Homeodomain Leucine Zipper Class I Genes in Arabidopsis. Expression Patterns and Phylogenetic Relationships[1][w]

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Members of the homeodomain leucine zipper (HDZip) family of transcription factors are present in a wide range of plants, from mosses to higher plants, but not in other eukaryotes. The HDZip genes act in developmental processes, including vascular tissue and trichome development, and several of them have been suggested to be involved in the mediation of external signals to regulate plant growth. The Arabidopsis (*Arabidopsis thaliana*) genome contains 47 HDZip genes, which, based on sequence criteria, have been grouped into four different classes: HDZip I to IV. In this article, we present an overview of the class I HDZip genes in Arabidopsis. We describe their expression patterns, transcriptional regulation properties, duplication history, and phylogeny. The phylogeny of HDZip class I genes is supported by data on the duplication history of the genes, as well as the intron/exon patterning of the HDZip-encoding motifs. The HDZip class I genes were found to be widely expressed and partly to have overlapping expression patterns at the organ level. Further, abscisic acid or water deficit treatments and different light conditions affected the transcript levels of a majority of the HDZip I genes. Within the gene family, our data show examples of closely related HDZip genes with similarities in the function of the gene product, but a divergence in expression pattern. In addition, six HDZip class I proteins tested were found to be activators of gene expression. In conclusion, several HDZip I genes appear to regulate similar cellular processes, although in different organs or tissues and in response to different environmental signals.

Homeodomain Leu zipper (HDZip) proteins constitute a large family of transcription factors characterized by the presence of a DNA-binding homeodomain (HD) and an adjacent Leu zipper (Zip) motif, which mediates protein-dimer formation. The HDZip proteins are apparently unique to plants but related to HD proteins of other eukaryotes. Genes encoding HDZip proteins have been isolated from several vascular plant species, e.g. from Arabidopsis (*Arabidopsis thaliana*; Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992), rice (*Oryza sativa*; Meijer et al., 1997), and the fern *Ceratopteris richardii* (Aso et al., 1999), as well as from the moss *Physcomitrella patens* (Sakakibara et al., 2001). The Arabidopsis HDZip genes have been grouped into four different classes, HDZip I to IV, on the basis of sequence similarity criteria and supported by the intron/exon patterns of the genes (Sessa et al., 1994). The HDZip I and II proteins interact with similar pseudopalindromic binding sites (BSs; CAATNATTG; Sessa et al., 1993; Meijer et al., 1997; Frank et al., 1998; Johannesson et al., 2001), whereas slightly different sequences are recognized by HDZip III and IV proteins, (GTAAT(G/C)ATTAC) and (TAAATG(C/T)A), respectively (Sessa et al., 1998b; Abe et al., 2001, 2003; Ohashi et al., 2003). A prerequisite for DNA binding is the formation of a protein dimer (Sessa et al., 1993, 1998b), either a homodimer or a heterodimer formed by proteins of the same class (Sessa et al., 1993; Gonzalez et al., 1997; Meijer et al., 1997, 2000; Frank et al., 1998; Johannesson et al., 2001). Different HDZip proteins either activate or repress transcription (Aoyama et al., 1995; Meijer et al., 1997, 2000; Sessa et al., 1998a; Steindler et al., 1999).

The functionally well-characterized HDZip class III includes five genes—*REV/IFL1*, *PHB/ATHB9*, and *PHV/ATHB14*, which direct the development of the apical meristem, the vascular bundles, and the adaxial domains of lateral organs (Zhong and Ye, 1999; McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003), and *ATHB8* and *ATHB15*, which have been suggested to direct vascular development (Baima et al., 2001; Ohashi-Ito and Fukuda, 2003). Among the 16 HDZip IV genes (Schrick et al., 2004), *GL2/ATHB10*,

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ATML1, and *PDF2* appear to be involved in establishing cell fates in epidermal cells and to regulate cell-layer-specific gene expression (Rerie et al., 1994; Di Cristina et al., 1996; Lu et al., 1996; Masucci et al., 1996; Ohashi et al., 2002; Abe et al., 2003) and *ANL2* to affect anthocyanin accumulation of the leaf subepidermal layer and cell identity in the root (Kubo et al., 1999). Both HDZip III and IV proteins, in addition to the HD and the Leu Zip domain, contain a steroidogenic acute regulatory protein-related lipid transfer domain hypothesized to bind a steroid-like ligand (Ponting and Aravind, 1999; Schrick et al., 2004).

The HDZip I and II genes are less well characterized with regard to function. These genes likely share a common origin and are distantly related to the HDZip III and IV genes (Chan et al., 1998). The functional information available on HDZip I and II genes suggests that at least some of the genes are involved in mediating the effects of external conditions to regulate plant growth and development. Three HDZip genes have been reported to have a role in light responses: *ATHB2* (HDZip II) as a mediator of the red/far-red light effects on leaf cell expansion in the shading response (Carabelli et al., 1993, 1996; Steindler et al., 1999); *ATHB1* (HDZip I) in the de-etiolation of dark-grown seedlings (Aoyama et al., 1995); and *ATHB16* (HDZip I) in blue-light signaling and in regulation of leaf cell expansion (Wang et al., 2003). Phenotypic analyses of plants with increased expression levels of *ATHB1*, *-3*, *-13*, *-20*, or *-23* (HDZip I) suggest that these genes are involved in the regulation of cotyledon and leaf development (Aoyama et al., 1995; Hanson, 2000; Hanson et al., 2001), and *ATHB13* potentially as a mediator of sugar signaling (Hanson et al., 2001). Several HDZip I genes have been proposed to be involved in abscisic acid (ABA)-related responses. *ATHB5*, *-6*, *-7*, and *-12* are either up- or down-regulated by water deficit conditions and/or externally applied ABA (Söderman et al., 1996, 1999; Lee and Chun, 1998; Johannesson et al., 2003), and the phenotypic analyses of Arabidopsis plants with elevated levels of *ATHB5*, *-6*, *-7*, or *-12* suggest that these genes regulate growth in response to ABA and/or water deficit conditions (Himmelbach et al., 2002; Hjellström et al., 2003; Johannesson et al., 2003; Olsson et al., 2004).

