HD-Zip I and II, Transcription Factors Involved in the Adaptive Response to Environmental Stress

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ABSTRACT

The response to abiotic stress results in a dramatic change of the whole plant transcriptome. This regulation takes place mostly at the transcriptional level for which the role of transcription factors turns out to be essential. Here we compile the knowledge acquired about the participation of HD-Zip I and II transcription factors in the response to abiotic stress, such as water deficit, low temperature, osmotic stress and different illumination conditions. We finally analyze the effect of transgenic model plants that over express some of the HD-Zip members and infer their potential use as biotechnological tools for the obtaining of tolerant transgenic crops.

CONTENTS

ENVIRONMENTAL CONDITIONS AFFECT PLANT DEVELOPMENT

Development in multicellular organisms results from growth and differentiation and is determined by a specific program of gene expression. In plants, environmental factors have a great influence on development *via* different signal transduction pathways that amplify the original stimuli and ultimately result in the activation or repression of certain genes. The synthesis of most eukaryotic proteins is regulated at the transcriptional level. Such a coordinated regulation depends on the activity of a group of proteins generally called transcription factors, which are able to enhance or reduce the rate of transcription by facilitating the assembly of the initiation complex. Transcription factors (also called *trans*-acting elements) specifically interact with DNA sequences (*cis*-acting elements) situated in the proximal promoter region of the target gene or with more distal response elements (Taiz and Zeiger 1998). Although transcription is the most important point of regulation, post-transcriptional silencing *via* different mechanisms also takes place in plants (Baulcombe 2004).

It has been estimated that *Arabidopsis* and rice have between 1300 and 1500 transcription factors encoding genes (Riechmann *et al*. 2000; Goff *et al*. 2002). Several experiments of transcriptome analysis have revealed that a large number of these transcription factors are induced or repressed by a range of environmental stresses. The response regulatory network turned out to be very complex and stress tolerance and resistance seem to be most controlled at the transcriptional level (Chen and Zhu 2004), what depends largely on the action of transcription factors. Moreover, the transcriptome comparison of plants under alternative stress treatments and even those including the combination of different stresses shed light on the functional basis of multiple stress tolerance (Mittler 2006; Swindell 2006).

A considerable number of transcription factors families have been implicated in plant stress responses, considering that their expression is induced or repressed under different stress conditions (Shinozaki *et al*. 2003). Each of these stress-related transcription factors family exhibits a distinctive DNA binding domain, such as NAC, ERF/AP2, Zn-finger, DOF, Myb, WRKY, b-Zip and HD-Zip. In this review we talk about HD-Zip I and II proteins, which belong to the Superfamily of Homeodomain Containing Proteins.

Review

HOMEOBOXES AND HOMEODOMAINS

Homeobox containing genes were first identified in the study of homeotic mutants in *Drosophila* and turned out to be present in evolutionary distant organisms, including animals, fungi and plants (Gehring 1987). The homeobox (HB) is a 180 bp consensus DNA sequence present in a number of genes involved in developmental processes. It encodes a 60 amino acid protein motif, the homeodomain (HD), which folds into a characteristic DNA-binding structure (Qian *et al.* 1989; Gehring *et al.* 1994; Tsao *et al.* 1995). The compilation of known HD sequences indicates that seven positions are occupied by the same amino acid in more than 95%, ten other positions are conserved in more than 80%, and 12 additional ones present only two amino acids in more than 80%. The HD folds into a characteristic three α-helix structure. Helix I and II are connected by a loop, while helix II and III are separated by a turn which makes this region of the HD bear a resemblance to prokaryotic helix-turn-helix transcription factors. Most HDs are able to bind DNA as monomers with high affinity, through interactions established by helix III (the so-called recognition helix) and a disordered N-terminal arm located beyond helix I (Kissinger *et al.* 1990; Otting *et al.* 1990; Wolberger *et al.* 1991; Gehring *et al.* 1994; Bellaoui *et al.* 2001).

