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ORIGINAL PAPER

Plant homeodomain-leucine zipper I transcription factors exhibit different functional AHA motifs that selectively interact with TBP or/and TFIIB

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Abstract
Key message **Different members of the HD-Zip I family of transcription factors exhibit differential AHA-like activation motifs, able to interact with proteins of the basal transcriptional machinery.**
Abstract Homeodomain-leucine zipper proteins are transcription factors unique to plants, classified in four subfamilies. Subfamily I members have been mainly associated to abiotic stress responses. Several ones have been characterized using knockout or overexpressors plants, indicating that they take part in different signal transduction pathways even when their expression patterns are similar and they bind the same DNA sequence. A bioinformatic analysis has revealed the existence of conserved motifs outside the HD-Zip domain, including transactivation AHA motifs. Here, we demonstrate that these putative activation motifs are functional. Four members of the *Arabidopsis* family were chosen: AtHB1, AtHB7, AtHB12 and AtHB13. All of them exhibited activation activity in yeast and in plants but with different degrees. The protein segment necessary for such activation was different for these four transcription factors as well as the role of the tryptophans they present. When interaction with components of the basal transcription machinery was tested, AtHB1 was able to interact with TBP, AtHB12 interacted with TFIIB, AtHB7 interacted with both, TBP and TFIIB while AtHB13 showed weak interactions with any of them, in yeast two-hybrid as well as in pull-down assays. Transient transformation of *Arabidopsis* seedlings confirmed the activation capacity and specificity of these transcription factors and showed some differences with the results obtained in yeast. In conclusion, the differential activation functionality of these transcription factors adds an important level of functional divergence of these proteins, and together with their expression patterns, these differences could explain, at least in part, their functional divergence.

Keywords Homeodomain-leucine zipper · AHA motif · Transactivation motif · Carboxy-terminal domain · HD-Zip I subfamily

Abbreviations

AD	Activation domain of GAL4
AHA	Aromatic and large hydrophobic residues embedded in an acidic context
BD	Binding domain of GAL4
CTR	Carboxy-terminal region
HA	Hemagglutinin
HD	Homeodomain
HD-Zip	Homeodomain-leucine zipper
HSF	Heat stress transcription factor
IB	Immunoblotting
LZ	Leucine zipper
NTR	Amino-terminal region
TBP	TATA binding protein
TF	Transcription factor
TFIIB	Transcription factor IIB

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Introduction

Regulation of gene expression in plants occurs mostly, but not exclusively, at the transcriptional level. The initiation of the transcription process involves a RNA polymerase (II for mRNAs) and a basal machinery composed of several proteins (TFIIA, -B, -D, -E, -F, y -H) able to recruit the polymerase. Transcription factor IID is itself a protein complex containing the TATA binding protein (TBP), responsible for the nucleation of the basal transcriptional machinery (Venters and Pugh 2009). Transcription factors (TFs) are modular proteins able to recognize and bind specific DNA sequences in the regulatory regions of their targets, inducing or repressing their expression. They generally exhibit a DNA binding domain and a protein–protein interaction domain. Specific transcription factors interact with the basal machinery directly or through other proteins, like the Mediator complex, which acts as a bridge (Venters and Pugh 2009).

Transcription factors play key roles in the regulation of gene expression in all life kingdoms. They are classified in families according to structural and functional features. Bioinformatic analyses predicted the existence of about 1,500–2,000 TFs in *Arabidopsis* and rice (Mitsuda and Ohme-Takagi 2009; Riechmann et al. 2000; Xiong et al. 2005). This represents about 5–10 % of these plants genes, a clearly larger proportion when compared to that of *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, indicating a more significant role of transcriptional control in the regulation of gene expression. Some families of TFs have a larger number of members in plants than in other kingdoms and there are several families unique to plants (Riechmann et al. 2000). Among the last, the HD-Zip family is composed of four subfamilies, I to IV, the members of which exhibit a homeodomain (HD) associated to a leucine zipper (LZ). The HD recognizes and binds the target DNA while the LZ acts as a dimerization motif, which is a prerequisite for DNA binding. In *Arabidopsis*, subfamily I has 17 members encoding proteins of about 35 kDa involved in abiotic stress responses (Ariel et al. 2007).

The HD-Zip domain is highly conserved in proteins from *Physcomitrella patens* to dicots and monocots but recently we reported the existence of uncharacterized conserved motifs outside the HD-Zip (Arce et al. 2011). In the previous work, we showed that the carboxy-terminal region (CTR) is responsible, at least in part, for the different functions exerted by these TFs in plants (Arce et al. 2011). We found putative phosphorylation, sumoylation and transactivation motifs, mostly located in the CTRs and to a minor extent in the amino-terminal regions. Transactivation activity was experimentally demonstrated for HD-Zip subfamily I proteins (Meijer et al. 2000; Wang et al. 2005) although the role of the CTRs in such transactivation function was only proven for AtHB1, AtHB12 (Arce et al. 2011; Lee et al. 2001) and more recently for Oshox22, HvHox2 and Vrs1 (Sakuma et al. 2013; Zhang et al. 2012). This activity was acknowledged for the CTRs of three HD-Zip I members but no deep and/or comparative analysis was performed to establish the specific motifs and amino acids responsible for such activity.

AHA motifs were first described as activation motifs present in tomato HSF transcription factors (heat stress factors) by Treuter et al. (1993). These authors used reporter assays in protoplasts with HSF24, HSF8 and HSF30 TFs and described these motifs as short, essential peptides exhibiting a characteristic pattern of aromatic and large hydrophobic residues embedded in an acidic context (AHA). Döring et al. (2000) analyzed the activation motifs present in tomato HSFA1 and HSFA2 TFs and concluded that an amphipathic and negatively charged helix is the contact region with components of the basal transcription complex.

Conserved motifs fitting the AHA description were informatically detected in HD-Zip TFs (Arce et al. 2011). Aiming to elucidate if such motifs are actually functional, four members of the HD-Zip I subfamily, characterized in our and other laboratories (Aoyama et al. 1995; Cabello et al. 2012; Cabello and Chan 2012; Hanson et al. 2001; Olsson et al. 2004; Söderman et al. 1996; Son et al. 2010), were selected for an in-depth analysis. AtHB7 and AtHB12 are paralogs belonging to group I while AtHB1 and AtHB13 belong to groups III and V, respectively (Arce et al. 2011). The genes coding for these four TFs were partially characterized; their expression is regulated by abiotic stresses and they exhibit different functions (Henriksson et al. 2005). AtHB7 and 12 were described as negative developmental regulators in front of abiotic stress and ABA response (Söderman et al. 1996; Olsson et al. 2004); AtHB13 is involved in sugar response metabolism and in the response to freezing, drought and salinity (Cabello et al. 2012; Cabello and Chan 2012; Hanson et al. 2001), and AtHB1 has been described as a gene involved in leaf development (Aoyama et al. 1995). These TFs bind in vitro with maximal affinity the same pseudopalindromic sequence, CAATNATTG (Johannesson et al. 2001; Palena et al. 1999). Even though their specific function and interacting partners, which could cooperate in controlling gene regulation, are largely unknown, they are the best characterized among the HD-Zip I subfamily and for this reason they have been chosen for this study. In addition, these TFs belong to three different clades of this TFs subfamily.