In this article, we explore the phylogenetic relationships and the duplication history of the HDZip class I members. We examine the diversity in gene structure within the HDZip-encoding motif and the expression patterns of class I genes. Furthermore, we study the effect of water deficit stress and light on the transcript levels of HDZip I genes. We also experimentally test transcriptional regulation properties of the six HDZip I proteins, ATHB1, -5, -6, -7, -12, and -16. The phylogenetic relationships between HDZip class I members are discussed in the context of the functional diversity among the genes.

RESULTS

Identification of HDZip Class I Sequences in Arabidopsis

A search of Arabidopsis databases allowed the identification of a total of 26 different genes with homeobox sequences highly similar to the HDZip class I/II homeoboxes, which encode Leu Zip domains located in a position typical for the HDZip proteins (Fig. 1). Nineteen of these sequences correspond to previously characterized HDZip I and II genes,

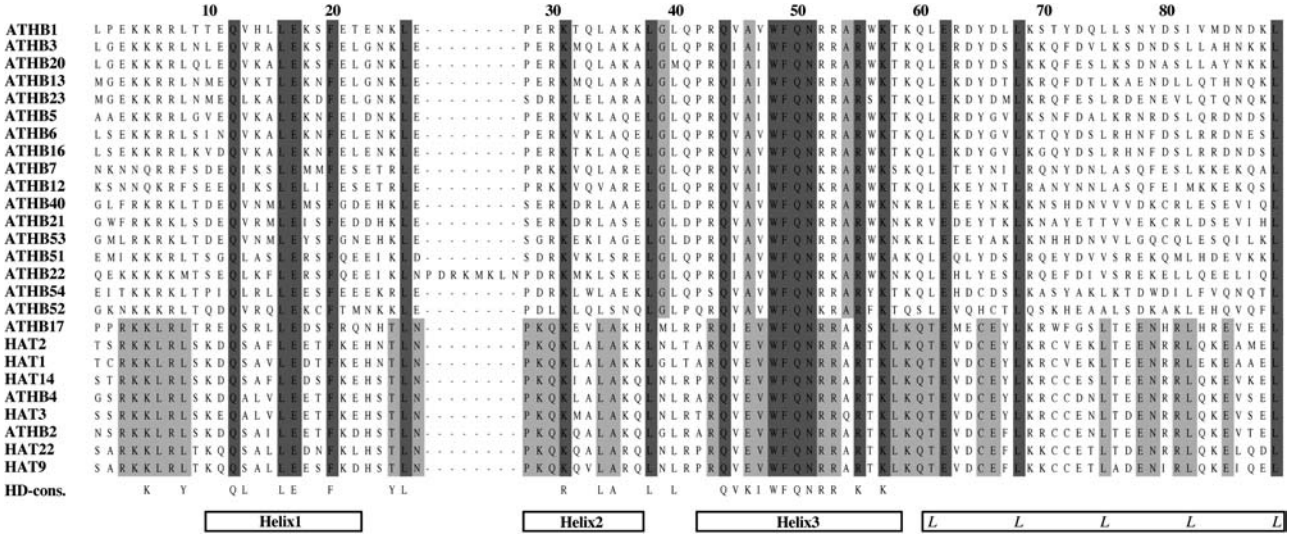

Figure 1. Alignment of the amino acid sequences of HDZip class I and II proteins. Gaps are indicated by dashes. Conserved positions in a HD consensus sequence (Bürglin, 1994) are indicated below the alignment. Residues conserved in both classes are depicted in dark gray, residues conserved only within HDZip I or II are depicted in light gray, and residues showing variation between and within the classes are depicted in white. The three α-helices of the HD and the Leu residues of the Leu Zip are shown below the alignment.

whereas seven represent novel putative HDZip genes. We refer to these novel genes as *ATHB21*, *-22*, *-40*, *-51*, *-52*, *-53*, and *-54* (Table I). An alignment of the deduced amino acid sequences of the HDZip domains of the previously characterized HDZip I and II and the novel HDZip proteins is shown in Figure 1. The sequences were alignable without insertions or deletions over the HD, with the exception of ATHB22, which has an eight-amino acid insertion between helix 1 and helix 2. The HDs of the HDZip proteins contain the five invariant amino acids (L_{16}, W_{48}, F_{49}, N_{51}, and R_{53}), except that of ATHB22 (K_{53}), and seven out of eight highly conserved residues (F_{20}, L_{26}, L_{40}/M_{40}, I_{45}/V_{45}, I_{47}/V_{47}, R_{55}, and K_{57}) of the HD consensus sequence defined on the basis of a compilation of 346 HD sequences from a range of different eukaryotes (Bürglin, 1994). The Leu Zip domains in all HDZip I and II proteins are in identical positions, C terminal to the HD. The HDZip domains of HDZip I and II proteins are similar to each other in sequence, although a number of amino acid positions distinguish HDZip I from II (Fig. 1). The amino acid at position 46 is invariant within the HDZip I and II, but distinct between the classes. Several other amino acids, e.g. the ones at positions 6, 25, 29, 30, 58, and 61, are invariable within the HDZip II and differ from HDZip I amino acids, which show variation at these positions. ATHB21, -22, -40, -51, -52, -53, and -54 are more similar to the HDZip I than to the HDZip II proteins and are thus thought to belong to HDZip class I.

Phylogenetic Analyses of HDZip Class I Proteins

Phylogenetic analyses, including all HDZip I and II proteins, confirmed that HDZip I and II, as previously defined by Sessa et al. (1994), are monophyletic (data not shown). To assess the phylogeny of the HDZip I proteins, we performed a maximum parsimony analysis based on the HDZip domain amino acid sequences shown in Figure 1. The HDZip II protein HAT1 was included in the analyses as an outgroup. The single most parsimonious tree (373 steps) resulting from this analysis (Fig. 2A) demonstrates that the HDZip I genes form six monophyletic subclasses—α (*ATHB3*, *-13*, *-20*, and *-23*), β (*ATHB1*, *-5*, *-6*, and *-16*), γ (*ATHB7* and *-12*), δ (*ATHB21*, *-40*, and *-53*), ε (*ATHB22* and *-51*), and ϕ (*ATHB52* and *-54*)—supported by bootstrap values over 50%. Bayesian analyses, based on the nucleotide sequences corresponding to the HDZip domain, resulted in trees with major topologies very similar to the maximum parsimony tree (data not shown). In the Bayesian tree, the monophyletic subclasses α to ϕ were all present: the α to ε clades with very high support values (posterior probability above 97%), but with lower support values for the association of *ATHB5* to clade β (57% posterior probability) and clade ϕ (53% posterior probability). The Bayesian analysis differed from the parsimony analysis in that, in the Bayesian tree, *ATHB1* did not associate with clade β, but instead appeared as a paralog to the α and β clades, with low support (58% posterior probability).

