised macroarray for expression studies from genes that contain HDE sequences in their promoters. This expression analysis was aimed at probing the validity of HDE sites rather than analysing complex expression profiles resulting from experiments with a whole genome DNA microarray such as those generated e.g. by AtGenExpress (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). This was feasible because the HDE sequence is a relatively long (9 bp) target site in comparison with many other transcription factor binding sites. Pattern searches identified over 4000 HDEs throughout the *Arabidopsis* genome. Mapping of their locations with respect to putative ORFs showed that 2800 of these HDEs occurred in an exon (655 HDEs), in an intron (522 HDEs) or within 1 kb of 5′ or 3′ non-coding sequence (807 and 816 HDEs, respectively). Although functional regulatory motifs can occur also within exons, introns and 3′ regions, the major part of the transcriptional control of gene expression lies in the promoter regions. Putative target genes were sampled from each chromosome with an HDE site in the promoter region within a distance of 700 bp from the start of the ORF. DNA array analysis of the transcript levels of a 238 of these putative target genes revealed that 25 of them are activated or repressed in transgenic *Arabidopsis* that express *CpHB-7*. Whether the genes are direct targets of CpHB-7 remains unclear: transcriptional regulation is probably due to the interaction of a large complex of transcription factors. It was, however, noticed that those clones with induced expression are mostly repressed by ABA/drought/salt treatment and those with reduced expression in transgenic plants are broadly induced by ABA/drought /salt, indicating that CpHB-7 modifies ABA responsive gene expression as a negative regulator. Of particular interest was the identification of a protein kinase (SnRK2.2), which is highly homologous to the guard cell-specific ABA response regulator AAPK from *Vicia faba* (Li *et al*., 2000). ABA-activated SnRK2 members have recently been characterised and phylogenetic analysis predicted SnRK2.2 to be activated by ABA (Kobayashi *et al*., 2004). A second protein kinase was also identified and found to be similar to the wheat PKABA1 protein kinase mRNA, which is upregulated by cold-temperature treatment, dehydration and ABA (Holappa and Walker-Simmons, 1995). These findings suggest that CpHB-7 is likely to function in ABA responsive pathways, negatively regulating ABA-responsive gene expression at the transcriptional level.

The observation that *CDeT6-19* was transcriptionally activated in transient assays, whereas other well characterised ABA/dehydration responsive genes from *Arabidopsis* were repressed by *CpHB-7* ectopic expression underlines the importance of other components required for promoter regulation. The possibility of heterodimerisation of CpHB-7 with other HDZip proteins *in planta* is supported by yeast two hybrid analysis of CpHB-7 complex formation with CpHB-4/-5/-6 (Deng *et al*., 2002). The leucine zipper motif allows a network of interacting HDZip factors to mediate responses to environmental stimuli of different kinds, and integrates information on environmental conditions to regulate target genes. Also, given the close proximity of the other *cis*-elements, we propose that CpHB-7 is likely to function in combination with ABRE and DRE binding proteins. Expression of mutant forms of *CpHB-7* that have the capacity to dimerise but have lost the DNA-binding activity may prove useful in understanding the mechanism of how CpHB-7 modulates ABA-induced gene expression.

CpHB-7 *and* ATHB-6 *share similar functional properties*

Physiological analysis demonstrated CpHB-7-mediated alterations of ABA responses in transgenic lines. Ectopic expression of CpHB-7 reduced the sensitivity of the plants towards ABA during germination. The observed phenotypic alteration is compatible with reduced ABA sensitivity and defines CpHB7 as a negative regulator of the hormone response. In addition, the CpHB7-expressing lines were affected in the regulation of stomatal aperture. The plants, however, responded indistinguishably from control lines to ABA in the regulation of vegetative growth as deduced from comparable inhibitions of root extension.

ATHB6 is a transcriptional activator that negatively regulates ABA responses in *Arabidopsis* (Himmelbach *et al*., 2002; Henriksson *et al*., 2005), the overexpression of which leads to a phenotype similar to that caused by ectopic expression of *CpHB-7* reported in this paper i.e., a reduced sensitivity to ABA during seed germination but no effect on root elongation, indicating that *ATHB6* and *CpHB-7* have a similar function. In addition to the similarities in ectopic expression phenotypes, *CpHB-7* showed also similar spatial and temporal expression pattern as *ATHB6*. Both transcripts rapidly respond to both dehydration and exogenously applied ABA (Deng *et al*., 2002; Söderman *et al*., 1999). Promoter assays revealed that both were expressed in a developmental pattern: GUS staining was found in developing cotyledons, leaves, roots, and pistils of the young inflorescence. Staining became less intense as the plants matured, indicating that both gene products function mostly in immature tissues.