In this manuscript, we present experimental data showing that these TFs are activators through different

AHA-like motifs. The results obtained in yeast suggested the existence of two AHA motifs in AtHB1 and only one for the other three TFs, while the assays in plants indicated the presence of only one motif in the four proteins. They directly interact with the basal transcriptional machinery, but in different ways. AtHB7 interacts with both TBP and TFIIB, AtHB1 interacts with TBP but not with TFIIB, AtHB12 is able to interact only with TFIIB while AtHB13 did not show significant interactions with any of these components. In addition, we demonstrate that the results obtained from experiments performed in yeast for these TFs were not always in accordance with those performed in plants and hence, a careful analysis must be done when a heterologous system is considered.

Materials and methods

Identification of AHA motifs in the CTRs

The definition of the AHA-like motifs present in AtHB1, AtHB7, AtHB12 and AtHB13 TFs was performed on the basis of a sequence comparison with HD-Zip subfamily I proteins from different plant species presenting a similar CTR. To retrieve these protein sequences, a blastp search was conducted with the full-length sequence of AtHB12, AtHB1 and AtHB13 TFs (representatives of groups I, III and V, respectively; Arce et al. 2011) against the NCBI non-redundant protein sequence database (default parameters were used, July 30, 2013; Altschul et al. 1990). Each set of sequences was aligned using the MAFFT multiple sequence alignment program (version 6.951b; Katoh and Toh 2008). The alignments were manually inspected to discard sequences with short or clearly divergent CTRs. After this filtering, a total of 37 sequences were analyzed for AtHB12 and AtHB7; 35 sequences for AtHB1; and 95 sequences for AtHB13. This represents an increase in the number of members analyzed per group in relation to the previously described (Arce et al. 2011).

The resulting multiple sequence alignments were summarized in sequence logos using the program Weblogo (http://weblogo.berkeley.edu/; Crooks et al. 2004). The sequences of the CTRs were also used to perform motif discovery using the MEME program (http://meme.nbcr.net/; Bailey et al. 2009).

Constructs

Constructs for yeast one and two-hybrid assays

pGBKT7::GAL4BD::AtHB1 and *pGBKT7::GAL4BD::AtHB1 ΔCTR* were previously performed and described (Arce et al. 2011).

pGBKT7::GAL4BD::AtHB7, *pGBKT7::GAL4BD::AtHB12* and *pGBKT7::GAL4BD::AtHB13* were performed by RT-PCR using total *Arabidopsis* RNA and specific oligonucleotides (Supplementary Table S1). The amplification products were cloned in the *pGBKT7* vector previously restricted with *Eco*RI/*Bam*HI.

pGBKT7::GAL4BD::AtHB7ΔCTR, *pGBKT7::GAL4BD::AtHB12ΔCTR*, *pGBKT7::GAL4BD::AtHB13ΔCTR*, *pGBKT7::GAL4BD::AtHB1Δ259–272*, *pGBKT7::GAL4BD::AtHB1Δ239–272*, *pGBKT7::GAL4BD::AtHB1Δ224–272*, *pGBKT7::GAL4BD::AtHB1 259–272*, *pGBKT7::GAL4BD::AtHB12Δ208–235*, *pGBKT7::GAL4BD::AtHB12 Δ227–235*, *pGBKT7::GAL4BD::AtHB12 208–235*, *pGBKT7::GAL4BD::AtHB7Δ229–258*, *pGBKT7::GAL4BD::AtHB7 229–258*, *pGBKT7::GAL4BD::AtHB13Δ275–294 pGBKT7::GAL4BD::AtHB13 275–294*, *pGBKT7::GAL4BD::athb1-W269A*, *pGBKT7::GAL4BD::athb1-W271A*, *pGBKT7::GAL4BD::athb1-W269A-W271A*, *pGBKT7::GAL4BD::athb1-G270A*, *pGBKT7::GAL4BD::athb1-F268A*, *pGBKT7::GAL4BD::athb13-W285A-W287A* and *pGBKT7::GAL4BD::athb13-D289A* mutant constructs were performed by PCR amplification using specific oligonucleotides (Supplementary Table S1) and the corresponding wild-type construct as probe; then, cloned in the *Eco*RI and *Bam*HI sites of *pGBKT7*.

pGBKT7::GAL4BD::AtHB1Δ239–253, *pGBKT7::GAL4BD::AtHB1Δ216–227*, *pGBKT7::GAL4BD::athb1-E264A* and *pGBKT7::GAL4BD::athb13-S282P-F284A* mutant constructs were carried out by PCR amplification and overlapping with the oligonucleotides listed in the Supplementary Table S1 and using as probes the constructs with the wild-type constructs. Mutated PCR products were cloned in *pGBKT7* fused to the BD of GAL4, following the technique described by Higuchi et al. (1988).

pGADT7::GAL4AD::HA::AtTBPII and *pGADT7::GAL4AD::HA::AtTFIIB*: the cDNAs corresponding to AtTBPII and AtTFIIB were amplified by RT-PCR (polyT was used for reverse transcription) from total RNA from *Arabidopsis* leaves with specific oligonucleotides (Supplementary Table S1). The amplification products were cloned in the *Bam*HI and *Xho*I sites of the pGADT7 vector (Clontech).

Clones for plant transient transformation

pBI122: this vector was constructed by inserting the His-tag and the multiple cloning site of vector *pQE32* and the *NOS* terminator in the *Xba*I and *Eco*RI sites of *pBI121*.

35S::GAL4BD::AtHB1, *35S::GAL4BD::AtHB12 and 35S::GAL4BD::AtHB13* were constructed as follows: the chimerical fusions *GAL4BD::AtHBX* were amplified from the corresponding constructs in *pGBKT7* using the oligonucleotides GAL4BDF and GAL4BDR (Supplementary Table S1) and cloned in the *Xba*I and *Sal*I sites for AtHB1

or *Xba*I and *BamH*I for AtHB12 and AtHB13 of the *pBI122* vector.

pMC::GAL1::GFP::GUS was constructed as follows: a fragment of the GAL1 promoter containing the *cis*-acting elements recognized by the GAL4 TF was amplified using yeast (*Saccharomyces cerevisiae* AH109) genomic DNA as probe and oligonucleotides prGAL1F and prGAL1R (Supplementary Table S1). The PCR product was cloned in the *Hind*III and *Pst*I sites for *pBluescript* SK(-) generating a construct named *pBS::prGAL1*. On the other hand, the TATA box of the CaMV 35S promoter was amplified from *pBI121* with the oligonucleotides 35Smin F and GUSNH2 R and cloned in *pGEM T-easy* (Promega) to get the construct *pGEM::TATAbox*. This last construct was restricted with *Pst*I and *Xba*I and inserted in the *pBS::prGAL1* construct. The entire artificial promoter was cloned in *pENR3C* and finally in the *pKGWFS7* vector to generate the construct by GATEWAY recombination.