PLANT HOMEOBOX GENES

In plants, the first identified HB containing gene was *KNOTTED1* (*KN1*), a maize gene for which dominant mutations affect leaf development (Vollbrecht *et al.* 1991). The knotted leaf phenotype is due to the ectopic expression in leaves of the *KN1* gene, whose activity is normally restricted to meristematic cells (Smith *et al.* 1992). Since the isolation of *KN1*, HD encoding genes have been identified in a wide range of distantly related plant species, revealing the existence of a large transcription factors superfamily throughout the plant kingdom (Ruberti *et al.* 1992; Schena and Davis 1992; Chan and Gonzalez 1994; Krusell *et al.* 1997; Nagasaki *et al.* 2001; Sakamoto *et al.* 2001; Smith *et al.* 2002). It is worth mentioning that although HDs in plants have been named after the previously identified proteins from the animal kingdom, no homeotic effect has been yet described as a result of the ectopic expression or mutation of any plant HD transcription factor.

Plant HD-containing proteins have been classified into several families and subfamilies according to their sequence conservation within the HD and other conserved domains. Based on the description of model plants genomes and the increasing knowledge of proteins from other species, the following six families have been identified: KNOX, BELL, PHD-finger, ZF-HD, WOX and HD-Zip (Chan *et al.* 1998; Ariel *et al.* 2007).

THE ASSOCIATION OF A HOMEODOMAIN WITH A LEUCINE ZIPPER IS UNIQUE TO PLANTS

Homeodomain-leucine zipper (HD-Zip) proteins constitute one of the homeodomain-containing transcription factors families. These proteins are characterized by the presence of a specific DNA-binding domain, the HD, associated to an adjacent dimerization motif, the leucine zipper (LZ). Both, HDs and LZs are common motifs present by themselves in other eukaryotic kingdoms, such as animals or fungi. However, the association of these two motifs in a single transcription factor is apparently a characteristic unique to plants. HD-Zip proteins can be classified into four subfamilies according to the conservation within the conforming domains, their target DNA sequences, gene structures and the roles they exert. A large number of members have been identified from different plant species, and their precise number has been determined only for those plants whose genomes have been fully described (Ariel *et al.* 2007).

FUNCTIONAL CHARACTERIZATION OF MEMBERS BELONGING TO SUBFAMILIES I AND II

In the past decade, it was suggested that HD-Zip I and II proteins are involved in the regulation of developmental processes associated with the response of plants to environmental conditions (Schena and Davis 1992). Since then, the experimental data obtained from expression studies and the use of transgenic plants overexpressing these transcription factors have supported this first hypothesis; it is by now known that members of this family are regulated by abiotic factors and it has been shown that their overexpression results in an altered plant developmental response to alternative environmental conditions.

HAT4/ATHB2 was described by Shena *et al.* (1993) as a developmental regulator. These authors obtained transgenic plants bearing constructs that alter this gene expression exhibiting a series of interesting developmental phenotypes, including changes in morphology and developmental rate. Notably, the observed phenotypical changes were dependent on illumination conditions. Meanwhile, an independent research group described that this gene is expressed during the vegetative and reproductive phases of plant growth as well as it is done by another member of subfamily II, *ATHB4*. A significant increment in transcripts levels for these two genes was observed in flowering and in dark-adapted plants. Remarkably, far-red-rich light treatment resulted in a rapid and strong induction of both genes expression in accordance with the observations of Schena *et al.* in transgenic plants (Carabelli *et al.* 1993). Later, further studies indicated that *ATHB2* expression is induced by the ratio of red/far red light and high levels of the corresponding transcript are present in rapidly elongating etiolated seedlings. Phytochromes A and B were shown to be involved in such regulation (Steindler *et al.* 1997). The observations done in phytochromes null mutants together with a strict correlation of this gene expression with FR-induced growth phenomena suggested a role for the *ATHB2* gene in mediating cell elongation (Carabelli *et al.* 1996). In 1999, the same research group showed that elevated *ATHB2* levels inhibit cotyledon expansion by restricting cell elongation in the cotyledon-length and width direction as well as enhance longitudinal cell expansion in the hypocotyl. Interestingly, these authors found that this gene is induced by shade, accordingly inhibiting specific cell proliferation such as secondary growth of the vascular system and lateral root formation. They finally proposed that *ATHB2* functions as a negative regulator of gene expression in the shade avoidance response (Steindler *et al.* 1999). *ATHB16*, another member of subfamily II regulates leaf development and the sensitivity to photoperiod in *Arabidopsis* (Wang *et al.* 2003). Similar results were obtained when the sunflower HD-Zip II encoding gene *HAHB10* was isolated and characterized (Rueda *et al.* 2005). Transgenic plants overexpressing it exhibit altered responses to illumination conditions resembling the behavior of *ATHB2/HAT4* transgenic plants.

