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A matter of quantity: Common features in the drought response of transgenic plants overexpressing HD-Zip I transcription factors☆

Facundo Romani, Pamela A. Ribone, Matías Capella, Virginia N. Miguel, Raquel L. Chan*

Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral, CONICET, Colectora Ruta Nacional 168 km 0, 3000, Santa Fe, Argentina

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ABSTRACT

Plant responses to water deficit involve complex molecular mechanisms in which transcription factors have key roles. Previous reports ectopically overexpressed a few members of the homeodomain-leucine zipper I (HD-Zip I) family of transcription factors from different species, and the obtained transgenic plants exhibited drought tolerance which extent depended on the level of overexpression, triggering diverse molecular and physiological pathways. Here we show that most HD-Zip I genes are regulated by drought in the vegetative and/or reproductive stages. Moreover, uncharacterized members of this family were expressed as transgenes both in Col-0 and *rdr6-12* backgrounds and were able to enhance drought tolerance in host plants. The extent of such tolerance depended on the expression level of the transgene and was significantly higher in transgenic *rdr6-12* than in Col-0. Comparative transcriptome analyses of *Arabidopsis thaliana* plants overexpressing HD-Zip I proteins indicated that many members have common targets. Moreover, the water deficit tolerance exhibited by these plants is likely due to the induction and repression of certain of these common HD-Zip I-regulated genes. However, each HD-Zip I member regulates other pathways, which, in some cases, generate differential and potentially undesirable traits in addition to drought tolerance. In conclusion, only a few members of this family could become valuable tools to improve drought-tolerance.

1. Introduction

Gene expression in plant cells is mainly governed by transcription factors (TFs). These regulatory proteins have a minimum of two domains: a DNA binding domain and a second domain that interacts with other proteins, including other TFs. The DNA binding domain recognizes and binds specific DNA sequences in the regulatory regions of their targets to activate or repress entire signal transduction pathways, changing the whole plant cell transcriptome.

In Arabidopsis, 5–10% of genes (depending on the consulted database) encode TFs, indicating that transcription is a key step in the regulation of gene expression [1]. These TFs were classified into families and subfamilies according to the structural features of their binding domains, gene structure, and presence of additional domains, motifs and functions. One of the largest TF families is the homeodomain (HD) family, which is characterized by the presence of a HD binding domain. The HD consists of a conserved 60-amino-acid motif folded into a bundle of three alpha helixes (I, II and III) joined by two loops and a turn. The domain interacts with the DNA through the high affinity interactions established by helix III (called the recognition helix) [2].

1.1. The homeodomain-leucine zipper I subfamily

In plants, HD-containing TFs were classified into several subfamilies, which were named according to the different motifs that are present in addition to the conserved homeodomain, PHD finger, ZF-HD, WOX, the TALE superclass (BELL and KNOX), DDT, NDX, LD, PINTOX, SAWADEE, and HD-Zip [3,4], and participate in a wide variety of developmental processes and responses to environmental conditions.

In the homeodomain-leucine zipper (HD-Zip) family, the HD binding domain is associated with a leucine zipper (LZ) domain, an association that is unique to plants even when both isolated domains are present in TFs of other kingdoms. The LZ, located immediately downstream of the HD, is a structure that folds into an alpha helix with a leucine in each seventh position, and allows the formation of homo- or heterodimers [5,6]. These TFs were found

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Abbreviations: HD-Zip, homeodomain-leucine zipper; ABA, abscisic acid; TF, transcription factor; OE, overexpressor.

* Corresponding author.
E-mail addresses: rchan@fbcb.unl.edu.ar, ial@santafe-conicet.gov.ar (R.L. Chan).

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in *Physcomitrella patens* and in all of the mono and dicot species in which they were searched for.

The HD-Zip proteins were classified into four subfamilies, I to IV, whose members differ in size and in the presence of other motifs in addition to the conserved HD-Zip, as well as in the responses and developmental pathways in which they are involved [7–9].

The HD-Zip I subfamily, whose members have a size of approximately 35 kDa, is composed of 17 members in *Arabidopsis thaliana* (*AtHB1/HAT5*, *AtHB3/HAT7*, *AtHB5–AtHB7*, *AtHB12*, *AtHB13*, *AtHB16*, *AtHB20–AtHB23*, *AtHB40*, and *AtHB51–AtHB54*) and 14 members in *Oryza sativa* (*OsHox4- OsHox6, OsHox8, OsHox12-OsHox14, OsHox16, and OsHox20-OsHox25* [7,10–12]. Using *in vitro* assays that were corroborated by *in vivo* assays in a few cases, it was shown that most HD-Zip I proteins bind the pseudopalindromic sequence CAAT(A/T)ATTG and that the dimerization through the LZ domain is absolutely required for DNA binding [13–15].

1.2. Expression patterns of HD-Zip I transcription factors

The expression patterns of many HD-Zip I TFs have been determined, particularly those from Arabidopsis. *AtHB1*, *AtHB5*, *AtHB6* and *AtHB16* were expressed in all plant organs. However, salinity stress and low temperatures down regulated *AtHB1*, *AtHB5* and *AtHB16* expression, whereas *AtHB6* expression was induced by the same factors [16,17]. Some HD-Zip I-encoding genes were regulated by illumination conditions, such as *AtHB1* and *AtHB16*, which were induced by darkness, and *AtHB16*, which was repressed by blue light, whereas other genes were unaffected [16].

AtHB5 and *AtHB20* were repressed during seed formation and then highly induced after one-two days of imbibition [18,19].

AtHB13 and *AtHB23* are paralogous genes that are expressed in the shoot meristem region; leaf junction; the basal part of the petals, sepals and stamens; and within the stigma [20,21]. However, their functions are different; *AtHB13* is essential for pollen hydration, whereas *AtHB23* has no defined function in this developmental process. Nevertheless, both TFs have a defined function as negative regulators of inflorescence stem elongation [22]. *AtHB12* and *AtHB7* are another pair of paralogous genes that exhibited clearly different expression patterns during development [23].

1.3. How do water deficits and excess affect HD-Zip I expression in model and non-model plant species?

The expression of genes encoding HD-Zip I TFs was affected by water availability and other environmental conditions. In particular, the expression of Arabidopsis HD-Zip I genes was analyzed in seedlings subjected to various environmental stresses [16]. Moreover, the expression of most of the studied members of this TF family was regulated in response to drought in different species [24–32]. The effects of a water deficit have been analyzed more often than the effects of water excess, likely because drought is a more harmful stress and produces enormous crop losses worldwide.

The Arabidopsis *AtHB12* and *AtHB7* genes and their rice homologous *OsHox22* and *OsHox24* were upregulated by ABA and water deficit [10,23,26,33–35]. *AtHB5* and *AtHB6* have also been shown to positively and negatively regulate the ABA responses, respectively [19,36].

The expression of the homologs *AtHB13* from Arabidopsis and *HaHB1* from sunflower was induced in response to drought, whereas *AtHB23*, which is considered to be the paralog of *AtHB13*, was down regulated by ABA and NaCl treatments [16,29,37].

Regarding the HD-Zip I TFs from non-model species, 21 out of 23 members of the soybean HD-Zip I genes identified by Chen et al. [38] were described as regulated by drought or salinity stresses. Later, a deeper analysis conducted by Belamkar et al. [39] detected 36HD-Zip I encoding genes. Moreover, a transcriptome study of soybean plants treated with different drought stress conditions during variable times conducted by the same authors indicated that several members showed a regulation by such stress conditions. A comprehensive survey of maize members showed that most of them were up or down regulated by water availability [39]. The *Nicotiana attenuata NaHD20* HD-Zip I TF was also related to ABA-mediated signaling of the water stress responses [40,41].

The resurrection plant *Craterostigma plantagineum* has four identified HD-Zip I TFs (CpHB4 to 7), and all them were regulated by drought and ABA; *CpHB6* and *CpHB7* expression was induced, whereas *CpHB4* and *CpHB5* expression was repressed [42]. The sunflower divergent members of clade I *HaHB4* and *HaHB11* were responsive to drought, and only the latter was responsive to flooding [25,43,44]. Unfortunately, no other members of this TF family were investigated with respect to flooding stress, including waterlogging and submergence; however, it is tempting to speculate that additional members from other species also have a role in such responses.

1.4. Ectopic expression and/or overexpression of HD-Zip I proteins in transgenic plants produced improved drought tolerance via different signal transduction pathways

Several Arabidopsis HD-Zip I TFs were functionally characterized using loss of function mutants. In most cases, significant morphological or developmental differences were not observed [45,46]; however, differential ABA sensitivity and other developmental characteristics, such as defects in pollen hydration and changes in hypocotyl elongation, were detected in these mutants [8,19,22].

On the other hand, HD-Zip I proteins have been ectopically expressed and/or overexpressed in *Arabidopsis thaliana* and other transformable species, and most, but not all, cases exhibited improved drought tolerance. Examples of such transgenic plants are those transformed with *AtHB6* and *CpHB7* from *C. plantagineum*, which reduced stomatal closure and the ABA-induced inhibition of germination [28,36].

HaHB1, *HaHB11* and *HaHB4* from sunflower were also ectopically expressed in transgenic Arabidopsis plants, causing tolerance to water deficits which extent depended on the expression level of the transgene [37,47,48]. However, these plants triggered different molecular and physiological mechanisms to address this stress. These mechanisms included increased membrane stability, main root elongation and senescence delay [25,29,49,48,50–52].

Other examples of drought tolerance generated by HD-Zip I TFs were described in patent applications or granted patents [24,25,31,51,52]. The ectopic and overexpression of the Arabidopsis *AtHB12* gene [52], the rice *OsHox5* gene and its homologues [31], the *Coffea arabica CaHB12* gene [24] and the maize ZmHdzip10 [27] and ZmHdzip4 [32] enhanced tolerance to drought, salinity stress or general abiotic stresses.