Clones for pull-down assays

GST::AtHB7 and *GST::AtHB13*: the complete corresponding cDNAs were cloned in the *EcoRI* and *XhoI* sites or the *pGEX4T1* vector (Amersham Biosciences).

GST::AtHB1: the cDNA was cloned in the *EcoR*I and *Sal*I sites of the *pGEX4T3* vector and then restricted with *BamH*I to be cloned in *pGEX3X* in the unique *BamH*I site. The orientation of the insert was determined by PCR and further sequencing.

All the recombinant GST fusions were cloned in BL21 Codon plus cells to optimize their expression.

Yeast culture and transformation

Saccharomyces cerevisiae AH109 or Y187 (Clontech) cells were grown in YPDA or synthetic minimal medium (SD) supplemented with an amino acids dropout solution deficient in Trp or Leu/Trp (Sherman and Wakem 1991). Yeast cells were transformed (one-hybrid assays) or co-transformed (two-hybrid assays) with the constructs indicated in the figures following the lithium acetate method (Gietz et al. 1992). AH109 or Y187 transformed cells were selected for tryptophan or leucine/tryptophan prototrophy on SD medium, respectively.

Yeast proteins extraction and western blot analysis

Total yeast proteins were extracted according to Kushnirov (2000) with minor modifications. Briefly, *Saccharomyces cerevisiae* AH109, previously transformed with the different constructs, was grown overnight on SD medium supplemented with an amino acids dropout solution deficient in Trp, 1 ml was transferred to fresh medium and incubated until the cells were in mid-log phase, and about 7.5 OD_{600} of cells were harvested by centrifugation. These cells were resuspended in 600 µl NaOH 0.1 M, incubated for 10 min at room temperature, pelleted, resuspended in 16 µl SDS sample buffer (100 mM Tris–HCl, pH 7, 10 % SDS, 5 % glycerol, 4 % β-mercaptoethanol, 0.0025 % bromophenol blue), boiled for 10 min and electrophoresed on 12 % polyacrilamide gels. Detection of the bands was performed by western blot using mouse anti-c-Myc antibodies (GenScript) and HRP-Rabbit anti-mouse antibodies (Invitrogen).

Transactivation evaluation in yeast

Transcriptional activation ability was determined either by measuring the β-galactosidase activity using ONPG as a substrate or evaluating growth ability on histidine-free media, as suggested by the manufacturer (Clontech).

Transient transformation of *Arabidopsis thaliana*

Five-day-old plants, grown on 0.25 × Murashige and Skoog basal medium supplemented with vitamins (MS, PhytoTechnology Laboratories™) plus 1 % sucrose, were used for *FAST transformation* according to Li et al. (2009). Briefly, *Agrobacterium tumefasiens* LBA4404 bacteria, previously transformed with the different constructs of interest were grown on YEB medium until an OD_{600} of 1.5. The cultures were centrifuged and washed with 10 mM $MgCl_2$, 100 µM acetosyringone and resuspended in the same solution. The transformation medium was prepared with 0.25 x MS, 1 % sucrose, 100 µM acetosyringone, 0.0025 % Silwett-77 and the bacterial culture (OD_{600} 0.5). *Arabidopsis thaliana* plants were infected with the transformation mixture in dark for 40 h. Then, the seedlings were washed once with 1 % bleach, three times with distilled water and finally harvested for RNA extraction and analysis.

Total RNA was extracted from seedlings with the TRIzol® reagent (Invitrogen™) and DNase-I treated (Promega, Madison, USA) according to the instructions of the manufacturer.

Total RNA (5 µg) were reverse transcribed using oligo$(dT)_{18}$ and M-MLV reverse transcriptase II (Promega). Quantitative RT-PCR (qRT-PCR) was performed with the Mx3005P Multiplex qPCR system (Stratagene, La Jolla, CA) in a 20 µl final volume containing 2 µl SyBr green (4×), 8 pmol of each primer, 2 mM $MgCl_2$, 10 µl of a 1/15 dilution of the RT reaction and 0,12 µl Platinum Taq (Invitrogen™). Fluorescence was measured at 78–80 °C

during 40 cycles. Specific primers were designed (Supplementary Table S1). Quantification of mRNA levels was performed by normalization with the actin and the GAL4BD::AtHBX mRNA levels according to the ΔCt method. All the reactions were performed with, at least, three replicates.

Expression and purification of recombinant proteins in E. coli

Expression of the recombinant proteins, GST::HD-Zip I was induced with 1 mM IPTG during 5 h at 28 °C. The cells were harvested and suspended in binding buffer (20 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 5 % glycerol, 1 mM DTT, 0.3 M ammonium sulfate and 1 mM PMSF), sonicated and purified through a glutathione-Sepharose (Amersham) column as suggested by the manufacturer.

Pull-down assays

The interaction of each HD-Zip protein with AtTBPII or AtTFIIB was tested as follows: cultures of yeast transformed with *pGADT7::GAL4AD::HA::AtTBP2* or *pGADT7::GAL4AD::HA::AtTFIIB* were incubated with the HD-Zip-GST fusions previously immobilized on glutathione-Sepharose during 2 h at 4 °C in 500 µl of binding buffer with continuous agitation. Then, the extracts were centrifuged and the proteins were released from the matrix by boiling the beads with SDS-PAGE loading buffer and electrophoresed on 12 % polyacrilamide gels. Detection of the bands were performed by western blot using mouse anti-HA antibodies (Invitrogen) and HRP-Rabbit anti-mouse antibodies (Invitrogen).

Results

The HD-Zip subfamily I transcription factors AtHB1, AtHB7, AtHB12 and AtHB13 act as activators in a yeast one-hybrid system

Previous reports indicated that AtHB1 and AtHB12 act as activators in yeast; it was demonstrated that the CTR, outside the conserved HD-Zip domain, is responsible for such ability (Arce et al. 2011; Lee et al. 2001). However, a comparison with other members of this family of TFs has not been tested. Aiming to answer if type I HD-Zip TFs are, in general, transcriptional activators or not, genetic constructs were prepared in which the coding regions of each of the four proteins (AtHB1, AtHB7, AtHB12 and AtHB13) were fused to the GAL4-binding domain (GAL4-BD). Yeast cells were transformed with these constructs, followed by *HIS3* gene activation analysis and β-galactosidase activity quantification.