Since *ATHB6* showed a similar expression profile and caused a similar ABA-insensitive phenotype in *Arabidopsis* when overexpressed, we looked more closely at the potentially functionally equivalent genes. A comparison of the two factors (Figure 6) revealed that the functional HD-Zip domain is highly conserved. It should also be noted that the deduced serine/threonine phosphorylation site reported by Himmelbach *et al*. (2002) in ATHB6 (KKRRLSINQV) was well conserved in CpHB-7. The target serine residue in the consensus motif was also found to be immediately adjacent to the predicted NLS.

A functional, near-perfect HDE sequence (CATTATTA) is present in the *ATHB6* promoter region, however overexpression of the *ATHB6* transgene did not interfere with regulation of the endogenous transcript (Himmelbach *et al*., 2002). In the same study, it was also stated that *ATHB6* overexpression had no effect on the expression of ABA-regulated genes *rd29b* and *rab18*. Interestingly, *ATHB6* was repressed in the transgenic

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CpHB-7  MDSCSVYGDDHPHLKNALLKNEASEDLDSLWPHDSSNPSFHGSASPAMVNFEDVRGEDSP 60
ATHB6   -------------MMKRLSSSDSVGGLISLCPTTSTDE--QSPRRYGGREFQSMLEG-YE 44
                     : : * ..::  .* ** *  *::   :..   .  :*:.:

CpHB-7  SPQKPIFPKIGNNAGKTRNSEELILDAFSSQKKRRLSSDQVRFLDKSFEVDNKLEPERKV 120
ATHB6   EEEEAIVEERGHVG--------------LSEKKRRLSINQVKALEKNFELENKLEPERKV 90
        . ::.*. : *: .                *:****** :**: *:*.**::********** 

CpHB-7  QLAKELGLQPRQVAIWFQNRRARYKTKLLEKDYDALKSNYDRLKEDFDALYSENEKLKTE 180
ATHB6   KLAQELGLQPRQVAVWFQNRRARWKTKQLEKDYGVLKTQYDSLRHNFDSLRRDNESLLQE 150
        :**:**********:********:*** *****..**::** *:.:**:*  :**.*  *

CpHB-7  VNTLAEKLLGKADHGDHRPIKEEPPFDDDVNKQTKASSAKSDVLDYSDADGGNQRSPTML 240
ATHB6   ISKLKTKLNGGGGE------EEEEENNAAVTTESDISVKEEEVSLPEKITEAPSSPPQFL 204
        :..*  ** * ...      :**   :  *..::. *  :.:*   ..   . . .* :*

CpHB-7  ELEHGDSSHVVGAISDYEDDDSLGRSLLLEAGNCLTKIEESFYDDLQPNSNCNLGFPVQN 300
ATHB6   EHSDGLNYRSFTDLRDLLPLKAAASSFAAAAGSSDSSDSSALLNEE-SSSNVTVAAPVTV 263
        * ..* . : .  : *    .: . *:   **.. :. ..:: ::  ..** .:. **

CpHB-7  NHGTWFWQV------------------- 309
ATHB6   PGGNFFQFVKMEQTEDHEDFLSGEEACE 291
          *.:*  *
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Figure 6. Protein sequence comparison between CpHB-7 and ATHB6. Sequence alignment of ATHB6 and CpHB-7 proteins including the locations of the putative NLS (boxed) and phosphorylation site (arrow). Sequences were aligned using Clustal W (http://www.ebi.ac.uk/clustalW).

plants expressing *CpHB-7* and the accumulation of endogenous ABA/abiotic stress responsive transcripts including *rab18* was also affected. Furthermore, although ABA treatment did not significantly affect the stomatal aperture of the Pro_{35S}:*CpHB-7* lines, a similar observation to that reported for the *ATHB6* overexpression lines, the ectopic expression of *CpHB-7* in untreated plants was similar to that observed in ABA-treated, wild-type plants, indicating that CpHB-7 expression may lead to a constitutive ABA response. These features distinguish *CpHB-7* from *ATHB6*. Therefore, although both genes have been shown to function as transcriptional activators, negative regulators of ABA responses and are similarly regulated, they cannot be considered to be functionally orthologous.

In summary we report that *CDeT6-19* represents one of the target genes of a developmentally regulated HDZip transcription factor, CpHB-7. Furthermore, *CpHB-7* is also shown to have the potential to regulate a broad spectrum of *Arabidopsis* genes, a subset of which is associated with ABA signalling and dehydration tolerance. We also show how transgenic plants that ectopically express *CpHB-7* display reduced sensitivity towards ABA during seed germination and stomatal closure. Taken together, the results presented in this study show that plants, like animals, use homeobox genes as transcription factors to control different developmental processes. In contrast to animal homeobox genes, which act in the control of embryonal development, the plant homeobox genes of the HDZip class appear to be important regulators of plant development and differentiation in response to environmental factors.

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