In 1994 *ATHB5*, *ATHB6* and *ATHB7* were isolated using a partial *ATHB3* clone as probe. Expression patterns of these genes were characterized in vegetative organs and also in flowers (Söderman *et al.* 1994). *ATHB7* expression was induced several-fold by water deficit, osmotic stress as well as by exogenous treatment with abscisic acid (ABA) and was rapidly reduced after removal of the hormone, indicating that the regulation is mediated strictly via ABA. Furthermore, no induction of *ATHB7* was detectable in the ABA-deficient mutant *aba3* subjected to drought treatment (Söderman *et al.* 1996). A similar behavior was shown by Lee and Chun (1998) for *ATHB12*, the *ATHB7* closest member of subfamily I. Both genes were shown to be negative developmental regulators in response to water deficit using transgenic plants either in WT genotype or in *abi* mutants (Olsson *et al.* 2004). Using a translational fusion with the reporter gene *GUS*, it was demonstrated that *ATHB6* (a member of subfamily I) expression is also up regulated by water deficit modulating hormone responses in *Arabidopsis*

(Söderman *et al*. 1999; Himmelbach *et al*. 2002). This research group also analyzed *ATHB13* over expressing transgenic plants and showed that they exhibit sugar-dependent alterations in cotyledon and leaf development (Hanson *et al*. 2001).

Five members of HD-Zip I and II subfamilies from *Craterostigma plantagineum* were isolated and characterized, all of them are up or down-regulated by water deficit in leaves and roots in early developmental stages as well as by ABA. The authors proposed that their results support the role of HD-Zip proteins in regulating programs of gene expression in *C. plantagineum* that lead to desiccation tolerance (Deng *et al*. 2002). A similar behavior in response to hydric stress was shown by the sunflower *HAHB4* subfamily I member (Gago *et al*. 2002) and later demonstrated that it confers drought tolerance when overexpressed in transgenic plants (Dezar *et al*. 2005a).

Summarizing these findings it is clear that the hypothesis suggesting that these TFs regulate development in response to environmental conditions was not wide off the mark.

FUNCTIONAL CHARACTERIZATION OF MEMBERS BELONGING TO SUBFAMILIES III AND VI

HD-Zip III and IV subfamilies have been more deeply described, especially in *Aarabidopsis* (Prigge *et al*. 2005; Nakamura *et al*. 2006). However, none of them participate in the response of the plant to environmental conditions. HD-Zip III members are functionally well characterized as development directors of the apical meristem, the vascular bundles, the adaxial domains of lateral organs and vascular development. IFL1 (INTERFASCICULAR FIBERLESS 1) regulates the spatial specification of interfascicular fiber differentiation; *ifl1* mutants exhibit abnormal stem interfascicular fibers (Zhong and Ye 1999). PHABULOSA and PHAVOLUTA, both members of subfamily III, are implicated in the perception of radial positional information in the leaf primordium; their dominant mutants (*phb* and *phv*) show a dramatic transformation of abaxial into adaxial leaf fate (McConnell *et al*. 2001; Emery *et al*. 2003). REVOLUTA, another member of this subfamily, is required for the initiation of the lateral shoot and the flower meristems, activating the expression of known meristem regulators (Otsuga *et al*. 2001). Ectopic expression of *ATHB8* increases the production of xylem tissue: a careful anatomical analysis of the transgenic plants indicated that the overexpression of this HD-Zip III protein promotes vascular cell differen-

Fig. 1 Members of the Plant HD Superfamily and physiological events in which HD-Zip proteins participate.

tiation (Baima *et al*. 2001) *ATHB15* promoter analysis strongly suggested that this gene is a pivotal transcriptional regulator responsible for early vascular development (Ohashi-Ito and Fukuda 2003).