A schematic representation of such constructs as well as the results can be observed in Fig. 1. AtHB13 showed the highest activity while AtHB12 was rather high too (compared with AtHB1/7). These results indicated that, on one side, all the four TFs are able to activate in yeast one-hybrid system and, on the other, the degree of activation capacity is rather different between them. To quantify more precisely the relationship between these activation capacities, we tried to detect the expressed proteins using anti-c-Myc antibody in western blots. Unfortunately, signals were very weak or directly null and so activation capacities were expressed as Miller units.

AHA-like motifs are necessary and sufficient for the activation ability of AtHB1, AtHB7, AtHB12 and AtHB13 transcription factors in yeast

Putative AHA motifs, located at the carboxy-terminal of the CTRs of HD-Zip subfamily I proteins from different species, were informatically detected (Arce et al. 2011; Supplementary Fig. 1). Consistently, transactivation activity has been associated to these four HD-Zip I TFs. Since this activity was dependent on each particular protein, we wondered which specific motifs were responsible for it. Several constructs were performed for each of the four HD-Zip I members selected (Fig. 2). In these constructs, successive and combined deletions of the putative AHA motifs or these motifs alone were fused to the GAL4 binding domain (BD), yeast cells were transformed, *HIS3* gene activation was analyzed and β-galactosidase activity was quantified.

AtHB1 presents two putative AHA motifs, located at positions 216 and 259 from the N-terminus, respectively. The LZ itself finishes at position 167. As it can be visualized in Fig. 2a, the deletion of the whole CTR of AtHB1 completely abolished the activation capacity and a similar result was obtained with the construct in which the last 48 aminoacids, containing both putative AHAs, were eliminated. The deletion of the last 12 aminoacids, containing one of the putative AHA motifs, caused a significant activity loss while the deletion of the last 33 residues, containing both putative AHAs and a linking segment between them, further diminished the activation level. Surprisingly, the deletion of only the second putative AHA (positions 216–227) did not considerably change the activation ability while the deletion of the intermediate region linking both AHAs produced a higher negative effect on transactivation, indicating that this linking segment may be important for the correct positioning and action of both AHA motifs providing an adequate distance between them or, alternatively, because it contains a relevant sequence

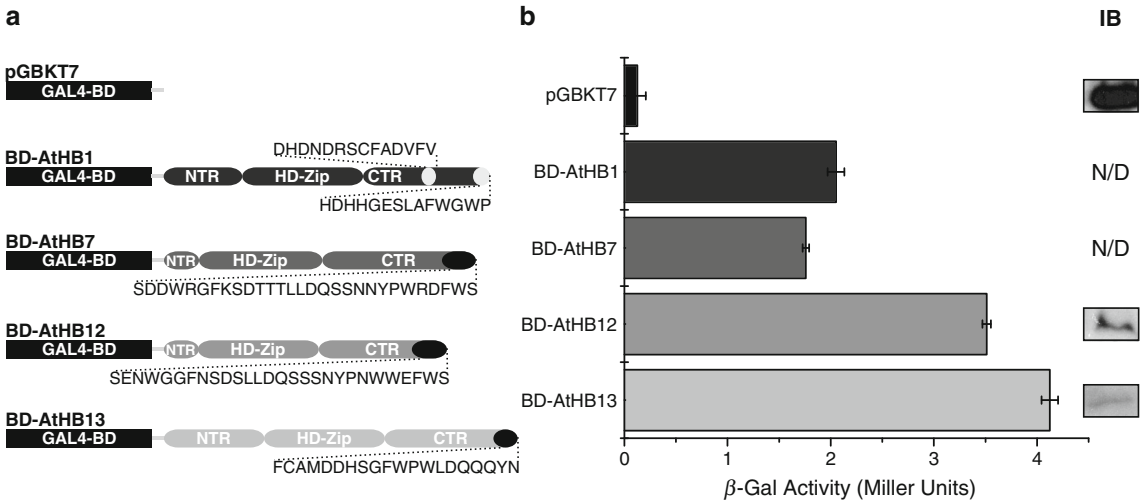

Fig. 1 AtHB1, AtHB7, AtHB12 and AtHB13 act as activators in a yeast one-hybrid system. **a** Schematic representation of the genetic constructs: the complete sequence of the four HD-Zip proteins were fused to the DNA-binding domain of GAL4 (GAL4-BD). The empty vector expressing only the GAL4-BD, pGBKT7, was used as negative control. **b** *β*-Galactosidase activity assay. The activity was quantified in Miller units and *error bars* represent standard deviation of three independent technical triplicates. *Right panel (IB)*: immunoblot signal obtained with anti-c-Myc after PAGE analysis

with activation ability unknown for other TFs. Expression levels of the mutated proteins could be detected in western blots showing differences when OD_{600} units were equilibrated. However, these differences strongly emphasized the conclusions since higher concentrations were observed in those constructs exhibiting the lowest activity (right panel in Fig. 2). Similar results were observed for the four TFs analyzed. Moreover, this disparity in protein concentration observed in the comparison of whole proteins and their mutants has already been reported in the literature for the HSF TFs (Döring et al. 2000; Kotak et al. 2004).

AtHB12 significantly lost its activation capacity when the last 8 aminoacids were eliminated and it was almost abolished when the deletion was of 27 aminoacids containing the whole putative AHA motif (Fig. 2b). Similar, but not equal, results were obtained after the analysis of AtHB7 constructs. For this TF, one AHA motif showed a significant role in transactivation activity; this motif is located between aminoacids 230 and 258 (Fig. 2c). Since AtHB7 and AtHB12 were considered as deriving from a unique gene duplication, this result was not surprising although some structural differences were detected between these two TFs (Arce et al. 2011).

AtHB13 showed the highest activity when compared with the other three members (Fig. 1). Only one putative AHA motif was detected in this protein and when it was deleted, consistently, almost all of the activity was lost, similar to what happened when the whole CTR was deleted (Fig. 2d).

The analysis of the putative isolated AHA peptides fused to the GAL4 BD indicated that all the four constructs were able to activate in the yeast one-hybrid system (Fig. 2), implying that these isolated motifs are responsible for the observed activation capacity. The AHA motif of AtHB1 and the whole protein showed similar activities, while for AtHB13 the isolated AHA displayed around half of the transactivation level of the entire TF. Surprisingly, the aminoacids 230–258 of AtHB7 and 208–235 of AtHB12 showed an enhanced activity compared to the complete TF.