Notably, in the few cases in which the mechanisms of drought tolerance conferred by HD-Zip I TFs were analyzed, such mechanisms exhibited different features. Some of them were ABA-dependent, whereas others were independent of the stress hormone. In some, stomata closure was the first response, while in others, it was not. Increased membrane stability was described in transgenic *HaHB1* or *AtHB13* plants, but did not exhibit the increased biomass and yield as *HaHB11* plants did [25,29,37].

It is important to note that drought tolerance was not assessed or described in several reports about HD-Zip I overexpressor plants (OE). However, there are at least two reports in which transgenic plants overexpressing a HD-Zip I did not exhibit drought tolerance; transgenic wheat, *Triticum aestivum*, overexpressing *TaHDZip*I-4 did not show improved tolerance, increased yield or other

beneficial agronomic trait [53]. Notably, the overexpression of the same wheat gene in transformed barley generated an aberrant phenotype characterized by notably smaller plants. In this case, drought tolerance was not tested because of the significant differences in the plants development and size [53]. Moreover, *OsHox22* OE rice plants exhibited more sensitivity to water deficits than control plants [54].

Even when these types of experiments contributed to our understanding of the functions of HD-Zip I TFs, it is necessary to be cautious in their interpretation because the observations may or may not be related to the native function of the gene within the source plant.

1.5. The importance of the conserved motifs present in the carboxy-termini of HD-Zip I proteins

As most HD-Zip I proteins bind *in vitro* the same pseudopalindromic DNA sequence CAAT(A/T)ATTG [7] and, in some cases, it was demonstrated that this sequence is also bound *in vivo* [15], we wondered why these proteins triggered different pathways upon overexpression. Experimental evidence from different research groups throughout the world proposed that the different carboxy-termini and uncharacterized motifs inside them (phosphorylation, sumoylation, transactivation, etc.) may have been partially responsible for the different phenotypes conferred to the transgenic OE [54–57]. However, among these motifs present in the carboxy-termini, only the transactivation property of a few Arabidopsis members was experimentally demonstrated (AtHB1, AtHB12, AtHB7, and AtHB13 [55,58]). This transactivation has been assigned to AHA-like motifs, i.e., aromatic and large hydrophobic residues embedded in an acidic context [55], which were first described in tomato HSF TFs [59,60].

Phylogenetic reconstructions of entire proteins, including the uncharacterized carboxy-terminal domains (CTDs), rather than only the conserved HD-Zip domain, separated the HD-Zip I proteins into six groups, I to VI [56]. To date, it has been suggested that the combination of expression patterns and the divergence of the conserved motifs present in the carboxy-termini of HD-Zip I TFs to promote interactions with different partners would determine the differential functions exerted by each of these proteins *in vivo*. As drought tolerance is exhibited by most HD-Zip I OE, our hypothesis was that these TFs have common targets that are responsible for this trait. The different pathways induced by these TFs could be a consequence of individual non-shared targets. To test this hypothesis, we analyzed the expression patterns of Arabidopsis members after severe drought treatments and compared the transcriptomes of OE plants. Although the publicly available microarray and RNA-Seq data from different research groups used OE plants and RNA from different developmental stages, growth conditions and tissues, they were still valuable tools for exploring the common pathways regulated by HD-Zip I TFs.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Heyhn ecotype Columbia (Col-0) seeds were grown directly on soil in a 22–24 °C growth chamber under a long-day photoperiod (16 h of illumination with a mixture of cool-white and GroLux fluorescent lamps, Sylvania, Madrid, Spain) at an intensity of approximately 150 $\mu E\, m^{-2}\, sec^{-1}$ in 8-cm diameter, 7 cm high pots for the time periods indicated in the figures. The *AtHB1* OE and mutant plants in Col-0 background as well as *HaHB4* and *HaHB1* OE plants were previously described [8].

Mutant *rdr6-12* (CS24286) plants were ordered from Arabidopsis Biological Resource Center (ABRC) stock.

2.2. Stable transformation of *Arabidopsis* rdr6-12 plants

Transformed *Agrobacterium tumefaciens* (LBA4404 strain) were used to transform Arabidopsis *rdr6-12* mutant plants by the floral dip procedure [61] with *35S:AtHB1* [56] and *35S:AtHB12* [23] constructs. Transgenic plants were selected on the basis of their resistance. Lines with one copy of the transgene were selected by segregation and two homozygous independent lines were used for further assays.

Transcript levels of the transgenes were evaluated by RT-qPCR. Samples were prepared from plants normally grown for 4-weeks. Two half leaves (leaves 11 and 12) from each plant were harvested and processed for RNA extraction. Four biological replicates were used for each RT-qPCR experiment; each replicate contained leaves from four different plants.

2.3. Water stress treatments

Arabidopsis thaliana ecotype Columbia (Col-0), *rdr6-12* mutant and transgenic plants grown as described above were subjected to different drought treatments.

Stress applied during the vegetative stage: the pots were saturated with water when the seeds were sown and no additional water was added until severe damage was observed (approximately 20 days later). Two leaves (leaves 7 and 8) from each plant were harvested and processed for RNA extraction. Four biological replicates were used for each RT-qPCR experiment; each replicate contained leaves from two different plants.

Stress during the reproductive stage: the plants were grown as described above and normally irrigated during the first 4 weeks; then, the irrigation was completely stopped. Samples were harvested (one rosette leaf per sample, leaf 11 or 12) ten days later. Four biological replicates were used for each RT-PCR experiment. Normally irrigated plants were used as controls for both treatments.

To evaluate survival rate after severe drought stress treatments, *AtHB1* OE and mutant plants in Col-0 background and *AtHB1* and *AtHB12* OE plants in *rdr6*-12 background were grown as described above for 3 weeks with normal watering, and then water stress applied by completely stopping the irrigation. When severe damage was observed, plants were watered again. Survival rate were evaluated as the percentage of plants able to recover themselves seven days after irrigation was restarted. Eight pots with four plants per genotype were used. A Binomial test were applied to calculate confidence intervals, and an ANOVA, followed by Tukey *post hoc* test was used for statistical significance.

2.4. Water loss and consumption measurements

For water loss, the *Arabidopsis thaliana AtHB1* OE and mutant plants were grown as described above for 3 weeks with normal watering, and then subjected to the water stress treatment by completely stopping the irrigation. Next, at least three rosette leaves from different pots considered as biological replicates were detached and weighed to assess the water loss at the periods indicated in the figure, as described in Jakab et al. [62].

For water consumption, the *AtHB1* OE and mutant plants were grown as described above. Then, watering was stopped until a mild drought stress level was reached, and a constant weight was maintained in all of the pots by adding water every two days as described in Cabello et al. [29]. The volume added to each pot/day was registered for 23 days and the average/day/genotype is shown in a graph in Fig. 4.

Four pots with four plants per genotype, as indicated in the figures, were used for water loss and water consumption experiments. An ANOVA, followed by Tukey *post hoc* test was used for statistical significance.

2.5. Evaluation of the transcript levels by RT-qPCR

Total RNA was purified from Arabidopsis leaves using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. One μg of RNA was reverse-transcribed using oligo$(dT)_{18}$ and M-MLV reverse transcriptase II (Promega). Quantitative real-time PCR (qPCR) was performed using a Mx3000 P Multiplex qPCR system (Stratagene, La Jolla, CA); each reaction contained a final volume of 20 μl that included 2 μl of SyBr green (4×), 8 pmol of each primer, 2 mM $MgCl_2$, 10 μl of a 1/15 dilution of the RT reaction and 0.1 μl of Taq Polymerase (Invitrogen). Thermocycler parameters were as follows: 94 °C 12″, 60 °C 12″, 72 °C 12″. Fluorescence was quantified over 40 cycles at 72 °C. Specific primers for each gene were designed and are listed in Supplementary Table 1. The mRNA levels were quantified by normalizing their levels to the levels of the *Actin* transcripts (*ACTIN2* and *ACTIN8*) using the ΔΔCt method. All of the reactions were performed with four biological replicates. An ANOVA, followed by Fisher's LSD *post hoc* test were used for the statistical analysis.

2.6. Sequence retrieval and phylogenetic analysis

The amino acid sequences of the HD-Zip I proteins from the different plant species described in Table 1 were obtained from the Phytozome v10.3 database [63]. The sequences that were not available in this database were retrieved from cited articles or the Patent Lens database (https://www.lens.org/). The AGI codes for the Arabidopsis and Rice HD-Zip I members were taken from Henriksson et al. [16] and Agalou et al. [10] and the corresponding sequences were retrieved from Phytozome.

The sequences were aligned using Molecular Evolutionary Genetics Analysis 6 (MEGA 6 [64]) with Pairwise Alignment or default parameters: Gap Opening Penalty (GOP) = 5, Gap Extension Penalty (GEP) = 0.1, and Multiple Alignment parameters: GOP = 10, GEP = 0.1.

The phylogenetic tree was constructed using the Neighbour-joining method, previously used in Agalou et al. [10], in MEGA 6 with 1000 bootstraps and other default parameters. *HAT22* (At4g37790; a member of the HD-Zip II subfamily) was included as an outgroup protein to root the tree.

2.7. Transcriptome comparisons

The dataset used in this study was obtained from cited publications. Cut-offs of a P-value < 0.05 and FC > 1.0 were applied, using the author's analysis for considering the differentially expressed genes. This analysis was difficult due to the use of different annotations and platforms. To reduce these problems, only AGI codes were used to identify each locus, and multiple references from microarrays were also considered. The lists of AGI codes were compared using in-house R-scripts. Venn diagrams were created with the VennDiagram package from the Comprehensive R Archive Network (CRAN) repository.