HD-Zip IV proteins are generally involved in epidermal cell fate determination and in the regulation of cell layer-specific gene expression. *GLABRA2* (*GL2*) is required for normal trichome development in *Arabidopsis*, mutation of this gene results in an abnormal trichome expansion (Rerie *et al*. 1994). Other members of this family affect anthocyanin accumulation of the leaf subepidermal layer and root identity. Abe *et al*. (2003) demonstrated that the 8 bp L1 box functions as a *cis*-acting element for outermost cell layer-specific gene expression in the shoot system and it was actually demonstrated that PDF2 (PROTODERMAL FACTOR 2) specifically binds to the L1 box *in vitro*. Although a knockout mutant did not display an abnormal phenotype, the double mutant *pdf2* and *atml1* shows severe defects in shoot epidermal cell differentiation. These authors evidenced that the two genes are interchangeable and play a critical role in maintaining the identity o L1 cells (Abe *et al*. 2003).

Fig. 1 exhibits a schematic representation of the processes in which animal and plant homeodomain containing proteins are involved pointing specially in those where HD-Zip proteins take part. Knowledge about functional characterization of members of subfamilies III and IV are detailed in the last two paragraphs while the mechanisms in which HD-Zip I and II participate in response to environmental factors and abiotic stress are partially described earlier and in more detail all throughout this review.

STRUCTURE AND PHYLOGENETIC ANALYSIS OF HD-ZIP I AND II TRANSCRIPTION FACTORS

HD-Zip proteins belonging to subfamilies I and II are about 300 amino acids long and their HD-Zip motif is located in the centre of the molecule. Subfamily I members from the same plant species are not conserved outside the HD-Zip and conservation inside this region is lower than in mem-
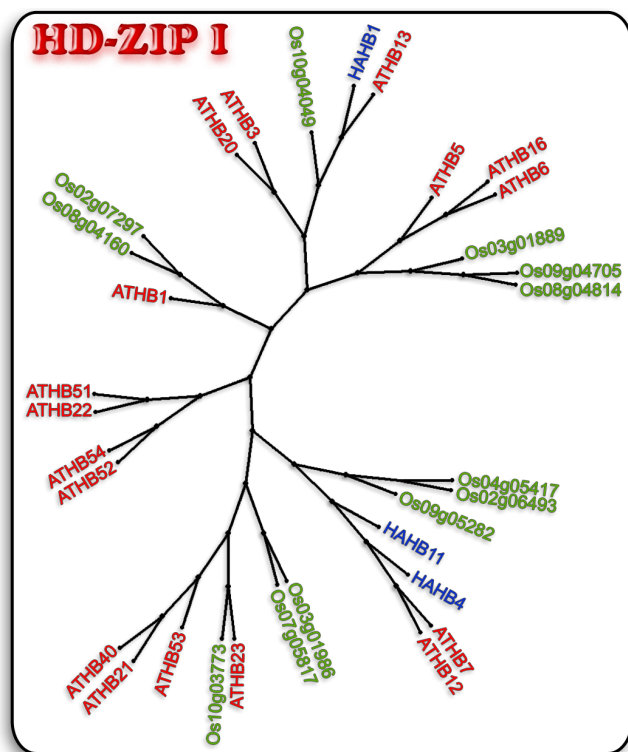