The different intron/exon organizations within the HDZip-encoding motif represented in HDZip I and II genes are shown in Figure 2B. The HDZip I genes have an intron/exon organization distinct from the HDZip II genes, which contain introns within the coding regions of helix 2 and helix 3 (Fig. 2B, letter E) that are not present in HDZip I genes. In the HDZip I genes, introns are in positions corresponding to helix 1 and the Leu Zip. Four different intron/exon patterns are present in the HDZip I genes (Fig. 2B, letters A–D): *ATHB52* and *-54* lack introns (A); *ATHB21*, *-40*, *-53*, *-22*, and *-51* have one intron after the codon for the fourth Leu in the Leu Zip (B); *ATHB3*, *-20*, *-13*, *-23*, *-5*, *-6*, *-16*, *-1*, *-7*, and *-12* have one intron after the codon for the fifth Leu (C); and *ATHB1* an additional intron within the region corresponding to helix 1 (D). In relation to the phylogeny of HDZip genes, the intron pattern B is common to all δ and ε genes. The α, β, and γ genes all have the C intron/exon pattern with the exception of *ATHB1*, which has an additional intron. Taken together, the analysis of the intron/exon organization shows that phylogenetically closely related HDZip genes share common intron distribution patterns within the HDZip-encoding motif, and the data thus support the results from the phylogenetic analysis presented in Figure 2.

Table I. *HDZip I genes in Arabidopsis*

Gene Name	Arabidopsis Genome Initiative ID
ATHB1/HAT5	At3g01470
ATHB3/HAT7	At5g15150
ATHB5	At5g65310
ATHB6	At2g22430
ATHB7	At2g46680
ATHB12	At3g61890
ATHB13	At1g69780
ATHB16	At4g40060
ATHB20	At3g01220
ATHB21	At2g18550
ATHB22	At2g36610
ATHB23	At1g26960
ATHB40	At4g36740
ATHB51	At5g03790
ATHB52	At5g53980
ATHB53	At5g66700
ATHB54	At1g27050

Genomic Organization and Duplication History of the HDZip I Genes

As several of the HDZip I genes appear as pairs of paralogs in the phylogenetic tree, we investigated whether traceable genome duplication events had contributed to the complexity of HDZip I. The chromosomal locations of the HDZip I genes in relation to the segmental duplication history of these regions, described by the Arabidopsis Genome Initiative (2000) and Blanc et al. (2003), were analyzed. The resulting

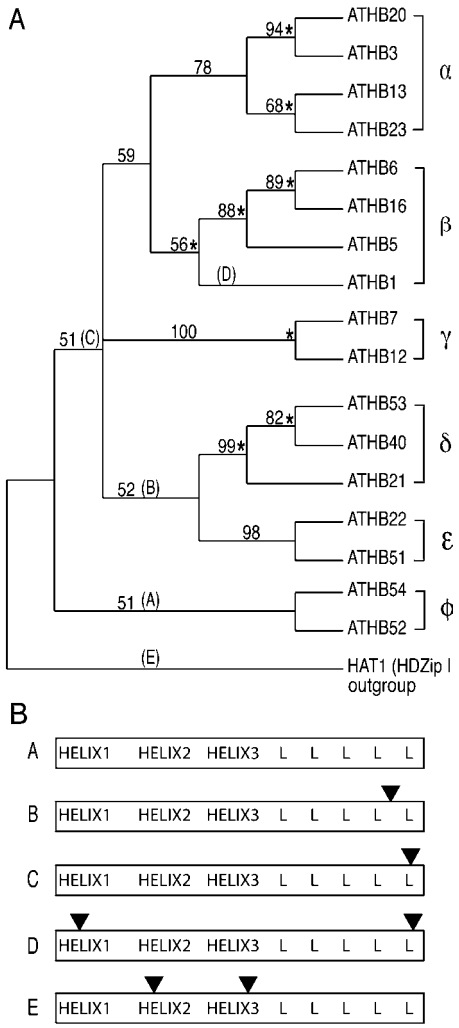

Figure 2. Phylogeny of the Arabidopsis HDZip class I sequences. A, The most parsimonious tree from the maximum parsimony analysis based on the amino acid sequences of HDZip domains. The HAT1 (HDZip II) sequence was used as an outgroup. The subclasses within the HDZip I are denoted α, β, γ, δ, ε, and ϕ. Bootstrap support is indicated on the branches of HDZip I sequences. Letters A to E indicate the intron/exon pattern within the HDZip domain illustrated in Figure 2B. The branch points supported by the data on the duplication history of the HDZip I genes, presented in the Figure 3, are marked with an asterisk (*). B, An illustration of the intron positions within the region corresponding to the HDZip domains of HDZip I and II (triangles indicate intron positions).

chromosome map (Fig. 3) shows that the HDZip genes are distributed evenly across the five chromosomes with a slight preference toward the ends of the chromosomes. According to the map, *ATHB13* and *-23*, *ATHB7* and *-12*, *ATHB6* and *-16*, *ATHB21* and *-40*, and *ATHB3* and *-20* constitute pairs of paralogous genes evolved from recent segmental genome duplication events. *ATHB5* and *-6*, as well as *ATHB40* and *-53*, have also evolved from segmental duplication events, albeit from older ones. Ancient segmental genome duplication events have potentially contributed to the formation of the paralogous genes *ATHB21* and *-53*, as well as *ATHB1* and *-5*. For four HDZip I genes, *ATHB22*, *-51*, *-52*, and *-54*, no traceable duplication history could be found, even though their genome positions are covered by duplicated blocks. The presented duplication history of HDZip I genes thus strongly supports the phylogenetic association of α-subclass members *ATHB23* and *-13*, *ATHB20* and *-3*, as well as the β-subclass members *ATHB5*, *-6*, *-16*, and *-1*, the γ-subclass members *ATHB7* and *-12*, and the δ-subclass members *ATHB21*, *-53*, and *-40*.