AHA motifs present in HD-Zip I proteins are functional in plants

Transactivation needs the joint action of different proteins, including those of the basal transcriptional machinery. HD-Zip subfamily I TFs exhibited such activity in yeast, as it was described above, but we wondered if these AHA motifs were or not functional in plants. Similar constructs to those tested in yeast were cloned in vectors suitable for plant transformation and *Arabidopsis* individuals were transiently co-transformed with them and with a construct bearing the GAL4 target sequence fused to *GFP* and *GUS* reporter genes. *GUS* transcripts were quantified by qRT-PCR and the results shown in Fig. 3. In contrast with the results for AtHB1 in yeast, the deletion of any of the segments having a putative AHA motif or the linking region resulted in a complete loss of activity (Fig. 3a). AtHB12 retained around 25 % of the activity when the last 9 or 29 aminoacids were eliminated while the deletion of the whole CTR showed a more dramatic loss. On the other hand, AtHB13 lost almost all the activation capacity when

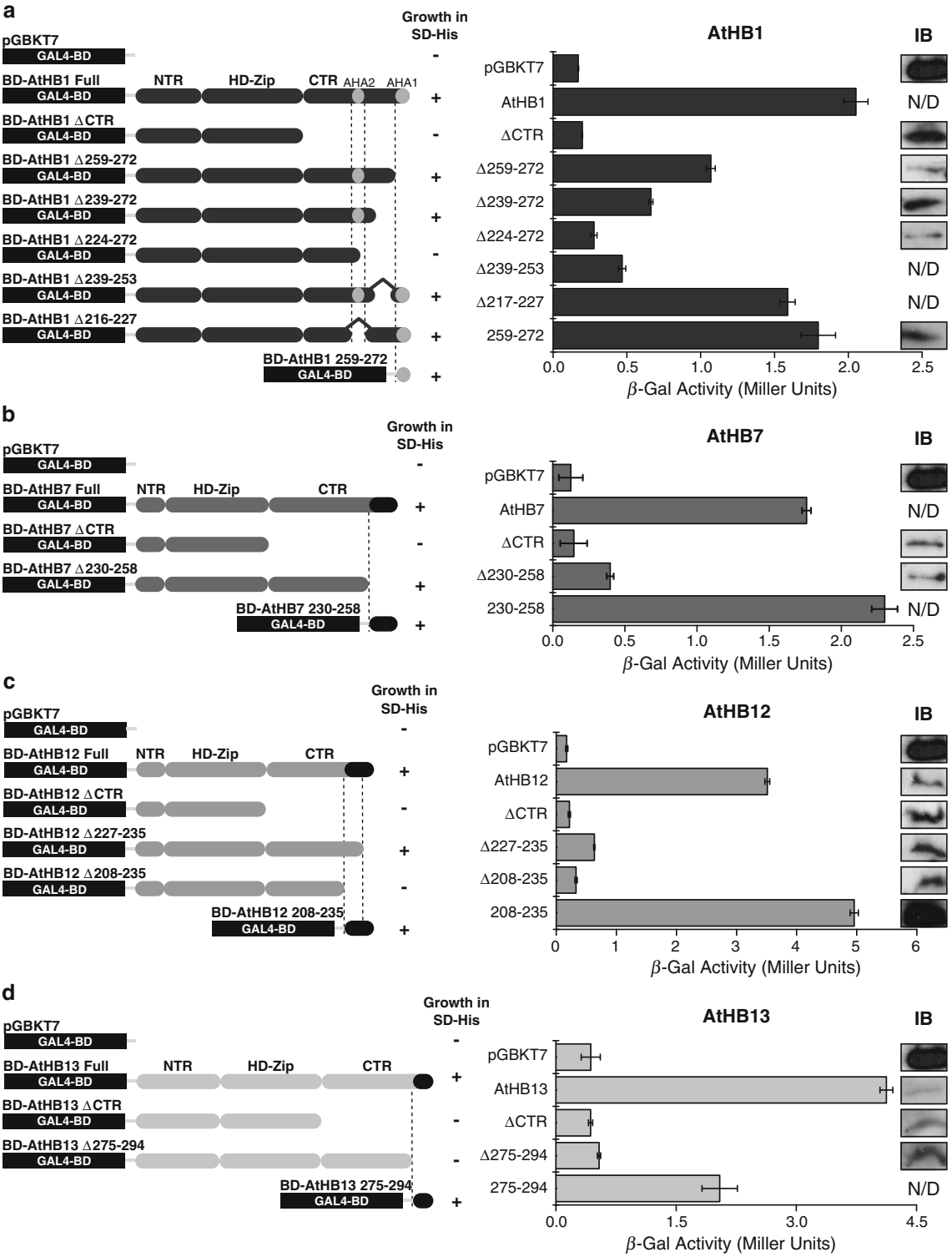

Fig. 2 AHA-like motifs are necessary for the activation ability of HD-Zip I transcription factors in yeast. *Left panels*: schematic representation of the genetic constructs tested in the yeast one-hybrid system and, on the right of each construction, the indication of growth in the selective medium (+, growth on SD-His plates; −, no growth). The complete and mutated sequences of the four HD-Zip proteins (**a** AtHB1, **b** AtHB12, **c** AtHB7, **d** AtHB13) were fused to the DNA-binding domain of GAL4 (GAL4-BD). The empty vector expressing only the GAL4-BD, pGBKT7, was used as negative control. *Right panels*: β-galactosidase activity assay. The activity was quantified in Miller units and *error bars* represent standard deviation of three independent technical triplicates. *Right panel (IB)*: immunoblot signal obtained with anti-c-Myc after PAGE analysis. **a** AtHB1, **b** AtHB12, **c** AtHB7, d AtHB13. *N/D* not detected, *AHA* aromatic and large hydrophobic residues embedded in an acidic context