The statistical analyses of the comparisons between pairs were conducted using Fisher's Exact Test [65] from the R stat package from the CRAN repository. A two by two contingency Table was constructed and then the odds ratio and p-values were extracted from the function. Later, the p-values were corrected for the number of combinations tested using the Benjamini-Hochberg correction [66].

Similar to the method described for comparisons between pairs, a two thousand replicate Monte Carlo simulation was designed to estimate the random superpositions between each combination of three experiments. The odds ratio was calculated and the p-value was estimated with a Poisson distribution and then corrected as described above; the other distribution tests were applied with similar results.

The Euclidean distance was calculated from odds-ratio calculated as described above using the Distance Matrix Computation package from R stat package from the CRAN repository. Cluster comparison dendrograms were created using the Hierarchical Clustering package from the same repository and the inverse of the distances calculated with the complete method.

The genes' descriptions were obtained from the Plant Genome Integrative Explorer Resource (PlantGenie [67]) and manually curated. Genes differentially expressed in at least one, two, three and four experiments (s1, d1, t1 and q1 respectively) were compared with the drought-related genes reported by Shanker et al. [68] using the same method as for the Venn diagrams and Fisher's Exact Test. The error bars were calculated using the 95% confidence interval provided by the same function. The same test was performed on the *CpHB7* macroarray data [28].

2.8. Simplified heatmap

Only the differentially expressed genes were considered when we compared the expression patterns extracted from the different platforms. The fold change was considered to be 1 if it was higher than 1 and −1 if it was lower than −1, due to the difficulty in comparing the expression levels in different platforms. This matrix was converted into an ExpressionSet class using the Biobase package [69] from the Bioconductor repository. Then, the heat map and hierarchical clustering were constructed using the default heatmap function in the R stat package from the CRAN repository.

2.9. GO term enrichment analysis

The Gene Ontology Enrichment analyses were conducted using the Cytoscape software environment for integrated models of biomolecular interaction networks [70]. Overrepresented categories of GO terms from genes in common groups were calculated using the BiNGO Cytoscape plugin [71] with default parameters and the entire *Arabidopsis thaliana* annotation as the reference set and s1 group.

Graphical representations were created with the Enrichment Map Cytoscape Plugin [72] using the following parameters: P-value cut-off 5.10^{-3}, FDR cut-off 5.10^{-2}, and similarity cut-off: Jaccard coefficient 0.25 and combined constant 0.5.

2.10. Coexpression analyses

For the coexpression analysis of the t1 group, the AGI codes were studied using exNet [67] from PlantGenie and the Affymetrics database, with default parameters. Only AGI codes with edges were represented using Cytoscape.

2.11. Analyses of the target promoters

Promoter sequences (1000 bp upstream from the +1) from all of the annotated *Arabidopsis thaliana* genes were extracted and compiled using the Bioconductor Annotation Data (TAIR10) package and the Full genome sequences for *Arabidopsis thaliana* (TAIR9) package, both from the Bioconductor repository.

An in-house R-script using the Biostrings package from the Bioconductor repository was developed to search for *cis*-elements in the promoters using the direct pattern and the

reverse-complement of all known *cis*-regulatory elements from AtcisDB in the Arabidopsis Gene Regulatory Information Server (AGRIS; http://arabidopsis.med.ohio-state.edu/AtcisDB/). Fisher's Exact Tests were performed to analyze the overrepresentation of common HD-Zip I-regulated gene groups over the whole genome or over group s1. For sequences longer than 9 bp, one mismatch was permitted.

Only *cis*-elements with a p-value < 0.05 were considered as significantly overrepresented in group t1 and are summarized in Table 3. The P-values were adjusted using the Benjamini-Hochberg correction [66] for testing multiple hypotheses (using only summarized motifs).

3. Results

3.1. Most HD-Zip I-encoding genes are regulated by water deficits

Although HD-Zip I TFs were widely associated with abiotic stress responses, particularly water deficits, and the expression of individual genes were analyzed in response to such stress, global experimental studies analyzing the expression patterns of all members have not yet been conducted. Informatics analyses using available microarrays, performed with plants of different ages, are shown in Fig. 1. These analyses indicated that all of the members are up- or down-regulated at least in one of the experiments conducted in soil or in agar, with roots or aerial parts and with different watering/drought treatments. Notably, the expression of some genes was down-regulated in one tissue/treatment and up-regulated in another. As an example, *AtHB5* expression in rosette leaves was repressed by the water deficit, but it was induced in roots (Fig. 1). *AtHB7* exhibited an opposite behavior; while others were always induced (*AtHB12*) or repressed (*AtHB13*), according to these experiments.

These data are certainly partial, and it has been reported that *AtHB13* expression is induced (instead of repressed) by drought [37] after longer treatments. Moreover, RT-PCR assays (not quantitative) demonstrated a general regulation of these genes by drought in Arabidopsis seedlings [16]; hence, RT-qPCR was performed using WT Arabidopsis plants subjected to severe water deficit either in vegetative or reproductive stages. The results indicated that the majority of the tested genes were up- or down-regulated after the stress treatments (Fig. 2). It is important to note that most of them, such as *AtHB1* and *AtHB5*, were repressed in one stage and induced in the other, while *AtHB7* and *AtHB12* were always induced. Notably, with the exception of these latter genes, all of the other genes were repressed in the reproductive stage and a significant proportion was induced in the vegetative stage. Interestingly, most members belonging to the same clade exhibited similar behaviors in both stages (Fig. 2).

3.2. The abiotic stress tolerance exhibited by HD-Zip I OE plants is independent of the clade belonging

A global exploration of HD-Zip I OE reported phenotypes related to abiotic stresses is summarized in Table 1. Interestingly, members from different species and carbon-metabolism pathways conferred tolerance to abiotic stress factors, such as drought and salinity, when they were overexpressed as transgenes, suggesting that the different structural features in these TFs do not have relevant roles in conferring such tolerance.

Although most of the assessed HD-Zip I TFs expressed as transgenes in OE plants were able to confer tolerance to water deficits (Table 1), not all of the HD-Zip I OE were evaluated for this trait, and, in a few cases, an opposite effect was observed.

Wondering if stress tolerance conferred by HD-Zip I members was associated to a particular group, TFs summarized in Table 1 were resolved in a new phylogenetic tree and classified in the same six clades (Fig. 3) previously described by Arce et al. [56]. Although, most HD-Zip I reported as able to confer drought tolerance belong to clade I, it seems that drought tolerance is not dependent on the TF clade belonging. The exceptions were clades IV and VI members, for which there are no available studies reporting drought response [73,74]. However, as shown in Figs. 1 and 2, the expression of clade IV TFs was also regulated by water deficits; clade VI does not include Arabidopsis HD-Zip I proteins.

3.3. Most HD-Zip I transgenic plants exhibit enhanced drought tolerance and the extent of such tolerance depend on the transgene expression level

Drought tolerance conferred by HD-Zip I TFs was mainly reported for genes from non-model species that were tested in Arabidopsis. Notably, with few exceptions, Arabidopsis members were not tested for their ability to confer such tolerance in Arabidopsis or

Table 1
Plants that ectopically express HD-Zip I TFs exhibit common traits. Summary of the characteristics of the reported HD-Zip I OE plants from different species and clades. The data were selected from the literature (Ref.: reference indicated in the last column) and include the structural (gene name, clade identity, and species) and functional (response to drought, salinity, pathogens, development, root growth and ABA) characteristics of transgenic plants expressing HD-Zip I TFs. Species: AT: *Arabidopsis thaliana*; OS: *Oryza sativa*; BN: *Brassica napus*; CP: *Craterostigma plantagineum*; CA: *Coffea arabica*; HA: *Helianthus annuus*; ZM: *Zea mays*; MT: *Medicago truncatula;* SL: *Solanum lycopersicum*. "?" indicates that the trait was not described.

Gene	Species	Transformed species	Clade	Drought	Salt	Pathogen	Development	Root	ABA	Ref.
OsHOX22	OS	OS	I	Sensitive	Sensitive	?	?	?	Sensitive	[54]
MtHB1	MT	MT	I	?	?	?	?	Large	?	[15]
HaHB4	HA	AT	I	Tolerant	?	Tolerant	Retarded	Short	?	[48,50]
HaHB11	HA	AT	I	Tolerant	?	?	Retarded	Large	Sensitive	[25,44]
CaHB12	CA	AT	I	Tolerant	Tolerant	Proposed	?	Proposed	Proposed	[24]
AtHB7	AT	AT	I	Tolerant	?	?	Retarded	Large	Sensitive	[23,26]
AtHB12	AT	AT	I	Tolerant	?	?	Retarded	Large	Sensitive	[23,26]
ZmHdz4	ZM	OS	I	Tolerant	?	?	?	Large	Sensitive	[32]
ZmHdz10	ZM	OS/AT	II	Tolerant	Tolerant	?	Retarded	?	Sensitive	[27]
BnHB6	BN	AT	II	Tolerant	?	?	?	?	Proposed	[30]
AtHB6	AT	AT	II	Proposed sensitive	?	?	Retarded	?	Insensitive	[104]
AtHB5	AT	AT	II	?	?	?	?	?	Sensitive	[19]
OsHOX5	OS	OS	III	Tolerant	?	?	?	?	?	[31]
CpHB7	CP	AT	III	Tolerant	?	Proposed	?	Large	Insensitive	[28]
AtHB1	AT	AT	III	?	?	?	?	?	?	[8]
HaHB1	HA	AT	V	Tolerant	Tolerant	Proposed	Slightly retarded	?	?	[29]
AtHB23	AT	AT	V	?	?	?	?	?	?	[22]
AtHB20	AT	AT	V	?	?	?	?	?	Insensitive	[18]
AtHB13	AT	AT	V	Tolerant	Tolerant	Tolerant	?	Proposed short	?	[29,105]