The Expression of Many HDZip Class I Genes Is Dependent on Water and Light Conditions

Previous reports have shown the transcript levels of *ATHB5*, *-6*, *-7*, *-12*, and *-16* to be influenced by ABA, water deficit stress, or different light conditions (Söderman et al., 1996, 1999; Lee and Chun, 1998; Johannesson et al., 2003; Henriksson, 2004). To examine the regulation of all HDZip I genes in these respects, northern-blot analyses were performed with RNA from 10-d-old seedlings exposed to ABA, NaCl, or low-temperature stress, or 4-d-old seedlings grown in different light conditions.

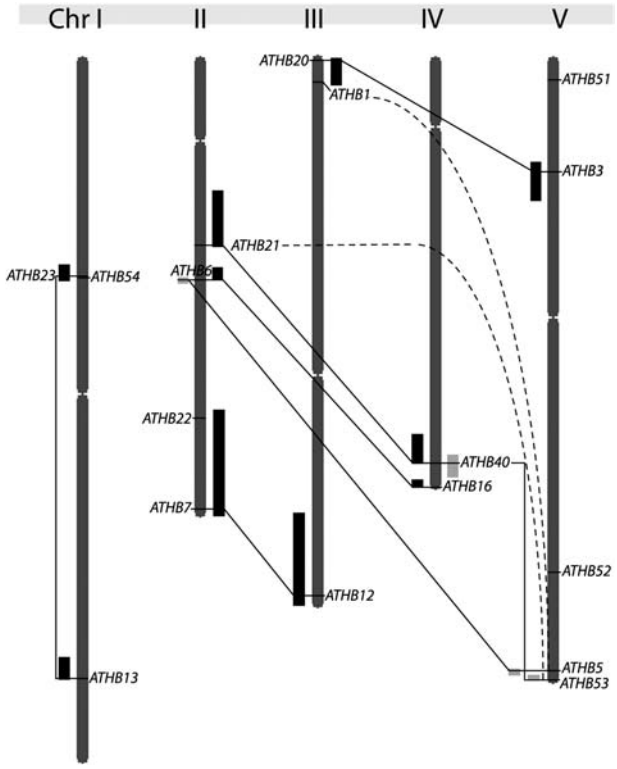

Figure 3. Chromosomal positions and duplication events for HDZip class I genes in the Arabidopsis genome. Black and gray boxes depict recent and old duplicated segments in chromosomes (Chr) I to V, respectively. Solid lines between boxes link the duplicated regions. Dashed lines link genes from ancient duplications and are of lower statistical significance than the others (Blanc et al., 2003).

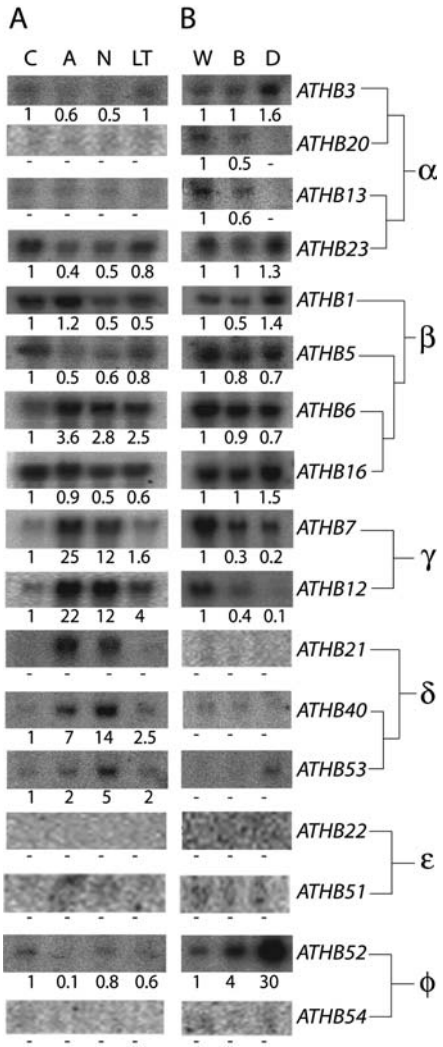

Figure 4. Expression of HDZip I genes in response to different environmental conditions. RNA gel blots containing RNA extracted from Col-0 seedlings (A) grown for 10 d on growth media without Suc in white light (60–90 μmol m^{-2} s^{-1}) under control conditions (C), exposed to 50 μM ABA (A), 100 mM NaCl (N), or low temperature (LT) for 4 h; or (B) grown for 4 d in white light (W; 80–100 μmol m^{-2} s^{-1}), blue light (B; 5–6 μmol m^{-2} s^{-1}) or in darkness (D) on media supplemented with 1% Suc. The RNA blots were hybridized to gene-specific probes. These hybridization signals are normalized to signals from a rRNA probe, rehybridized to the same RNA membranes, and expressed relative to the control sample for each gene (A) or to the white-light sample (B) and are given in numbers below the respective sample. Samples not determined due to nondetectable hybridization signals are indicated by (-).

Treatment of plants with ABA or NaCl (Fig. 4A) resulted in an up-regulation of *ATHB7* and *-12* transcript levels by a factor of 12 to 25 times the untreated control. The same treatments also increased the expression of *ATHB6*, *-21*, *-40*, and *-53* by more than 2-fold compared to the control. Further, this treatment reduced the expression of *ATHB3*, *-23*, *-5*, and *-52* to approximately one-half the level of the untreated control. Similar reductions of *ATHB1* and *-16* transcript levels were also shown in response to the salt treatment but not in response to ABA. In addition, *ATHB6*, *-7*, *-12*, *-40*, and *-53* transcript levels were up-regulated 2- to 4-fold by low-temperature exposure, whereas *ATHB1*, *-16*, and *-52* expression was reduced to one-half the control level by this treatment. The expression of the remaining HDZip class I genes was not affected by the treatments or undetectable in the experiments (Fig. 4A).