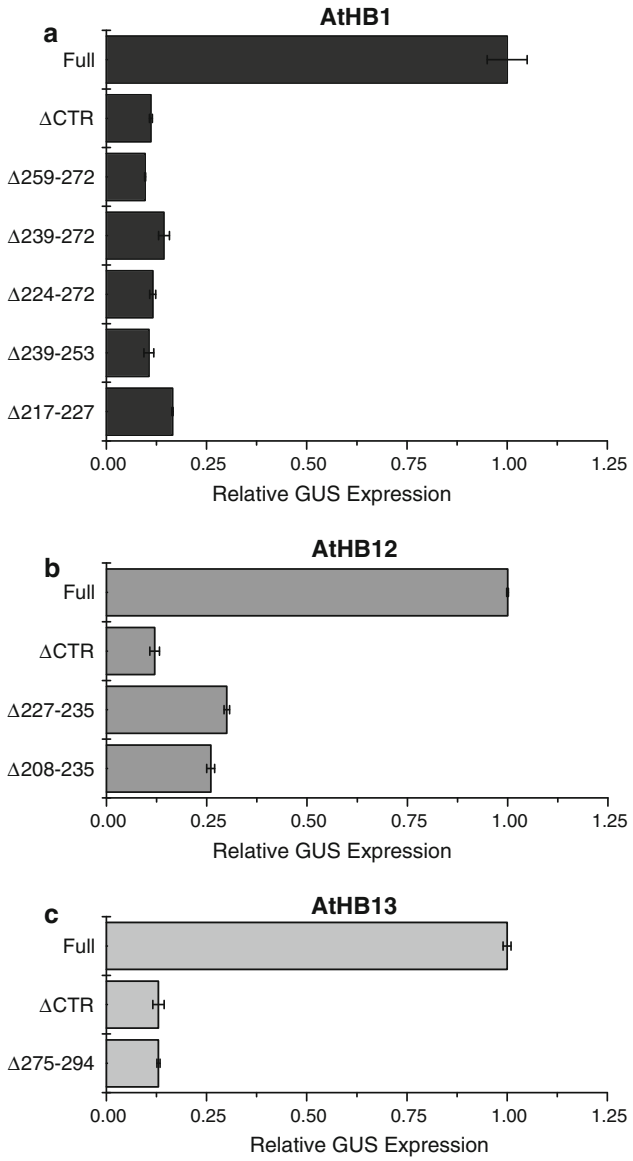

Fig. 3 AHA motifs of AtHB1, AtHB12 and AtHB13 are functional in plants. *Arabidopsis* seedlings were transiently co-transformed with constructs bearing *35S::GAL4BD::HD-Zip* (**a** AtHB1, **b** AtHB12, **c** AtHB13) and *pMC::GAL1::GFP::GUS*. *β*-Glucuronidase transcript levels were expressed as a proportion of that of the construct bearing the full transcription factor, arbitrary given a unitary value. *Error bars* represent standard deviation of three independent biological triplicates. *AHA* aromatic and large hydrophobic residues embedded in an acidic context

the only putative AHA (the last 19 aminoacids) was eliminated.

The role of the tryptophan residues in the HD-Zip I AHA motifs

AHA motifs were first described 20 years ago in HSF (Heat Stress Factors) TFs as motifs presenting aromatic residues, generally one or more tryptophans (W), and large hydrophobic residues embedded in an acidic context (Treuter et al. 1993). Tryptophans were acknowledged as important contributors to the activation ability and thus, we investigated the role of these aminoacids in the HD-Zip I AHAs. AtHB1, like other members of the same clade (Arce et al. 2011), exhibits the motif F**W**G**W**P within the AHA while AtHB13 belonging to group V, exhibits a F**W**P**W**L motif in the distal region of their CTRs. Genetic constructs in which distinct aminoacids were mutated to alanine (A) were performed and used to transform yeast. Figure 4 shows the results obtained.

The mutation of E264, F268, G270, W269 or W271, or both W to A in AtHB1 did not affect its ability to activate in yeast. Only the deletion of the complete CTR generated such effect, indicating that at least in yeast, these aminoacids are not essential to confer the activation capacity. On the other hand, the mutation of S282 and F284 or D289 in the CTR of AtH13 did not considerably modify its activation level, but the mutation of both W to A was sufficient to significantly diminish the activity. These results indicate that these residues play a crucial role in the structure/function relation of AtH13, but they are not the only determinants since the activity was not completely abolished.

In contrast, the effect of the W to A mutation in plants was the complete loss of activation capability, with the exception of the construction BD-athb1 W271A which retained a 30 % of activity. This suggested the existence of partially differing mechanisms of action of these TFs when expressed in the heterologous or the homologous systems.

AtHB1, AtHB7, AtHB12 and AtHB13 differentially interact with components of the basal transcriptional machinery

Transactivation frequently occurs via the interaction of a particular domain or motif of the transcription factor with a component of the basal transcriptional machinery. The TATA binding protein, TBP, and TFIIB are major components of such machinery. It has been shown that these components interact with the acidic activation domain (AHA-like motif) of VP16, a TF that exhibits also a disordered CTR (Stringer et al. 1990; Ingles et al. 1991; Lin et al. 1991; Shen et al. 1996). Aiming to test if AHA motifs present in the HD-Zip subfamily I proteins were interacting with one of these major basal components, genetic constructs were prepared bearing the cDNA of AtTBP2 (At1g55520) and AtTFIIB (At2g41630), respectively, fused to the activation domain of GAL4 (AD) and used separately to co-transform yeast with the four selected HD-Zip proteins fused to the DNA-binding domain of GAL4 (BD). The results obtained are shown in Fig. 5.