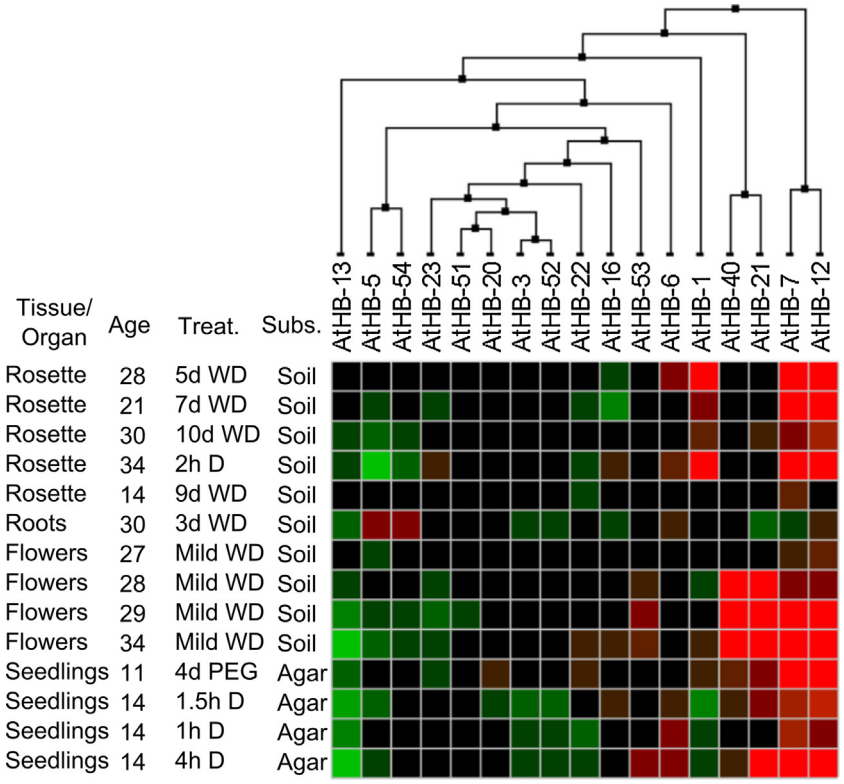

Fig. 1. The expression of HD-Zip I-encoding genes is regulated by water deficit. HD-Zip I-encoding genes were analyzed using selected microarray experiments with plants subjected to different drought treatments, according to the GENEVESTIGATOR datasets [103]. The heatmap, hierarchical clustering, period and type of stress applied, sampled tissue/organ, plant age and substrate (subs) are shown. WD: water depletion stress; D: desiccation stress; PEG: polyethylene glycol; Mild WD: mild water deficit.

in other species (Table 1). Considering that most Arabidopsis HD-Zip I TFs are regulated by drought in at least one stage/condition (Figs. 1 and 2), which suggests a role in the response to water deficit, it was tempting to state that Arabidopsis members can also generate drought tolerant plants upon overexpression.

AtHB1 was shown to be repressed by drought, ABA and NaCl in agar plate assays and microarrays [16] but a role in drought response was not assigned to this gene. However, the results shown in Fig. 2 indicated that in the reproductive stage, the expression of *AtHB1*was induced after a severe drought treatment applied to plants grown in soil. To test our hypothesis, transgenic *AtHB1* OE plants as well as *athb1-1* mutants, both in the Col-0 background, were subjected to drought treatment (Fig. 4).

The results indicated that after severe water deficit the OE plants were drought-tolerant compared with their controls, whereas the *athb1-1* mutants were more sensitive, both in the reproductive stage (Fig. 4). In the same sense, the OE plants consumed and lost less water during a mild drought stress assay than the WT plants. It is noteworthy that the expression level of *AtHB1*, tested in at least 40 independent transgenic lines, varied from 1 to 7 fold over the endogenous expression level (data not shown). A similar scenario was observed when *AtHB12* and *AtHB7* were expressed as transgenes [23]; the maximum overexpression detected was about 7-fold over the expression in WT. Considering the differences in survival rates after severe stress treatments, the drought tolerance conferred by these three genes to transgenic plants was rather limited (Fig. 4 and Re et al. [23]), suggesting that higher expression levels are necessary to observe more significant differences.

These and previous observations [37,47,48] led us to hypothesize that the drought tolerance extent was dependent on the transgene expression level and probably a threshold must be achieved/surpassed to observe significant tolerance differences.

To obtain Arabidopsis HD-Zip I higher expression levels, *rdr6-12* mutant plants with defective Post Transcriptional Gene Silencing (PTGS) [75] were transformed with *35S:AtHB1* and *35S:AtHB12* constructs. The new obtained genotypes were tested for the transgenes expression levels showing significantly differences (30 to 60- and 10 to 16- fold) with the untransformed *rdr6-12* background (Fig. 5B and E). Subjected to severe drought stress, both *AtHB1* and *AtHB12* OE plants exhibited significant tolerance allowing to evaluate the differences in survival rates between transformed and untransformed plants (Fig. 5C and F). However, pleiotropic effects such as growth retardation were also observed (Fig. 5A and D).

3.4. An analysis of the HD-Zip I OE transcriptomes reveals that these transcription factors have common target genes

To determine whether the common traits exhibited by some HD-Zip I OE were a consequence of shared molecular mechanisms triggered by these TFs, the available OE transcriptomes from high-throughput experiments were compared to identify the putative common target genes. To achieve this, a restrictive criteria of fold change (FC > 2.0) and statistical significance (p-value < 0.05) were used. Five transcriptomes were chosen for this analysis, all them from plants expressing the transgenes driven by the 35S CaMV promoter. Four out of the five were obtained from heterologous HD-Zip I OE in the *Arabidopsis thaliana* Col-0 ecotype, two from sunflower genes (*HaHB4* [49]; HaHB1, [29]) the third from *Coffea Arabica* (*CaHB12*, Cruz et al., personal communication) and the fourth from rice (*OsHox22*) [73] The other one was *AtHB13* also in Arabidopsis Col 0 plants [76]. Only the *CaHB12* OE transcriptome was obtained from an RNA-Seq experiment, whereas the others were obtained from microarray-based experiments (complete references of transcriptomic experiments are provided in Supplementary Table 2). All of the experiments analyzed in this

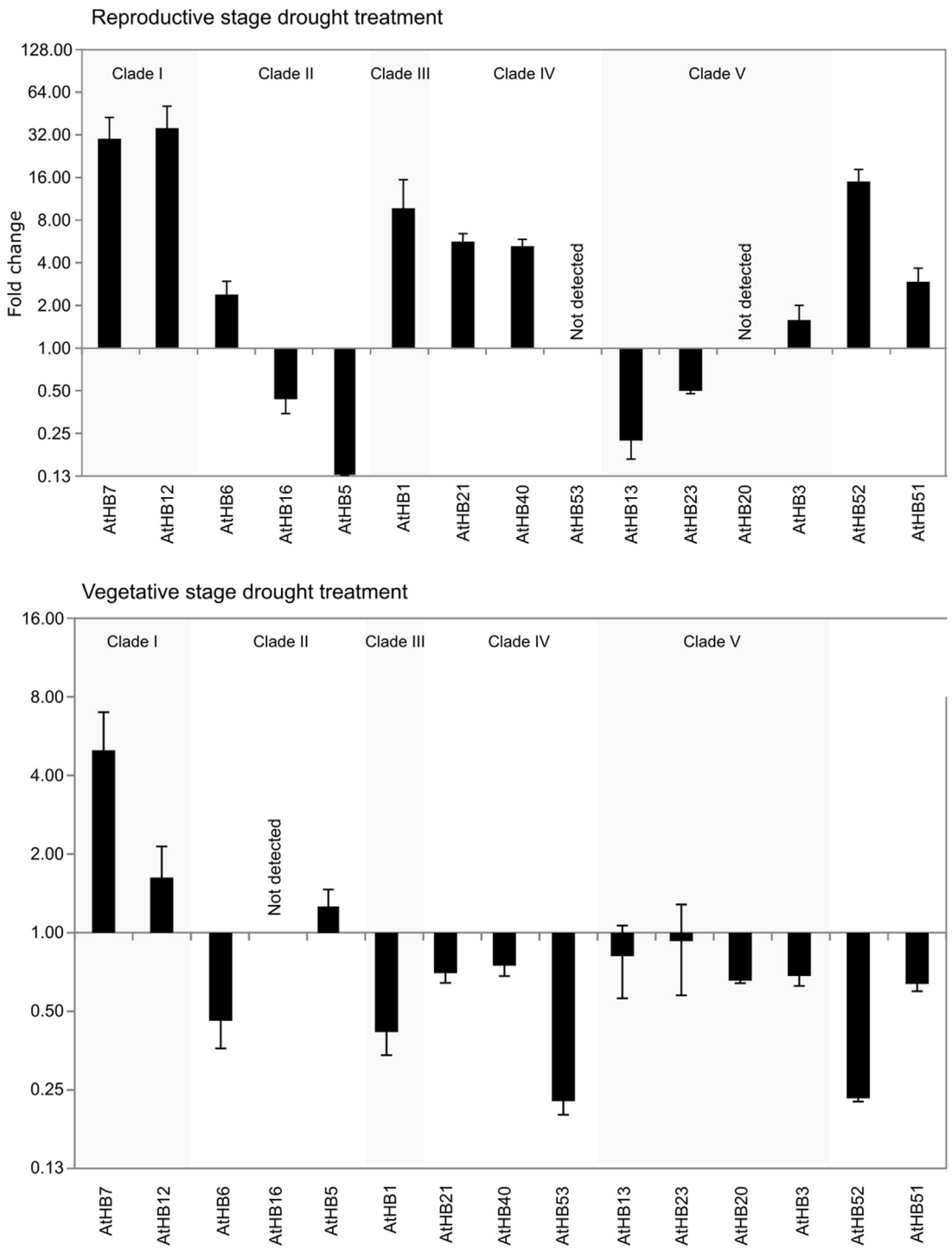

Fig. 2. The expression of HD-Zip I-encoding genes is regulated by drought during the vegetative and reproductive stages.
The levels of the *AtHB7*, −12, −6, −16, −5, −1, −21, −40, −53, −13, −23, −20, −3, −52 and −51 transcripts belonging to clades I to V of the HD-Zip I family were quantified by RT-qPCR and normalized to the levels of the endogenous *ACTIN* (*ACTIN2* and *ACTIN8*) transcripts. Leaf samples were harvested from plants subjected to drought during the vegetative (upper panel) or reproductive stage (lower panel) as described in the Methods. The values were related to the corresponding value of each gene measured in the samples taken from the control plants. Three biological replicates were performed for each experiment. The error bars represent SD calculated from three independent biological replicates.