Light conditions—white light, blue light, or darkness—affected the transcript level of *ATHB52* dramatically (Fig. 4B). Dark treatment resulted in 30 times increased expression compared to white-light conditions, and blue light in 4 times increased expression. *ATHB53* expression was up-regulated in darkness, but not in blue-light conditions. *ATHB3*, *-23*, *-1*, and *-16* also showed increases in transcript levels in dark-grown seedlings, as compared to white- and blue-light-grown seedlings, but the differences between conditions were small (Fig. 4B).

In contrast, *ATHB5*, *-6*, *-7*, *-12*, *-13*, and *-20* transcript levels were higher in white-light-grown seedlings than in seedlings grown in blue light or in darkness (Fig. 4B). The expression of *ATHB21*, *-22*, *-40*, *-51*, and *-54* was low or not detectable and did not show any difference between different light conditions (Fig. 4B). The transcript levels *ATHB1*, *-7*, *-12*, *-13*, and *-20* in blue light were one-half the level in white-light-grown seedlings (Fig. 4B).

The results show the majority of the HDZip I genes to be responsive to ABA and water deficit stress and to differ in their response to different light conditions.

HDZip Class I Genes Are Widely Expressed

The organ distribution of HDZip gene expression was determined by use of reverse transcription (RT)-PCR on RNA derived from 5- and 12-d-old seedlings, as well as from roots, leaves, stems, flowers, and siliques of adult plants (Fig. 5). Transcripts of all subclass β and γ genes, the subclass α gene *ATHB20*, and the subclass δ and ε genes *ATHB21* and *ATHB51* were broadly expressed, transcripts being present in all tissues examined. The α genes *ATHB3* and *-23* were expressed in all tissues examined, except leaves and flowers, respectively. *ATHB13* transcripts were detected in seedlings, leaves, and flowers, but not in roots, stems, or siliques. Members of subclasses δ, ε, and ϕ were all found to be expressed in seedlings and/or in different organs of adult plants. With the exception of *ATHB21* and *-51*, they showed organ specificity in their expression. Transcripts of *ATHB53* and *-40* were detected in roots and flowers and in the seedlings, roots, flowers, and siliques, respectively. Expression of the subclass ε gene *ATHB22* was only detected in seedlings and siliques. The subclass ϕ genes, *ATHB52* and *-54*, showed essentially complementary expression patterns, as *ATHB52* transcripts were detected in seedlings, roots, and flowers, and *ATHB54* transcripts in leaves, stems, flowers, and siliques. Overall, the results

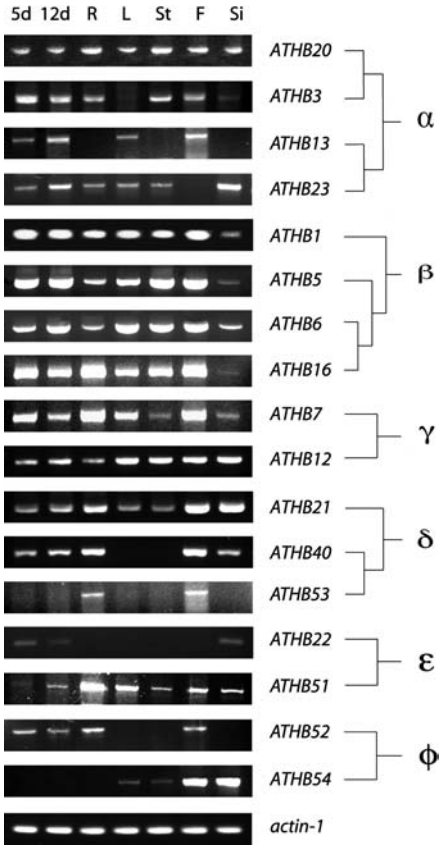

Figure 5. Expression of HDZip class I genes in different organs. The expression of HDZip I genes was determined by RT-PCR analysis. Total RNA was isolated from 5-d-old seedlings (5 d), 12-d-old seedlings (12 d), roots of 12-d-old seedlings grown in liquid medium (R), leaves (L), stems (St), flowers (F), and siliques (Si). The class I genes are divided into α, β, γ, δ, ε, and ϕ subclasses defined in Figure 2. *Actin-1* expression is shown as a control.

from RT-PCR analyses show that all 17 HDZip I genes in Arabidopsis are expressed and have broad expression profiles with transcripts being detected in most organs in different developmental stages.

HDZip Class I Proteins Function as Transcriptional Activators

HDZip proteins from different plant species have been shown to interact with the BS CAATNATTG (Sessa et al., 1993; Meijer et al., 2000; Johannesson et al., 2001) and to activate transcription in a BS-dependent manner (Sessa et al., 1993; Aoyama et al., 1995; Meijer et al., 2000).

We therefore analyzed the transcriptional regulation of a subset of HDZip I proteins either capable of binding to the consensus site in vitro (ATHB1, -5, -6, and -16) or with different apparent binding specificities in vitro (ATHB7 and -12; Johannesson et al., 2001). Transient expression assays on Arabidopsis leaves were performed using cobombardment of a reporter construct consisting of a minimal promoter with HDZip BSs driving the expression of a luciferase (*LUC*) reporter gene and transacting plasmids, expressing each of the proteins under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The transactivation activities of ATHB5, -6, -7, -12, and -16 relative to that of ATHB1 is shown in Figure 6. In each experiment, the control samples showed low transactivation levels with the wild-type BS, as well as with the mutated BS (mBS; data not shown). ATHB5 had a strong effect on LUC activity, the average relative transactivation value with BS was 1.21 ($\pm$0.40). ATHB6 and -16 had a weaker effect on LUC activity than ATHB5; the average transactivation values were 0.62 ($\pm$0.09) and 0.42 ($\pm$0.06), respectively. Both ATHB7 and -12 also enhanced LUC activity strongly. The average transactivation values with the BS were 1.23 ($\pm$0.17) and 1.96 ($\pm$0.86) for ATHB7 and -12, respectively. For all proteins tested, activation from the mBS reporter plasmid was low, ranging from 0.05 to 0.2. Together, these results demonstrate that ATHB1, -5, -6, -7, -12, and -16 function as transcriptional activators in Arabidopsis leaves by binding specifically to the HDZip I consensus sequence.