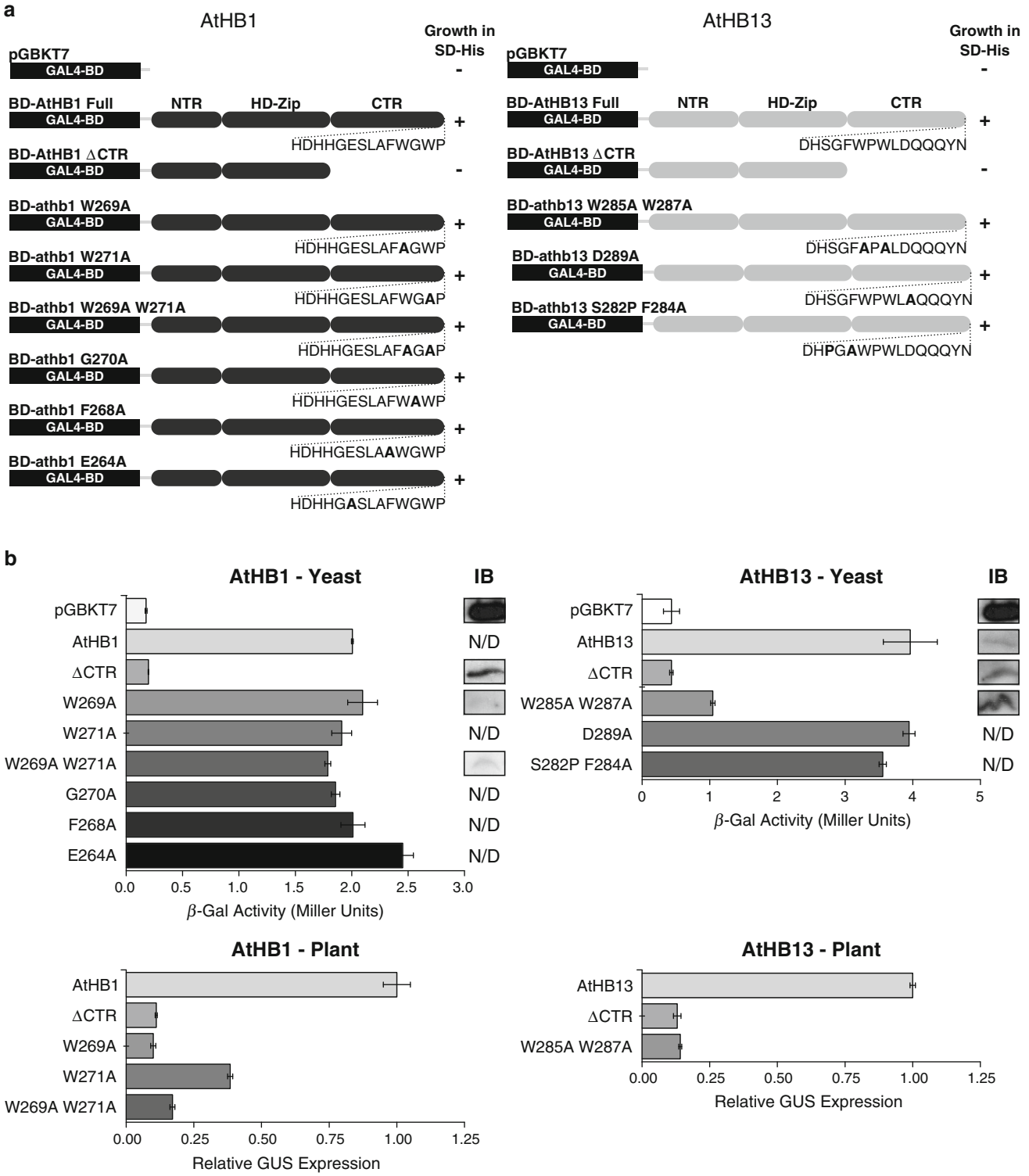

Fig. 4 Tryptophans in the AHA motifs play different roles in the HD-Zip I transcription factors analyzed. **a** Schematic representation of the genetic constructs tested in yeast and plant one-hybrid system and, on the *right* of each panel, the indication of growth in the selective medium (+, growth on SD-His plates; −, no growth). The complete, simple and double tryptophan mutated sequences of two HD-Zip proteins were fused to the DNA-binding domain of GAL4 (GAL4-BD). The empty vector expressing only the GAL4-BD, pGBKT7, was used as negative control. **b** β-Galactosidase activity assay and β-glucuronidase transcript levels in yeast and plants of the different constructs. The expression level of *GUS* was expressed as a proportion of that of the construct bearing the full transcription factor, arbitrary given a unitary value. *Right panel (IB)*: immunoblot signal obtained with anti-c-Myc after PAGE analysis. All the assays represent triplicates and were repeated at least three times. *N/D* not detected, *AHA* aromatic and large hydrophobic residues embedded in an acidic context

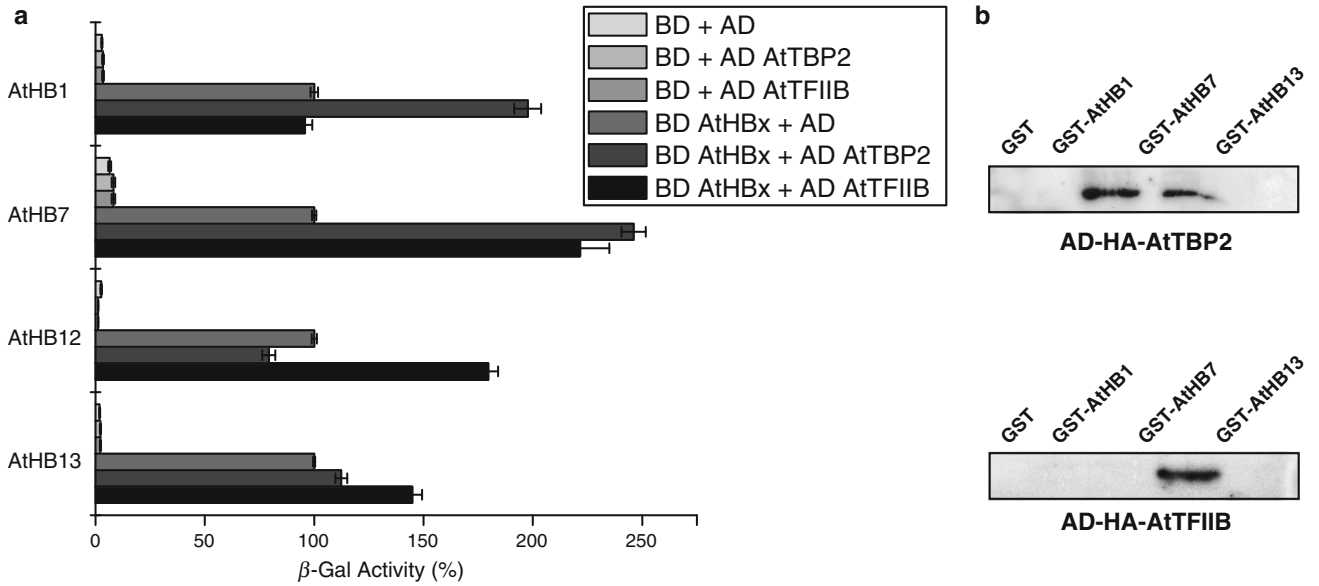

Fig. 5 AtHB1, AtHB7, AtHB12 and AtHB13 differentially interact with the basal transcription factors TBP and TFIIB. **a** β-Galactosidase activity assay. Full HD-Zip I proteins were fused to the binding domain (BD) of GAL4 while TBP and TFIIB were fused separately to the activation domain (AD) of GAL4 and a yeast two-hybrid system was used to test the interaction. The activity was expressed as a percentage of that of the construct bearing the full transcription factor fused to the DNA-binding domain (BD) of GAL4, arbitrary given a value of 100 %. *Error bars* represent standard deviation of three independent technical triplicates. **b** GST pull-down assay. Full-length AtHB1, AtHB7 and AtHB13 fused to GST were tested for interaction with AtTBP2 and AtTFIIB in pull-down assays

For the yeast two-hybrid assay, negative controls were performed using the BD co-transformed with the AD alone or fused to AtTBP2 or to AtTFIIB. AtHB7 and AtHB1 had twice the activation level when co-transformed with AD-AtTBP2 than when they were alone while AtHB12 and AtHB13 did not show significant differences, indicating that these TFs do not interact with TBP to exert their activity (Fig. 5a). The co-transformations with AD-TFIIB indicated clear interactions between this basal component with AtHB7 and AtHB12, no interaction with AtHB1 and a weak interaction with AtHB13.

These interactions were also tested in vitro. Three HD-Zip TFs were cloned as fusions with GST and expressed in bacteria. After induction with IPTG, the bacteria were lysed and the proteins bound separately to a glutathione-Sepharose matrix. Yeast extracts containing chimerical proteins of AD fused to hemagglutinin (HA) and TBP or TFIIB were passed through the matrix containing the GST fusions, and the eluates separated by PAGE. Western blots were performed to detect the interactions using anti-HA antibodies elicited in mouse. The results are shown in Fig. 5b and confirmed the results previously obtained in the yeast two-hybrid system.