comparison were conducted with leaves or seedlings from plants in the vegetative stage. However, there were differences between experiments, including selected tissue, plant developmental stage, platforms, differential expression analysis and technologies, and this fact can underestimate some of the common features, but rarely creates false positives. Fig. 6a shows that these HD-Zip I TFs, which belong to different phylogenetic clades, regulate common target genes. Fisher's Exact Test was conducted for transcriptome pairs and triads, and demonstrated that the results cannot be random (Supplementary Table 3). Conversely, increased similarities between the proposed homologs were not observed (Fig. 6b).

Based on these results, five groups of genes were created using genes that appear to be regulated in one (s1, 3327 genes), two

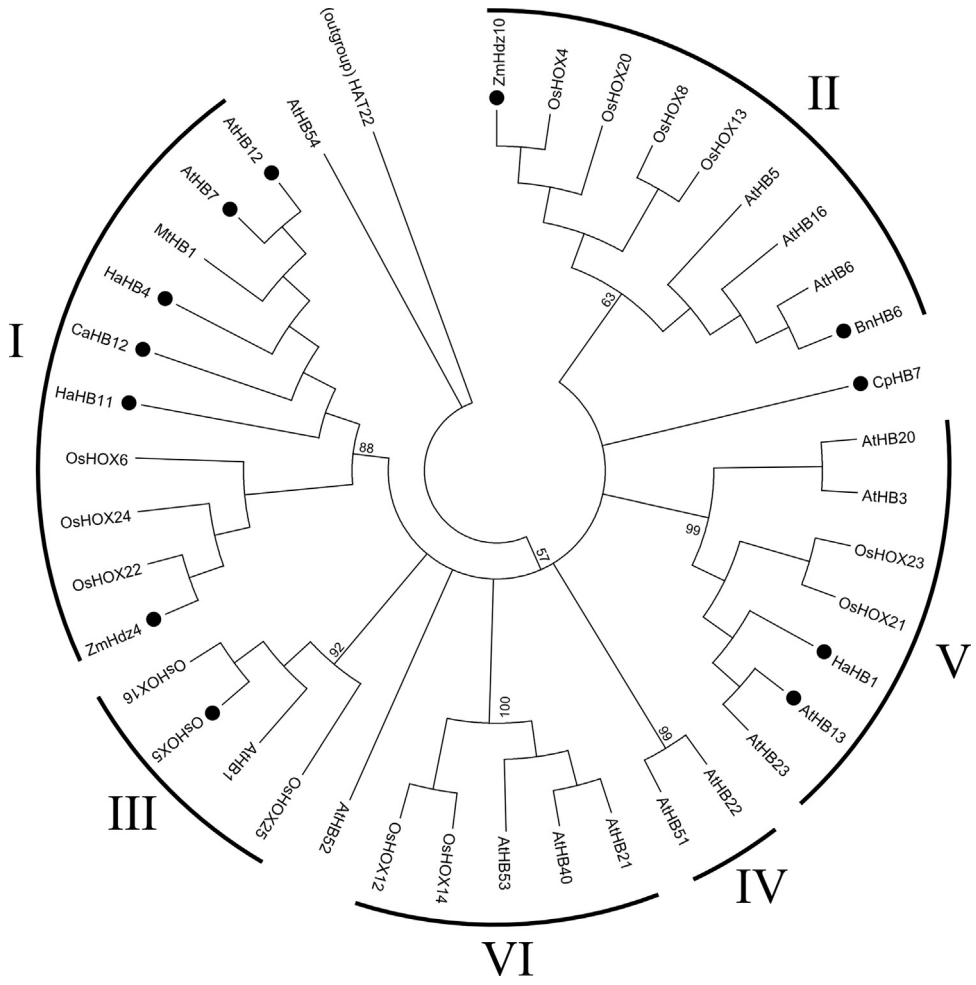

Fig. 3. Members of various HD-Zip I clades are able to confer drought tolerance. The phylogenetic tree of the HD-Zip I TFs was constructed with HD-Zip I members from *Arabidopsis thaliana*, *Oryza sativa* and those from different plant species mentioned in Table 1 as able to confer stress tolerance in order to classify them in clades. The tree was resolved with full length amino acid sequences using the Neighbour-joining method. The Roman numerals indicate the clade to which each protein belongs [56]. The dots represents drought tolerant phenotype reported as shown in Table 1. HAT22 (a HD-Zip II family member) was used as outgroup to root the tree. Only bootstrap values sustaining clades are shown, branches with lowers bootstrap than 50 were condensed.

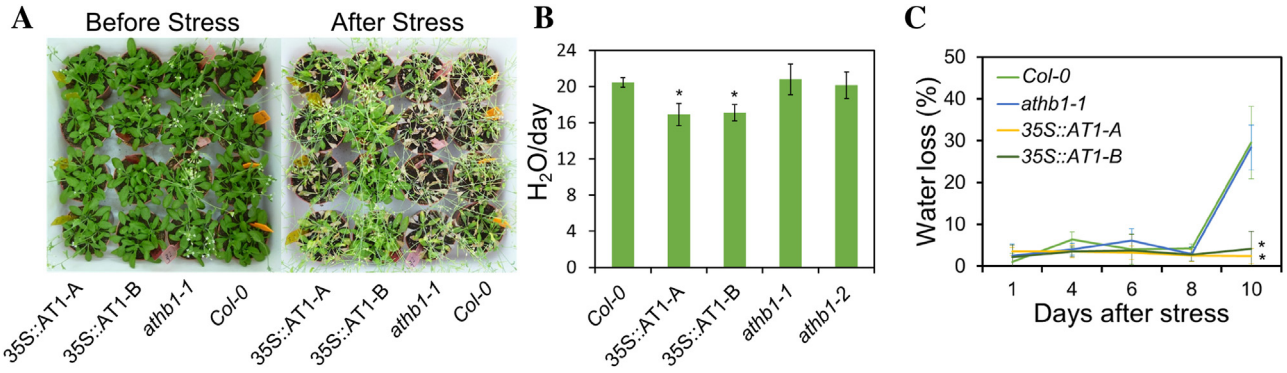

Fig. 4. *AtHB1* OE plants in Col- 0 background exhibit enhanced drought tolerance compared with their controls.
(A) Illustrative photographs of the *AtHB1* OE (*35S:AT1*, two independent lines) and mutant (*athb1-1*) plants and controls (Col-0) were taken before and after a severe drought stress treatment was applied to 3-week-old normally watered plants for 11 days. (B) The average daily water consumption of each genotype during a mild drought stress treatment is expressed as ml H_2O/day. (C) The water loss from the leaves was assessed during the severe drought stress treatment. The error bars indicate the standard deviation of at least three replicates. The asterisks denote the significant difference compared to Col-0, as evaluated by ANOVA, followed by Tukey's post hoc test. NS: not significant.

(d1, 463 genes), three (t1, 79 genes), four (q1, 15 genes) or all of the analyzed transcriptomes (w1, 2 genes). Remarkably, the genes that appeared to be differentially regulated in three or more experiments exhibited a high odds-ratio in Fisher's Test, indicating that clade identity did not determine the targets. The complete list of genes belonging to group t1 is shown in Supplementary Table 4. Further analyses were conducted with the t1 group that aimed to avoid the potential artifacts that were generated when the genes