DISCUSSION

The HDZip transcription factors are of ancient evolutionary origin. Their typical arrangement of a DNA-binding HD in close proximity to a protein dimer-promoting Zip domain has not been found in proteins outside the plant kingdom, even though both sequence elements are present in separate classes of

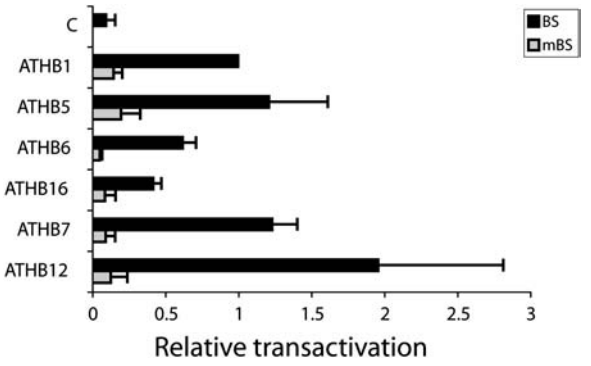

Figure 6. HDZip class I proteins activate gene expression. Transient expression assays on Arabidopsis leaves were performed by cobombarding three plasmids: (1) containing a transacting gene and a gene encoding for the GUS driven by the CaMV 35S promoter; (2) containing a gene encoding for GUS driven by the CaMV 35S promoter; and (3) a reporter plasmid containing six copies of the BS for the transacting gene (5′-CAATTATTG-3′) or a mBS of the sequence (5′-CAATTGTTG-3′) in the promoter of the LUC gene. After 20-h incubation, the leaves were assayed for LUC and GUS activities. The LUC activity was normalized with respect to the GUS activity for each experiment. The results presented are average values of duplicate samples from three independent experiments $\pm$ SD. The relative transactivation value for ATHB1 is set to 1. The leaves of the control (C) sample were bombarded with the reporter plasmid and the plasmid containing the GUS gene without the sequence of the transacting factor.

transcription factors in other eukaryotes. The juxtaposition of the HD and Zip elements, possibly a result of an exon capture event (Schena and Davis, 1994), is therefore thought to have occurred after the divergence of plants and animals. The cloning of genes, with features typical for HDZip genes of classes I, II, and III from the moss *P. patens* (Sakakibara et al., 2001), indicates that HDZip genes were present and the main classes of HDZip genes already established in early plant evolution. The phylogenetic analysis of the HDZip class I genes in Arabidopsis, presented in this article, is consistent with this notion and further shows that HDZip I has evolved by a series of gene duplications, many of which are of considerably later date. Pairs of paralogous HDZip genes in Arabidopsis can be traced to a major genome duplication event thought to have occurred between 20 to 60 million years ago, and part of the present complexity in the gene class derives from genome duplications estimated to have taken place in the period of establishment and expansion of the angiosperm lineage over 100 million years ago (Blanc and Wolfe, 2004, and refs. therein). The analysis further shows that the six subclasses of genes within HDZip I—α, β, γ, δ, ε, and ϕ—are of older origin and probably were already established in the early angiosperms.

In the phylogenetic analyses, the subclasses α, γ, δ, and ε were well supported, whereas the subclasses β (*ATHB1*, *-5*, *-6*, and *-16*) and ϕ (*ATHB52* and *-54*) received low support. However, the association of the subclass β members was independently supported by the intron/exon organization within the HDZip motif and the genome duplication data. Overall, the intron/exon organization within the HDZip motif and the genome duplication data are consistent with the presented tree. The phylogenetic associations of the subclass members are largely in accordance with the previously reported phylogenetic analysis of HDZip I to IV sequences in which 13 Arabidopsis HDZip I genes were included (Sakakibara et al., 2001), but the two analyses differ in the positions within the tree of *ATHB1*, *-52*, and *-54*.

Our data show that the HDZip class I in Arabidopsis includes 10 previously known genes and an additional seven sequences that are not previously described: *ATHB21*, *-22*, *-40*, *-51*, *-52*, *-53*, and *-54*. These novel sequences encode HDZip domains highly similar to the class I proteins, with the exception of ATHB22, which has an insertion of eight amino acid residues in the region between helix 1 and helix 2. An insertion in a similar position in the yeast (*Saccharomyces cerevisiae*) $\alpha 2$ protein (Hall and Johnson, 1987) does not significantly alter the three-dimensional structure of the HD (Wolberger et al., 1991). Therefore, this difference between ATHB22 and the other class I proteins may not be of functional significance with regard to DNA binding. As the seven new members, like the 10 previously known genes, are actively transcribed into RNA, we conclude that HDZip I includes 17 gene members and that pseudogenes are lacking in the class.

It is interesting to note that, to the extent that the duplication history of HDZip I can be traced, paralogous genes originating from duplication have been retained in the genome at high frequency, rather than lost by mutation. Thus, positive selection for an expansion of the gene class must have been present in the evolving plants. As evident from data presented in this article and previously published data, this expansion of the class is associated with an extensive divergence in expression patterns and, thus, in the regulatory properties of the genes, both between and within subclasses, as well as a divergence in function of the gene products. As might be expected, differences in protein function are most pronounced between subclasses, whereas the transcription factors within each subclass appear more highly conserved in function.

The subclass γ genes *ATHB7* and *-12* in many aspects are very similar to each other. Both ATHB7 and -12 bind to the CAATNATTG sequence to activate transcription in Arabidopsis, but fail to interact with this sequence in in vitro DNA-binding assays (Johannesson et al., 2001). The two genes are also highly similar in their regulation (Söderman et al., 1996; Hjellström et al., 2003; Olsson et al., 2004). Transgenic plants with elevated levels of *ATHB7* or *-12* expression show similar phenotypic alterations, thus indicating extensive similarities in gene product function in relation to the regulation of growth in response to water availability (Hjellström et al., 2003; Olsson et al., 2004).