Aiming to answer if the AHA motif present in AtHB1 is necessary for the interaction with AtTBP2, the constructs of AtHB1Δ259–272 and athb1-W269A-W271A fused to GAL4 binding domain were co-transformed with AD-AtTBP2 and the β-galactosidase activity was quantified. The yeast two-hybrid assays indicated that the mutation of W269 and W271, or the deletion of the segment comprised between positions 259 and 272, completely abolished the interaction between AtHB1 and AtTBP2 (Supplementary Fig. 2).

In conclusion, these HD-Zip TFs are capable of interacting with the basal transcriptional machinery but each protein establishes different interactions, which could partially explain the functional divergence of these proteins, as was previously proposed (Arce et al. 2011).

Discussion

HD-Zip subfamily I transcription factors were first identified in *Arabidopsis* (Ruberti et al. 1991; Schena and Davis 1992). Since this first identification, performed when the *Arabidopsis* genome was still unknown, the knowledge about these regulatory proteins has significantly increased. The 17 members of *Arabidopsis* have been classified in six groups according to phylogenetic relationships and gene structure, including introns number and location (Henriksson et al. 2005). More recently, a new phylogenetic reconstruction with 178 HD-Zip subfamily I proteins from different species was performed (Arce et al. 2011). In this case, *Arabidopsis* members were classified in five groups, defined considering the presence of conserved motifs in the CTRs. We decided to use the latter classification since it

was obtained with a larger number of sequences and it took into account the potential functionality of the CTRs.

Expression patterns of these members in front of some abiotic stress factors were determined showing that most of them are similarly regulated. The characterization of knockout and overexpressor plants involving HD-Zip proteins from several species indicated that each member takes part in different signal transduction pathways. These observations led us to hypothesize that other features besides the high conservation in the HD-Zip domain were responsible for the divergence in their functionality. Applying bioinformatics and experimental approaches in yeast and plants, we put in evidence the important role played by motifs present in the CTRs of HD-Zip I TFs (Arce et al. 2011). Among other putative motifs, AHA-like activation sites were detected at the carboxy-terminal of the CTRs. This type of sites was first described by Treuter et al. (1993) for the HSF TFs from tomato.

The importance of AHA motifs in HD-Zip TFs *in planta* has been indirectly demonstrated in the previous studies. Sakuma et al. (2010) identified *HvHox2*, a putative paralog of the gene *VRS1* known for being responsible for the six-rowed phenotype in barley plants bearing the recessive allele *vrs1*. These two genes, both encoding HD-Zip I proteins, differ particularly in their CTR. HvHox2 exhibits 14 additional amino acids compared with VRS1, which form a conserved motif when compared with proteins from other species. When we examined the sequence of this motif we noticed that it exhibits the characteristics of an AHA motif.

The HD-Zip I Tendril less (TL) mutant from garden pea has been shown to generate plants in which leaflets take the place of tendrils. This phenotype has been also observed when the gene codes for a protein lacking 12 amino acids (Hofer et al. 2009). Notably, these 12 aminoacids contain an AHA-like motif.

Here, we demonstrated that putative AHA motifs are functional activation motifs for the four HD-Zip I tested. However, the activation ability as well as the functionality of the W in these proteins showed marked differences. In addition, differences were observed for the same AHA motif between plant and yeast tests.

In this sense, AtHB1 almost completely lost its ability to activate in plants when the last 12 aminoacids were deleted while in yeast a deletion of 48 residues or the whole CTR was necessary to obtain the same result. It is tempting to speculate that the transcriptional activity of AtHB1 in yeast could be exerted through less specific protein–protein interactions. A similar observation was performed for AtHB13. In contrast, AtHB12 without any of its putative AHAs retained about 25 % of the activation in plants, as was also found in yeast.

A similar scenario was observed when mutations of different aminoacids present in the AHA motifs were evaluated. Several AtHB1 point mutants did not change the ability of this TF to activate in yeast; however, the effect in plants of W to A mutations was almost a total loss of this ability. In the case of AtHB13, the results in yeast and plants were closer but still exhibited a discrepancy with W285 and W287; once again, the effect of the mutation was severe in plants but not so severe in yeast. These results highlighted that the useful heterologous yeast system cannot always be directly extrapolated to the plant system. The differences observed between these two systems strongly suggest that the proteins that interact with HD-Zip subfamily I TFs in yeast and in plants are not functionally identical, thus, their sensitivity to W mutations is variable. Similar discrepancies between yeast and plants have been reported by Meijer et al. (2000). These authors showed the rice HD-Zip subfamily II member, Oshox1, acts as a repressor in rice and as an activator in yeast. Further work must be carried out to elucidate these points.

In spite of these discrepancies, the four HD-Zip TFs analyzed behaved as activators both in yeast and in plants. A study performed by Ohta et al. (2000) with ERF TFs showed activation activity for ERF2 and ERF4 in yeast and plants and repressor activity for ERF3 in plants which could not be evaluated in the yeast system. The yeast system for transactivation assays of plant TFs has been largely used and validated. For example, this activity was described for APETALA1 using this system exclusively (Cho et al. 1999). No contradictory results were observed for the HD-Zip TFs tested here between yeast and plants but the differences noted must be carefully analyzed and, taking into account that the plant system tested is homologous, the results obtained in it are more trustworthy.

Analogous results were obtained with *Arabidopsis* HSFs TFs, particularly with class A members (Kotak et al. 2004). These proteins presented different activation capabilities among members and had similar, but not identical behaviors in yeast and tobacco protoplast assays in relation to the functionality and number of AHA motifs.

We also demonstrated that AtHB1, AtHB7 and AtHB12 could differentially interact with two basal transcription factors, AtTBP2 and AtTFIIB. This capacity might indicate that transcriptional activation exerted by these HD-Zip TFs may be through directly recruiting the basal transcriptional machinery to the promoter of a target gene and/or stimulating transcription. This ability has also been attributed to several transcriptional activators (Pugh 1996).

In a previous study, a putative ortholog of AtHB7 and AtHB12 from *Medicago truncatula*, MtHB1, was shown to repress the transcription of its target *MtLBD1 in planta* (Ariel et al. 2010). Recently, it was also shown that the HD-Zip I AtHB5 negatively regulates *BODENLOS* expression (De Smet et al. 2013). These results put in evidence that HD-Zip I TFs may not only exhibit activation

capability as it was shown in this and previous works (Lee et al. 2001), highlighting the complex and still poorly understood mechanisms of action of these TFs.

In conclusion, the four HD-Zip proteins analyzed behave as activators. Each of these proteins presents a peculiar arrangement of AHA motifs and W play different roles but seem to be very important in all cases to their functionality in plants. In addition, each TF interacts with different components of the basal machinery to exert its function. It is tempting to speculate that the divergence in the CTRs is partially responsible for the different functions assigned to these TFs in part through the interaction with different partners. Future effort will be focused on identifying such partners.

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