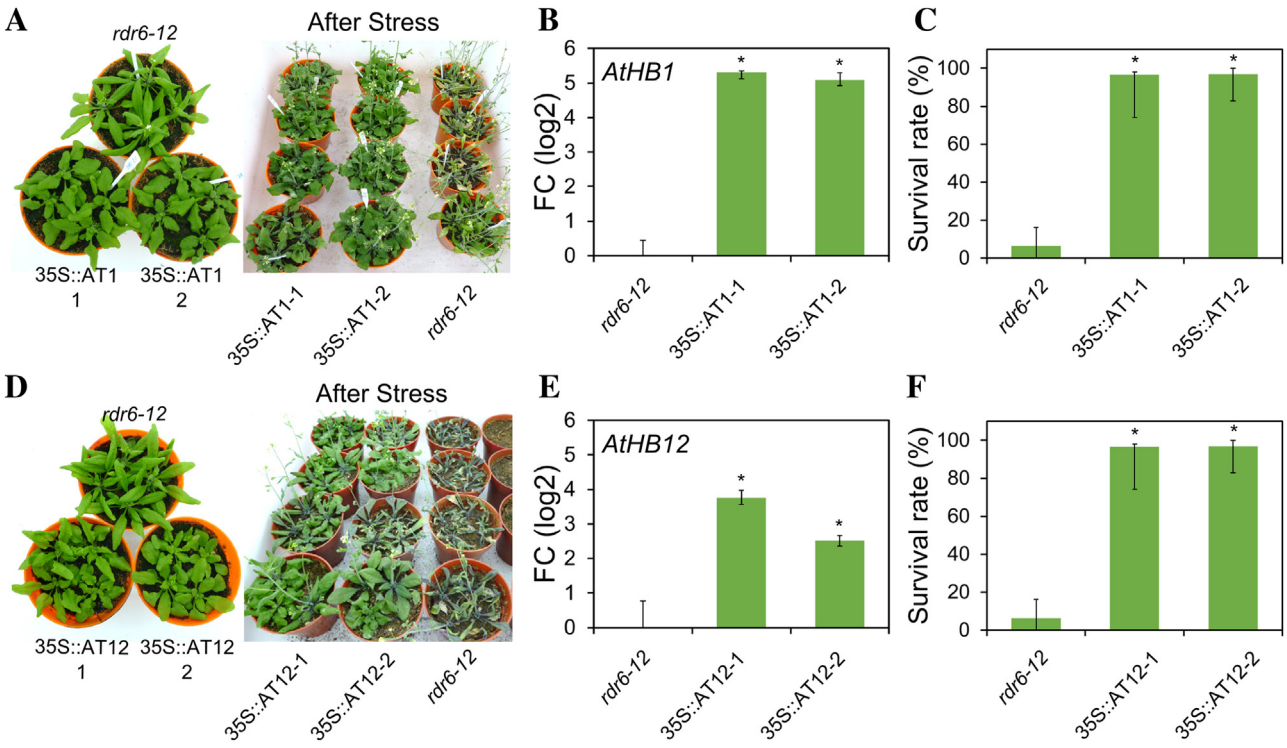

Fig. 5. *AtHB1* and *AtHB12* OE plants in *rdr6-12* background exhibit high expression levels and drought tolerance. A and D Illustrative photographs of 3-week-old *AtHB1* OE (*35S:AT1*, two independent lines) and *AtHB12* OE (*35S:AT12*, two independent lines) plants and controls (*rdr6-12*). B and E *AtHB1* and *AtHB12* transcript levels quantified by RT-qPCR in OE plants and normalized to the levels of the endogenous *ACTIN* (*ACTIN2* and *ACTIN8*) transcripts and then with *AtHB1* and *AtHB12* transcript levels in the *rdr6-12* mutant plants. The error bars represent SD calculated from three independent biological replicates. C and F Survival rates of OE and *rdr6-12* mutant plants subjected to severe drought and recovered (see Methods). The error bars indicate a 95% confidence interval. The asterisks denote significant differences when compared to *rdr6-12* mutant plants evaluated by ANOVA, followed by Tukey's post hoc test. NS: not significant.

from the d1 group were chosen, which could be derived from transcriptomes from different species.

To extend the analysis and further test the hypothesis of common regulated genes, the proposed putative targets of CpHB7 [28] were searched in the already created groups. The experiment conducted with *CpHB7* [28] was a macroarray limited to a sample of 41 genes, all of which contain the pseudopalindromic sequence CAAT(N)ATTG in their regulatory regions. Because of this and intending to avoid biases, these results were not included in the first survey. Not surprisingly, the comparison between the 41 putative targets of CpHB7 and those included in the five groups described above showed a match of 53.7% with the s1 group, which includes all of the regulated genes, as well as a match of 12.2% with the d1 group. This latter result indicated a high overlap between CpHB7 targets and those included in the groups.

A second analysis was focused on the direction (up or down) of the differential expression of the putative targets that appeared to be regulated in at least two experiments. The results indicated that the same regulated genes were induced or repressed, depending on the HD-Zip I TF. Notably, the direction of the interaction showed a higher similarity when the OE transcriptomes analyzed were from HD-Zip I TFs from the same clade than when they were from different clades. The analyses performed with the d1 group are shown in Supplementary Fig. 1.

3.5. Common target genes regulated by HD-Zip I TFs belong to specific biological processes

Based on the transcriptomes of the OE plants, the genes that were regulated by the HD-Zip I TFs were subjected to Gene Ontology (GO) term enrichment analysis by comparing the genes from t1 and s1 groups against the whole genome annotation. Many GO terms shared by s1 and t1 groups were mainly associated with relevant pathways, such as stress (biotic and abiotic) responses and hormones. Moreover, few GO terms only appeared in the t1 group, such as wax and indole glucosinolate biosynthesis and starch metabolic processes. A second GO term analysis comparing the t1 group with the s1 group (instead of the whole genome) showed that many GO terms were also enriched compared with the terms derived from single experiments (Fig. 7 and Supplementary Table 5).

The enriched GO terms were specifically related to hormone responses (jasmonic acid, salicylic acid, ethylene, gibberellin and ABA) and biotic and abiotic stresses (defense response and water deprivation), indicating a close relationship between the observed OE phenotypes and the common putative target genes.

To demonstrate that the common genes regulated by HD-Zip I TFs may be relevant for determining the shared phenotypes in OE plants, a comparison between the s1, t1 and q1 genes with those previously identified as drought-related was conducted [68]. The results revealed that while the s1 genes were not particularly related to the drought response, the genes from the d1 and t1 groups showed a stronger correlation (Fig. 8a). A Fisher's test was applied to the comparison of the groups and indicated that the target genes shared by more transcriptomes increased their connections with the drought-related genes (Fig. 7b).

A coexpression analysis of the common putative target genes was conducted to identify the regulated pathways (Supplementary Fig. 2). Among these pathways, the ethylene-related pathway was the most relevant. The down-regulation of ethylene synthesis and sensing was previously reported as responsible for the senescence delay and drought tolerance exhibited by the HaHB4 OE [49] and other transgenic plants reported in the literature [77]. Although ethylene synthesis and sensing events were not analyzed in other

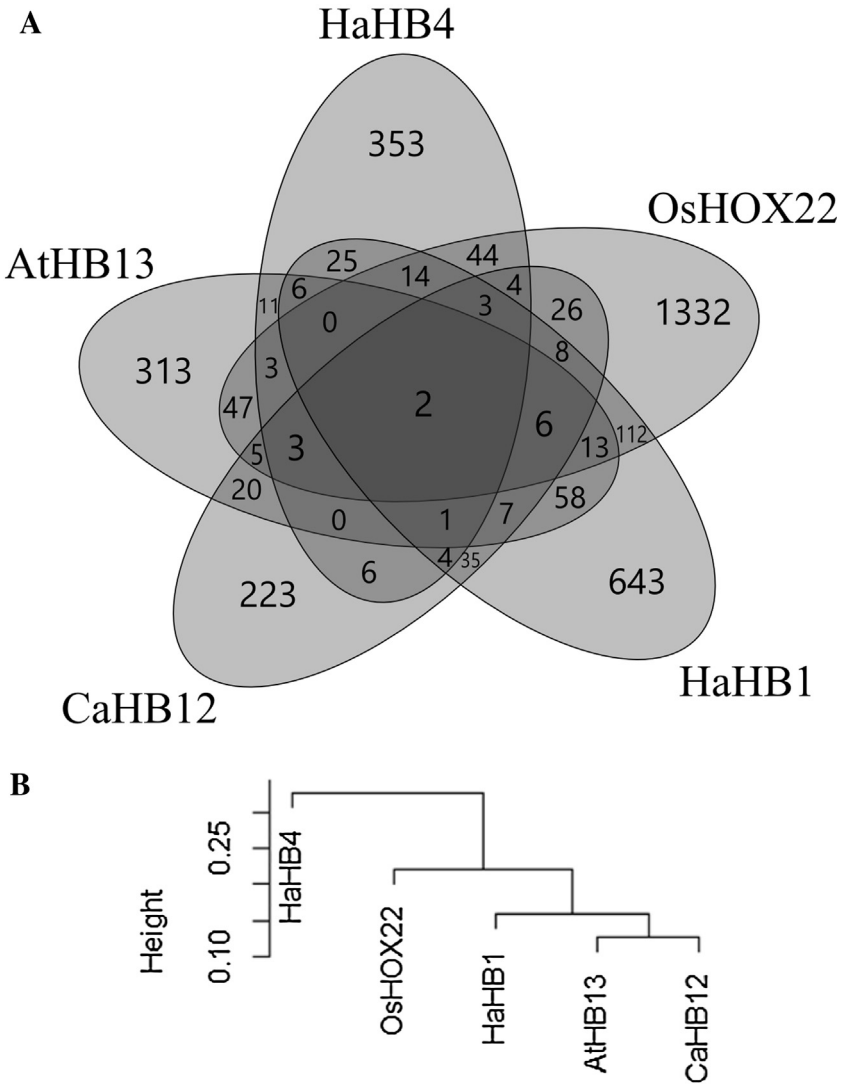

Fig. 6. Comparisons of the transcriptomes of OE plants indicate the presence of common HD-Zip I-regulated genes.
Venn diagram of the genes that were differentially expressed in the transcriptomes of HD-Zip I OE plants; the name of each TF is indicated outside the diagram. The numbers inside the diagram indicate the number of genes forming each group. B Dendrogram of the hierarchical clustering of genes regulated by more than one HD-Zip I TF using the Euclidean distance in bases from the Fisher's Exact Test odds-ratio.

HD-Zip I OE plants, they could be one of the keys to understand some of the common phenotypic characteristics exhibited by these plants as a result of water deprivation.