Fig. 3 Phylogenetic tree of plant HD-Zip II sequences. Phylogenetic tree of plant HD-Zip proteins based on the alignment of sequences from *Arabidopsis* (red), rice (green) and sunflower (blue) by using the program Tree-Puzzle 5.2 (program designed and described by Schmidt 2002).
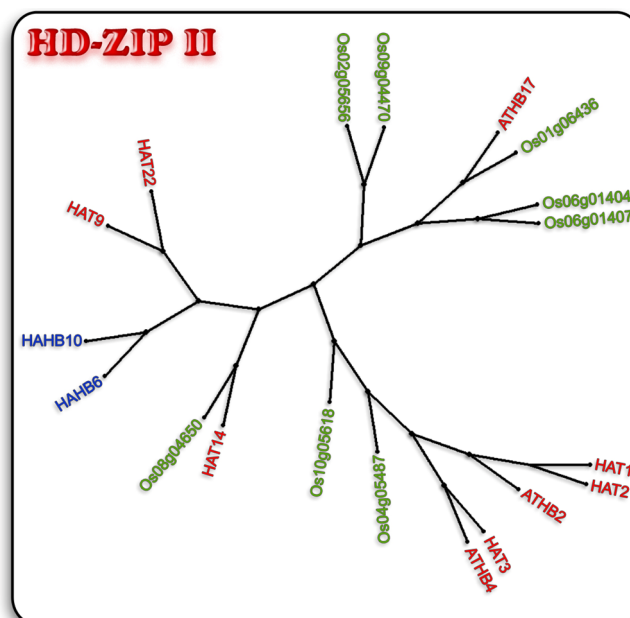
bers of subfamily II (Schena and Davis 1994). Most members of subfamily I exhibit between 75 and 85% homology with the HD consensus sequence. However, there are some divergent members as HAHB4, OSHOX6, HBLZP, ATHB7 and ATHB12 presenting lower homology values (49-65%). Regarding the LZ, no conserved residues outside the leucines positioned in d_1 to d_6 were detected in phylogenetic analysis, neither additional conserved motifs outside the HD-Zip. On the other hand, HD-Zip II proteins exhibit two external conserved motifs, the CPSCE (a conserved cysteine-containing motif within a variable region) adjacent downstream the LZ, and a common N-terminal consensus. The CPSCE is probably responsible for redox cell state sensing. Under oxidizing conditions, these proteins form multimers through intermolecular disulfide bonds. Mutation of these cysteins to serines activates these transcription factors allowing DNA binding even in oxidizing conditions and suggesting that a redox-dependent mechanism may operate *in vivo* to modulate the activity of these transcription factors in response to metabolic and/or environmental signals (Tron *et al*. 2002).

A protein phylogenetic analysis in *Arabidopsis* enabled Henriksson and coworkers (2005) to classify HD-Zip I members in a set of subclasses. Considering the gene structure, the intron/exon distribution within the HD-Zip encoding region is in complete accordance with the phylogenetic sub-classification. **Figs. 2** and **3** show a phylogenetic tree constructed with the sequence of HD-Zip I proteins or HD-Zip II proteins respectively from *Arabidopsis*, rice and sunflower using the Tree-Puzzle 5.2 program (Schmidt *et al*. 2002).

HD-ZIP I AND II BIND RELATED DNA SEQUENCES IN THEIR TARGET GENES

In contrast to the rest of HD-containing proteins, HD-Zip transcription factors only bind DNA as dimers. This dimerization occurs as the result of the interaction of two leucine-zippers (Sessa *et al*. 1993; Palena *et al*. 1999). The removal of the LZ or the introduction of extra amino acids between the zipper and the HD causes a complete loss of binding, indicating that the relative orientation of the monomers is essential for an efficient recognition of DNA (Gonzalez *et al*. 1997; Palena *et al*. 1998).