Functional data on the δ, ε, and ϕ genes is as yet lacking. Our expression data, though, imply that a significant divergence in the regulatory properties of the genes has occurred both between and within these subclasses. The δ genes *ATHB21*, *-40*, and *-53*, like *ATHB7* and *-12*, show an increase in transcript levels in response to ABA or water deficit treatments. In addition, *ATHB40* and *-53*, but not *ATHB21*, are reported to be regulated by auxin (Son et al., 2005). The ABA and water deficit stress response is supported by available microarray data using the Genevestigator database (Zimmermann et al., 2004). Further, the expression of the ϕ gene, *ATHB52*, differs dramatically between different light conditions and, according to microarray data, is enhanced in response to auxin, 1-aminocyclopropane-1-carboxylic acid, and ethylene (Genevestigator; Zimmermann et al., 2004).

The four β genes—*ATHB1*, *-5*, *-6*, and *-16*—encode proteins that interact with the consensus sequence CAATNATTG in in vitro DNA-binding assays (Johannesson et al., 2001) and act as transcriptional activators in Arabidopsis. The expression patterns of the β genes are similar at the organ level, but differ at the tissue level as well as in their responses to ABA (Söderman et al., 1999; Wang, 2001; Johannesson et al., 2003; Figs. 4 and 5). The similarities in phenotypes of transgenic plants expressing either *ATHB6* or *-16* at elevated levels indicate that proteins encoded by these two genes are highly conserved in function (Wang

et al., 2003; Henriksson, 2004), whereas *ATHB1* and *-5* cause different phenotypic alterations to the plant (Aoyama et al., 1995; Johannesson et al., 2003) and thus differ in gene product function. Microarray data (Genevestigator) indicate that the β genes may also depend on light conditions for their expression. This is not confirmed by our northern-blot data, which show the β genes to be largely unaffected by different light conditions. However, our mutant data have demonstrated that *ATHB5*, *-6*, and *-16* function in the regulation of light-dependent developmental phenomena (Wang et al., 2003; Henriksson, 2004). Thus, the three genes have partly overlapping functions in the plant growth response to ABA and light, but also differ in aspects of this function.

The two gene pairs of the α-subclass, *ATHB3/20* and *ATHB13/23*, show similarities as well as differences in their expression patterns with regard to both distribution and response to environmental conditions. All four gene products interact with DNA with similar sequence preferences (Johannesson et al., 2001) and the genes cause similar pointed-cotyledon phenotypes to transgenic plants when expressed at elevated levels (Hanson, 2000; Hanson et al., 2001). Therefore, the products of the α genes show extensive functional similarities.

In summary, HDZip class I has evolved by a series of gene duplications to a considerable complexity. The evolution of the class has been associated with an extensive divergence between genes in expression patterns both large scale, affecting the organ distribution of gene activity, and at the cellular/tissue level within organs. It appears that the majority of the genes within class I have evolved to be differentially responsive to alterations in growth conditions, most notably water availability and light conditions. The evidence for functional divergence at the gene product level is more limited even though the divergence in amino acid sequence is quite considerable. As the phenotypic effects of ectopic expression of the genes differs between subclasses and, at least in one case (β), also within the subclass, the capacity of the different HDZip I transcription factors to regulate downstream target genes must differ. This is consistent with the difference between proteins in the specificity in DNA binding that is documented (Johannesson et al., 2001), but likely also reflects other functional differences between proteins, as β proteins with similar DNA-binding preferences produce different phenotypic alterations when produced ectopically in transgenic plants. A selective capacity to form heterodimers is one possible such difference, as documented among the β proteins (Johannesson et al., 2001). It should be noted, however, that the phenotypic effects of ectopic expression of the different class I genes also show considerable similarity between subclasses α, β, and γ. Transgenic plants expressing *ATHB1*, *-3*, *-6*, *-7*, *-12*, *-13*, *-16*, *-20*, or *-23* at elevated levels all show a reduction in cell expansion in different organs and with somewhat different consequences for organ development. It is thus possible that the HDZip I genes may act to regulate similar or partly overlapping sets of target genes, and their biological functions may be distinct mainly as a consequence of the differences in their expression properties.

It is a tempting hypothesis that the selective advantage for the plant in the retention of an increasing number of HDZip I genes may lie in an increased capacity of the plant to fine tune its growth response to changes in environmental conditions.

MATERIALS AND METHODS

Screening of Databases, Sequence Alignments, and Phylogenetic Analyses of HDZip Sequences

Available Arabidopsis (*Arabidopsis thaliana*) databases were searched by use of the BLAST algorithm (TBLASTN) to identify HDZip sequences in the Arabidopsis genome. Intron/exon predictions of genomic sequences were confirmed by comparison with available expressed sequence tags. Where expressed sequence tags were not available, corresponding cDNAs where amplified from first-strand cDNA derived from pooled RNA extracted from various tissues and growth conditions. The amino acid sequences of the HDZip domains of class I and II proteins were aligned using the Sequence Alignment Editor Version 2.0 (Se-Al) software (http://evolve.zoo.ox.ac.uk) and refined manually, omitting the less-conserved C termini of the Leu Zips (Fig. 1). The nucleotide sequences, corresponding to the aligned amino acid sequences (Fig. 1), were aligned using ClustalW (Thompson et al., 1994), followed by manual adjustments. Maximum parsimony analyses were performed by use of PAUP 4.0b10 software (Swofford, 2002) and the amino acid alignment shown in Figure 1. One thousand replicates of random addition with tree bisection reconnection branch swapping were performed with MulTrees on. The shortest tree resulting from the analysis was saved. We estimated the support for each clade using 10,000 replicates of nonparametric bootstrap, with simple additions of taxa and tree bisection reconnection branch swapping, saving one tree per replicate. For the Bayesian analyses, the appropriate model for DNA sequence evolution was examined with Mrmodeltest 1.1b (http://www.ebc.uu.se/systzoo/staff/nylander.html). The Bayesian analyses were performed with MrBayes 3.0b (Huelsenbeck and Ronquist, 2001), with vague or uninformative prior probability distributions for all parameters of the likehood model, as implemented by default in MrBayes. Four parallel Markov chains, of which three were heated with 0.20°C-temperature increments, were run for 1 million generations starting from random trees. Every one-hundredth tree was sampled. The number of generations needed to reach stationarity was empirically determined by checking for convergence of the likelihood scores; trees sampled before reaching stationarity were discarded. A 50% majority rule consensus tree was calculated for the sampled trees using PAUP. As the time to reach stationarity is unknown a priori, we repeated the analysis three times to ensure that convergence had been achieved.