In addition to the ethylene-related pathways, group t1 showed an enrichment in GO terms associated with transcriptional regulation, involving eleven TFs. Notably, five of them belong to the AP2/ERF (APETALA2/Ethylene Response Factors) family: ERF104, ERF105, ERF2, ERF13 and RAV2 (At5g61600, At5g54490, At5g47220 and At1g68840, respectively). Those TFs, particularly ERF2, are repressed in four out five HD-Zip I OE transcriptomes (Supplementary Fig. 3a). *HaHB4* and *HaHB1* OE plants RNAs were tested for ERF2 and expression levels by RT-qPCR. The results indicated a clear downregulation corroborating the *in silico* analysis (Supplementary Fig. 3c). Interestingly, with the exception of RAV2, which belongs to a different subfamily, the other TFs are part of group IX (or B-3) of ERF TFs. ERF2 and −13 belong to subclade a, whereas −104 and −105 belong to subclade *b* [78].

Other TFs that were well represented in the groups belong to the NAC (NAM/ATAF1/2/CUC2), JAZ (Jasmonate Zim-domain), ARR (Arabidopsis Response Related), and WRKY families. It is well known that TFs from these families play important roles in abiotic stress signaling [79–81].

Genes showing induction or repression in at least three drought-tolerant OE (*HaHB4*, *AtHB13*, *CaHB12* and *HaHB1*) and the opposite behavior in *OsHox22* OE (drought-sensitive) were analyzed to identify the targets that were directly associated with drought tolerance in the HD-Zip I OE. Only four genes matched with this criterion: *IPSI* (encoding an Inositol-3-phosphate synthase isozyme 1, At4g39800), a member of the *GRP* family (encoding a glycine-rich protein associated with senescence, At2g05540), *XTH25* (encoding a Xyloglucan Transferase Hydrolase, At5g57550) and *PSK3* (encoding a signal peptide Phytosulfokines 3 associated with pathogen defense, At3g49780). Among these 4 putative targets, the best characterized is *XTH25*. It belongs to a family of genes that are upregulated by abiotic stress and are involved in cell wall remodeling by causing growth arrest [82]. XTH OE plants showed tolerance to salinity and drought stresses, likely by strengthening the cell wall to avoid water loss [83]. The expression levels of *XTH25* in the HD-Zip I OE transcriptomes are shown in Supplementary Fig. 3b. This gene is upregulated in three out of four drought-tolerant OE

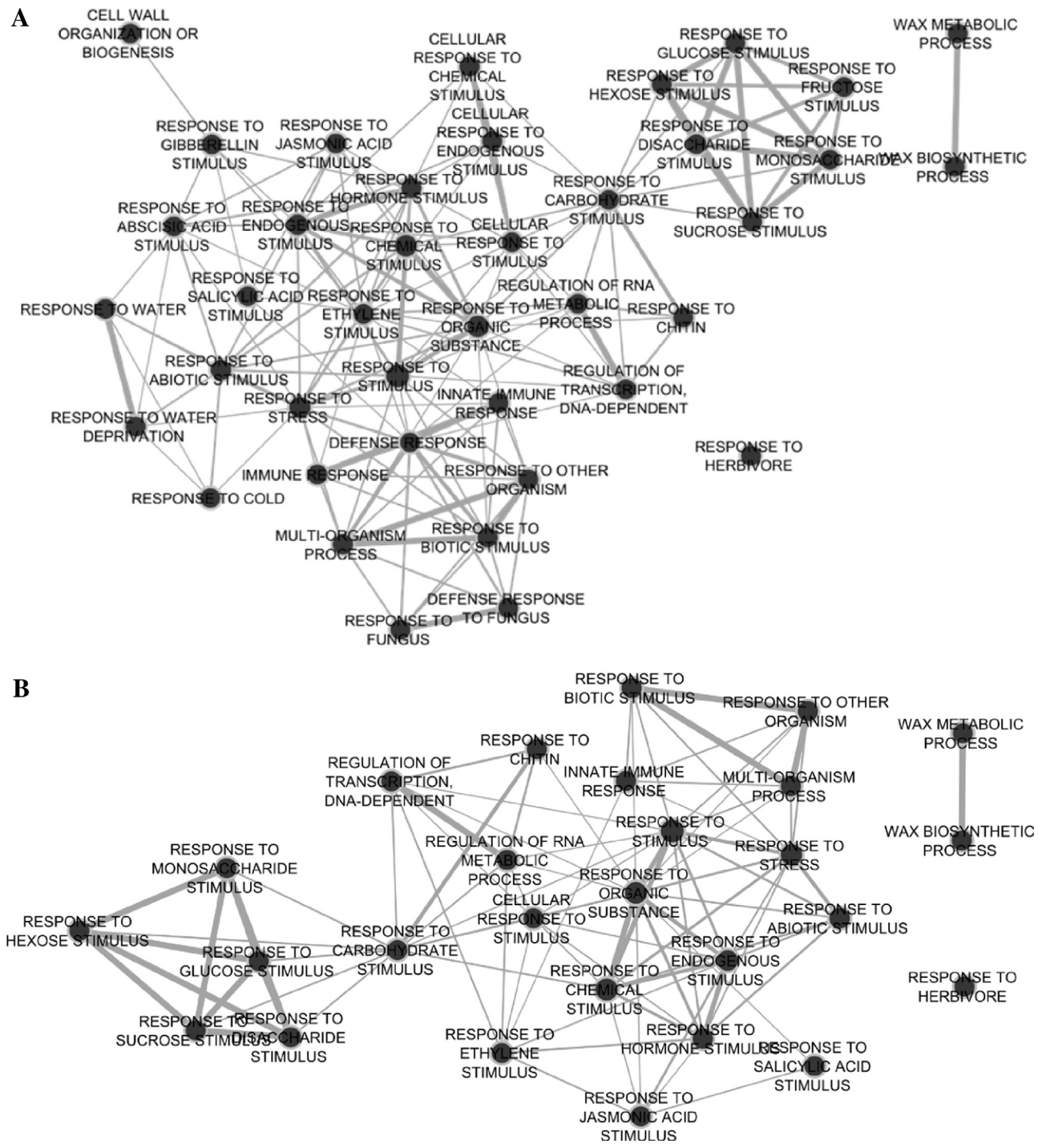

Fig. 7. Analysis of the GO terms for the genes in group t1. Map of the enrichment in the GO terms of the genes in the t1 group compared with the whole genome annotation (A) or with the s1 group (B). Bonferroni-corrected p-value < 5.10^{-3}, FDR < 5.10^{-2}, similarity cut-off: Jaccard coefficient 0.25 and combined constant 0.5.

plants and repressed in *OsHox22* OE; however, the evaluation in *HaHB1* OE did not show statistical significance. Validation of the in silico results by RT-qPCR indicated that XTH25 expression is up-regulated in *HaHB4* and *HaHB1* OE plants with statistical significance (Supplementary Fig. 3d).

3.6. Promoter regions of the common HD-Zip I-regulated genes

The promoter regions of different groups of targets were analyzed for the presence of the pseudopalindromic DNA sequence (CAATNATTG) that is bound *in vitro* by most HD-Zip I TFs. Sequences 1000 bp upstream of the +1 site of genes from the s1, d1, tl and q1 groups were considered in the analysis. The results showed that the target sequence was more frequent in the t1 and q1 groups than in a random sample, but with no statistical significance (Table 2).

First, the t1 group was analyzed to detect the enrichment of other *cis*-acting elements in the putative targets promoters. DRE and DRE-like *cis*-acting elements (TACCGACAT and DRCCGACNW respectively), which are recognized by TFs involved in drought responses, were enriched in t1, with high statistical confidence. Additionally, ABRE-like (BACGTGKM) and the more general G-box

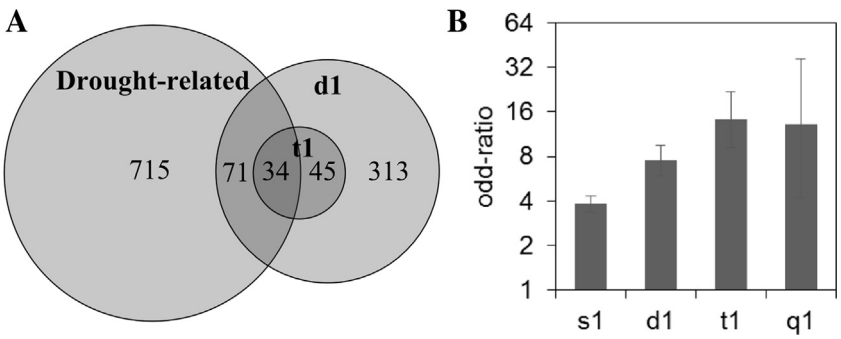

Fig. 8. The genes that are regulated by HD-Zip I TFs are closely related to those regulated by drought. Venn diagram comparing the genes reported to be regulated by drought [68] and those forming the t1 and d1 groups. B Odds-ratios for the comparisons between the drought-regulated genes [68] and the genes forming all of the groups classified in this work, with the exception of group w1, which only has two members and, hence, was not statistically significant. All odd-ratios display a two-sided Fisher's Exact Test P-value < 5.10^{-5}. The error bars indicate the 95% confidence intervals.

Table 2
Promoter analysis of the HD-Zip I targets. The odd-ratios (odd rows) and percentages (even rows) corresponding to the *cis*-elements that were enriched in the promoters of genes belonging to groups q1, t1, d1 and s1 (formed by putative targets of HD-Zip I TFs) are shown. In the first column, the sequence of each element analyzed is shown in brackets below the name of the *cis*-acting element. In the column header, the number of genes forming each group is indicated. The asterisks indicate the Benjamini-Hochberg-corrected [66] p-values. *p-value < 0.25, **p-value < 0.05, ***p-value < 0.01.

	q1 (15)	t1 (79)	d1 (463)	s1 (3327)	Ref.
HD-Zip BD (CAATNATTG)	*3.913	1.505	*1.343	***1.357	[13]
	13.30%	5.13%	4.58%	4.62%	
ABRE-like BD (BACGTGKM)	*2.700	**1.731	***1.559	***1.235	[106]
	60.00%	38.46%	34.64%	27.46%	
G-BOX (CACGTG)	**3.668	**1.959	***1.545	***1.245	[107]
	60.00%	32.00%	25.27%	20.37%	
DRE BD (TACCGACAT)	*19.91	**7.664	*2.605	**1.750	[108]
	6.67%	2.56%	0.87%	0.59%	
DRE-like BD (DRCCGACNW)	*2.105	**1.822	*1.256	*1.068	[109]
	26.67%	23.08%	15.90%	13.53%	

(CACGTG) elements were also enriched in the analyzed group, both of which are associated with ABA signaling.