HD-Zip I and II proteins bind to a 9-bp pseudopalindro-

Fig. 2 Phylogenetic tree of plant HD-Zip I sequences. Phylogenetic tree of plant HD-Zip proteins based on the alignment of sequences from *Arabidopsis* (red), rice (green) and sunflower (blue) by using the program Tree-Puzzle 5.2 (program designed and described by Schmidt 2002).
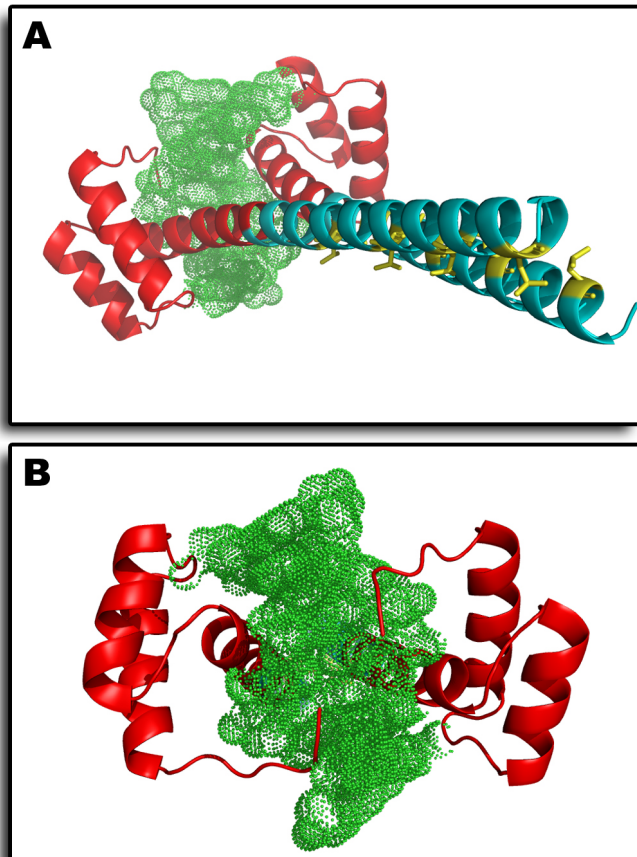
Fig. 4 Schematic representation of the interaction between an HD-Zip protein and its target DNA sequence. Crystallographic models for a b-Zip transcription factor (PDB ID:1gu4) and the HD of ENGRAILED were combined into a single homology model for the HD-Zip HAHB4 using the package Modeller 9v1. Different restrains were applied while building the model; monomers were supposed to be identical and the secondary structure of HD helices was constrained as well. Moreover, DNA was considered as a rigid body. Pictures were made with Pymol v0.99 software; DNA is represented as green dots showing van der Waals radii, HD is depicted in red and LZ in cyan as cartoons, and LZ leucines as yellow sticks.

mic sequence of the type CAAT(N)ATTG, which can be regarded as composed of two partially overlapping TNA TTG sequences (Sessa *et al*. 1993). It has been postulated that each monomer interacts with one of these half-sequences in a way that resembles the interaction of monomeric animal HDs with DNA (TAATNN). HD-Zip I and II proteins prefer different nucleotides at the central position of the recognition sequence (A/T and G/C, respectively). The specificity for binding at the central position seems to be conferred in part by amino acids 46 and 56 of helix III (ala and trp in HD-Zip I; glu and thr in HD-Zip II), together with a different orientation of the conserved arg55 in both proteins, which would be directly responsible for the interaction (Sessa *et al*. 1997).

Up to now, no members from the HD-Zip family have been crystallized. **Fig. 4** shows a virtual chimera between a typical HD and the LZ from a b-Zip protein, binding as a dimmer its DNA target sequence (Sali and Blundell 1993; Eswar *et al*. 2006).

Hydroxyl radical footprinting protection and interference techniques have been employed to analyze the interaction of the sunflower HD-Zip proteins HAHB4 and HAH B10, which belong to classes I and II, respectively, with target sites containing A/T or G/C base pairs at the central position. The results are indicative of a different orientation of each HD present in the dimer respective to the TNATTG half-sequence it recognizes. The nucleotide present at the central position of each strand in both target sites would be in part responsible for this behavior (Tron *et al*. 2005).

GENES ENCODING HD-ZIP I AND II PROTEINS ARE REGULATED BY ENVIRONMENTAL FACTORS

In *Arabidopsis thaliana* 17 members of subfamily I and 9 of subfamily II have been identified. These genes are distantly related to the subfamilies III and IV members and share a common origin. They are not as well functionally characterized as other plant HD containing transcription factors but the available information indicates that they would mediate the effects of environmental conditions to regulate growth and development in plants. Examples of such regulation are given in **Table 1**.