Chromosomal Locations and Duplication History of HDZip I Genes

The chromosomal location of each of the HDZip I genes was determined by use of http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp. The localization of each gene in relation to the major chromosomal duplication events in the Arabidopsis genome was determined by use of the tools provided by http://wolfe.gen.tcd.i.e./athal/dup, and as defined by Blanc et al. (2003).

Plant Material and Growth Conditions

Arabidopsis (ecotype Columbia) was used in all experiments. Seeds were surface sterilized as described by Söderman et al. (1996) and seed dormancy broken by 3 to 4 d of cold treatment (4°C). The seeds were either plated on 0.5× Murashige and Skoog (MS) medium (Duchefa Biochemie) supplemented with 0.8% agar, 2.5 mM MES-KOH, 1% Suc, pH 5.7, or placed in liquid

0.5× MS medium supplemented with 4% Suc, pH 5.7, and grown on a rotary platform under continuous light at 20°C for 12 d. For cultivation of adult plants, 2-week-old seedlings were transferred from the solid MS medium to a soil-vermiculite mixture and grown under long-day conditions (16 h of light, 8 h of darkness) for 1 to 3 weeks.

The water deficit stress and ABA treatments were applied to 10-d-old seedlings grown on Suc-free 0.5× MS medium in white light (60–90 μmol m^{-2} s^{-1}). The seedlings were exposed to either 50 μM ABA (mixed isomers; Sigma), 100 mM NaCl, low temperature (2°C–4°C), or were left untreated for 4 h.

To investigate the effect of different light conditions on transcript levels of HDZip I genes, seeds were plated on MS medium supplemented with 1% Suc, cold treated for 4 d, exposed to light for 4 h, transferred to darkness for 21 h, and thereafter transferred to white light (80–100 μmol m^{-2} s^{-1}), blue light (5–6 μmol m^{-2} s^{-1}), or left in darkness for 4 d.

RNA Extraction, Gel-Blot Analyses, and RT-PCR

Total RNA was isolated according to Verwoerd et al. (1989) or Chang et al. (1993) from 4-, 5-, 10-, and 12-d-old seedlings from roots of 12-d-old seedlings grown in liquid culture and from tissues of 4- to 5-week-old soil-grown plants.

Samples of 20 μg total RNA were separated in denaturing formaldehyde agarose gels and blotted to Hybond-XL membranes (Amersham-Pharmacia Biotech). These membranes were prehybridized, hybridized, washed, and stripped, according to the manufacturer's instructions. Probes corresponding to the 3′-end of the HDZip class I genes and an rRNA probe (Söderman et al., 1996) were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). The final washes were conducted in 0.1× SSC and 0.1% (w/v) SDS at 65°C. The hybridization signals were visualized with a BAS2000 image plate reader (Fuji Photo Film) and quantified with Image Gauge version 3.3.

RT-PCR was performed using the Access RT-PCR system (Promega) and 0.1 μg of total RNA, according to the manufacturer's instructions. Primers were designed to flank introns where possible. The RNA used for the RT-PCR reactions with primers designed to amplify *ATHB52*, which lacks introns, was DNase I (Promega) treated according to the manufacturer's instructions. In negative control reactions, where RNA was exchanged with water, neither of the presented RT-PCR experiments resulted in PCR products (data not shown). Primers designed for Arabidopsis *actin-1* (At2g37620) were used in control reactions. (See supplemental data for *HDZip I* and *actin* primers used.)

Transient Expression Analysis

The transactivating plasmids, for transient expression assays, were constructed by replacing the *ATHB1-VP16* sequence from the derivative of pMON721 (Aoyama et al., 1995) with *ATHB5*, *-6*, *-7*, *-12*, or *-16* cDNA sequences. The coding sequences were amplified by PCR using the following primer pairs: *ATHB5*, 5′-CGGGATCCCAAGAGATCACGTGGAAG-3′ and 5′-CGGGATCCTTACGAATTCCACTGAT-3′; *ATHB6*, 5′-CGGGATCCCAAGAGATTAAGTAGTTCAG-3′ and 5′-CGGGATCCTCAATTCCAATGATCAAC-3′; *ATHB7*, 5′-CGGGATCCCACAGAAGGTGGAGAAT-3′ and 5′-CGGGATCCTCATGACCAAAAATCC-3′; *ATHB12*, 5′-CGGGATCCCGAAGAAGGAGATTTTTTC-3′ and 5′-CGGGATCCTTATGACCAAAACTCCCA-3′; and *ATHB16*, 5′-CGGGATCCCAAGAGACTAAGCAGCTCA-3′ and 5′-CGGGATCCTCAAGTCCAATGATCTG-3′. The HDZip-coding sequences as well as the gene encoding β-glucuronidase (GUS; Jefferson et al., 1987) were fused to the CaMV 35S promoter. Derivatives of pGEM (Aoyama et al., 1995) were used as reporter plasmids in the transient expression assays. The reporter plasmids contained six copies of the binding sequence (5′-CAATTATTG-3′) or a mutated version of the sequence (5′-CAATTGTTG-3′) upstream of the −46 CaMV 35S promoter driving *LUC* gene expression.

Leaves from 3- to 4-week-old soil-grown plants were used for the transient expression analysis. Transformations were performed with particle bombardment as described by Sessa et al. (1998a); 1.2 μg of the reporter plasmid, 0.4 μg of the transactivating plasmid, and 0.2 μg of the empty transacting plasmid (derivative of pMON721 without *VP16* or *ATHB* coding sequence to enhance the GUS activity) were cobombarded into the leaves. The leaves were incubated for 20 h at 20°C in constant light and total cell extracts were assayed for LUC and GUS activity, respectively, as described by Sessa et al. (1998a). The values for LUC activities were normalized with respect to the GUS activities. ATHB1 was used as a positive control and the LUC:GUS ratio for ATHB1 was set to 1. The results shown are averages of duplicate samples from three independent experiments ±SD. The leaves of the control were bombarded with the reporter (1.2 μg) and the GUS (0.6 μg) plasmid only.

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