4. Discussion

4.1. The regulation of common genes could explain the drought tolerance phenotypes exhibited by the HD-Zip I transgenic plants

HD-Zip I TFs play crucial roles in plant development and adaptation to environmental conditions. These proteins have evolved to exquisitely regulate different events, and duplicated genes in Arabidopsis even have differentiated functions [7,9]. These functions are governed by the expression patterns and interactions that the encoded proteins establish with other proteins, triggering the differential regulation of the target genes. Despite the fact that each member of this TF family plays a natural role, when they were overexpressed as transgenes, some of them triggered similar phenotypes, specifically an enhanced response to water deprivation compared with the control plants. Although it was very likely that the induced drought-tolerance phenotype was associated with the common differentially regulated genes, the transcriptomes of HD-Zip I OE have not yet been analyzed together.

In this study, we analyzed the expression patterns of the HD-Zip I TFs in response to drought and the transcriptomes of the transgenic plants, which allowed us to propose that the tolerance to water deprivation exhibited by most HD-Zip I OE may be due to the action of common genes that are regulated by these TFs.

The expression of most members of the family was up- or down-regulated in response to water deprivation. Even members of clade IV, which had not previously been analyzed under these conditions, were regulated. In accordance with this result, *AtHB21* and *-40*, which belong to clade IV, were identified because they recognize the promoter of *COX5b-1* (cytochrome-c oxidase zinc binding subunit; At3g15640), which conferred drought tolerance when overexpressed [74].

4.2. Common genes regulated by HD-Zip I proteins

The analyses of five HD-Zip I OE plants allowed us to classify the target genes into different groups. Group t1 includes 79 genes and was chosen for further analysis because its members can be associated with biological processes related to drought tolerance with high confidence.

Among these 79 genes, several were associated to senescence delay, such as the *ERF* genes. In the same way, *SAG21* (At4G02380) is also part of group t1 which was reported to be regulated by the TFs NAC29 and NAC72. The *NAC29* mutant and *NAC72* OE plants exhibited enhanced drought tolerance compared with the controls [84,85]. Other genes that were included in group t1 and associated with senescence delay were *JAZ7*, recently described as a negative regulator of dark-induced senescence mediated by H_2O_2 [81], and NAC type TFs [86].

Regarding membrane stabilization, group t1 includes *LTP4* (Lipid Transfer Protein-4; At5g59310) and *PR4* (Pathogen Related-4; At5g04720), a gene described as playing a role in this process after biotic or abiotic stresses [87,88].

Group t1 also includes genes related to cell wall modifications, such as *LTP4* (mentioned above), *XTH22*, *XTH25*, *XTH27* (three Xyloglucan Transferase-Hydrolases; At5g57560, At5g57550, At2g01850 respectively), *EXPA1* (Expansin-A1, At1g69530), and peroxidases, such as *PER21* (At2g37130). These proteins were reported to cause growth arrest by promoting cell wall modifications [82]. Other cell-wall-associated genes were proposed as induced by TaHDZipI-2 in barley, modifying cell wall deposition and generating dwarfism. Finally, genes associated with the crosstalk between biotic and abiotic stress responses were also present

in group t1. Among them, *CHI* (Chitinase class 4-like protein; At2g43570) and *PR2* (Beta-1,3-Glucanase 2 or Pathogen Related-2: At3g57260) were demonstrated to be HaHB1 and AtHB13 targets, both of which confer drought tolerance [37].

Other genes belonging to group t1 that may be related to drought stress are *CHX17* (Cation/H(+) antiporter-17; At4g23700), whose soybean homologue, *SALT3*, was recently reported to be involved in the natural adaptation of soybean to salt stress [89,90], *WRKY75*, which is involved in root development [91] and *WRKY46*, which participates in osmotic stress responses and stomatal movement [92].

4.3. What are the common features of HD-Zip I TFs putative target genes?

One of the experimental approaches to answer this question consisted of analyzing the presence of the pseudopalindromic sequence bound by most HD-Zip I TFs *in vitro* (CAATNATTG) and other *cis*-acting elements in the regulatory regions of the putative targets. The results indicated a slight enrichment in these sequences, although it was expected to be stronger due to the artificial interactions produced by overexpression. Although the expectation was not met, the results were in agreement with other reports in which the enrichment in the pseudopalindromic sequence in putative HD-Zip I targets was not so evident [93,94]. It is important to note that most reports showing the interaction between HD-Zip I TFs and the pseudopalindromic sequence were conducted *in vitro* and that AtHB7 and −12 were unable to bind this, or other, but not a different DNA sequence [13]. It is possible that HD-Zip I TFs bind to shorter versions of the pseudopalindrome *in vivo*, such as AATNATT, which are widely distributed in promoter regions. Alternatively, other *cis*-elements, such as the G-box and DRE, which are bound by putative HD-Zip I partner proteins, were more enriched in the OE transcriptomes. In this sense, HD-Zip I TFs were proposed as fine regulators of gene expression, and in some cases, they exhibited other antagonizing functions, particularly those related to hormone sensing functions [95,96]. In particular, some ABA receptors were described to be putative targets of AtHB7 and AtHB12 [94]. In accordance, the t1 group includes *PYL6* (At2g40330), a TRAF-like protein-encoding gene (At3g28220), and *ERF105*, which is proposed to interact with ABI2, ABI5 and ABI1 [97].

Altogether, it can be suggested that in OE plants occurs artificial scenarios due to the excess of a normally regulated TF. Taking into account that HD-Zip I TFs probably bind similar target sequences, it is likely that when these TFs are overexpressed, they occupy the endogenous HD-Zip I binding sites. Alternatively, the transgenes may be titrating the endogenous TFs by heterodimerization. In accordance with this latter hypothesis, Harris et al. proposed a model in which *Triticum aestivum* HD-Zip I members interact between them depending on the stress condition [98].

4.4. The drought tolerance in HD-Zip I OE plants requires a transcript level threshold

The reports in the literature indicated that the extent of the drought tolerance conferred by most HD-Zip I TFs was dependent on the expression level of the transgene [37,48]. Similarly, it was reported that successive drought stress treatments in WT plants increased the expression of HD-Zip I TF-encoding genes, among others [99]. These latter observations could be related with the OE plants.

One of the questions was whether any HD-Zip I TF was able to confer drought tolerance because in many cases, this trait was not tested in the OE plants. For example, *AtHB1* was solely assigned a developmental role, which was associated with the illumination conditions [8,100]. Nevertheless, the experiment described here indicated that this TF is capable of conferring water stress tolerance (Figs. 4 and 5), which is associated with its regulation by this condition during the reproductive stage (Figs. 1 and 2). This result tempted us to propose that any HD-Zip I would confer drought tolerance if the transgene was expressed at sufficient levels. On the other hand, it has been shown that *AtHB7* OE exhibited a rather low tolerance to drought [23]. In these and other OE plants in which the transgene was from the same species as the host plant, the expression levels of the transgene were no more that 5-7-fold higher than the natural genes [8,53]. Remarkably, when the same transgene was expressed in heterologous systems, drought tolerance was assessed and described. For example, when *AtHB7* gene was expressed in tomato or peanut, it conferred significant drought tolerance [101,102].

These observations led us to suggest that the drought tolerance phenotype is only achieved when the transgene expression level surpasses a threshold. The drought tolerance observed in transformed *rdr6-12* mutant plants supported this hypothesis. These plants exhibited higher expression levels of *AtHB1* and *AtHB12* transgenes than those achieved in Col 0 background accompanied by stronger drought tolerance phenotypes.

In other words, transgenic plants with low expression levels did not exhibit significant differential tolerance compared to controls, whereas plants with very high expression levels improved the tolerance achieved with low expression levels, indicating that a minimum level must be reached. It is noteworthy that when the transgene is expressed at very high levels, deleterious effects appeared that prohibited the drought tolerance assessments ([53] and data not shown).

These observations also indicated that HD-Zip I OE plants trigger silencing mechanisms limiting expression when the transgene is from the same species whereas such mechanisms do not take place when the transgene is heterologous. Additional studies are required to elucidate why such mechanisms occur, especially because miRNAs targeting these genes have not been identified in the databases.

5. Concluding remarks

Together, the available reports and this study show that the HD-Zip I OE plants exhibit enhanced drought tolerance and trigger some common mechanisms to confer this tolerance. Even when each OE displays differential traits, water deficit tolerance appears as a main trait in all of these plants. This common trait could be partially explained by the regulation of the common targets.

This work proposes a new insight, in which the common HD-Zip-regulated targets trigger common pathways, and suggests the existence of specific and non-specific interactions with target genes that could help us understand the natural functions of HD-Zip I TFs.

However, drought responses are not always beneficial for crops, and some of these responses appeared when the expression level of the transgene is higher than desired, leading to pleiotropic effects accompanied by yield penalties. A few HD-Zip I OE plants exhibited enhanced productivity through unknown mechanisms, which is not a shared trait conferred by HD-Zip I TFs; hence, it must be a result of unknown and likely specific interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.03.004.

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