Similar but not identical roles in light responses were described for *ATHB2/HAT4*, from *Arabidopsis* and *HAHB10*, from sunflower, both members of the subfamily II. Their expression pattern is quite alike, since they are both present in photosynthetic tissues. The overexpression of both genes in *Arabidopsis* plants confers a very similar phenotype, which reveals that they are involved in the elongation of the hypocotyls. It has been clearly stated that *ATHB2* participates in the shade avoidance response: plants expressing a *HAT4/ATHB2* antisense construct are shorter and develop slower than normal, whereas those constitutively expressing a *HAT4/ATHB2* sense construct show a shade avoidance phenotype characterized by elongated hypocotyls and petioles, as well as earlier flowering (Schena *et al*. 1993; Ohgishi *et al*. 2001). *HAHB10*, another member of this family is up regulated by etiolation in seedlings. When this gene was overexpressed in *Arabidopsis*, leaf shape and color, growth rate, flowering time and life cycle were affected under standard growth conditions. Remarkably, these plants in the stage of seedlings are almost insensitive to darkness and in the vegetative and reproductive stages to low illumination intensities. These altered responses to changes in illumination quality and intensity, indicate a participation of this gene in shade avoidance. Transduction pathways in which these genes are involved are not elucidated and due to the complexity of this type of response, further efforts should be devoted to unravel the functional participating mechanisms (Rueda *et al*. 2005).

Regarding the action of HD-Zip I encoding genes in response to light, *ATHB1* and *16* were also proposed to be regulators of different developmental events in response to

Table 1 Examples of HD-Zip I and II encoding genes regulated by abiotic agents.

Gene name	Subfamily	Plant	Environmental factor regulating expression	Reference
ATHB2/HAT 4	II	AT	Light	Schena *et al*. 1993; Steindler *et al*. 1997
HAHB10	II	HA	Light	Rueda *et al*. 2005
ATHB1/ATHB16	I	AT	Light, salt, low temperature	Wang *et al*. 2003; Henriksson *et al*. 2005
ATHB13	I	AT	Sugar signaling	Hanson *et al*. 2001
ATHB7/ATHB12	I	AT	ABA, salt, low temperature	Söderman *et al*. 1996; Lee and Chun 1998
HAHB4	I	HA	ABA, drought, salt	Gago *et al*. 2002
ATHB6/ATHB40/ATHB53	I	AT	ABA, salt, low temperature	Söderman *et al*. 1999; Himmelbach *et al*. 2002; Henriksson *et al*. 2005
ATHB5/ATHB21	I	AT	ABA, salt	Henriksson *et al*. 2005
ATHB52	I	AT	Low temperature	Henriksson *et al*. 2005

light quality and intensity. The first of them participates in the de-etiolation of dark-grown seedlings (Aoyama *et al.* 1995), whereas the second one is involved in blue-light signaling and leaf cell expansion regulation (Wang *et al.* 2003). Both genes are induced also by darkness, as well as *ATHB3, 23, 52* and *53*. Blue-light conditions also stimulate the expression of *ATHB52* and inhibit the expression of *ATHB1*, while *ATHB5, 6, 7, 12, 13* and *20* are down-regulated both by darkness and blue-light conditions (Henriksson *et al.* 2005).

Treatment of *Arabidopsis* plants with 10 μM ABA or 100 mM NaCl resulted in an up-regulation of *ATHB7* and *12* transcript levels by a factor of 12-25 times in relation to the untreated control. Expression of *ATHB6, 21, 40*, and *53* also increased after these courses of treatment but by a lower factor (approximately 2-fold) in comparison to the levels measured in control grown plants. Repression of *ATHB3, 5, 23* and *52* to approximately the half of its normal expression was observed in the same treated plants. *ATHB1* and *16* had their transcript levels reduced when plants were treated with salt but not in response to ABA.

Low temperature (2-4ºC during 4 hrs) exposure produces up regulation of *ATHB6, 7, 12, 40* and *53* (2-4–fold), while *1, 16* and *52* expression was reduced under the same conditions to the half of its normal expression (Henriksson *et al.* 2005). *ATHB5, 6, 7* and *12* are either down or up regulated by water deficit conditions (Söderman *et al.* 1996; Lee and Chun 1998; Henriksson *et al.* 2005). The overlapping, similar or distinct expression of HD-Zip I genes in response to NaCl, low temperature and water deficit is a sign that they are all involved in the water status maintenance of the plant, since these three types of stress decrease the availability of water to plant cells (Verslues *et al.* 2006).

The results presented by Henriksson *et al.* (2005), in addition to previously reported ones indicate that the majority of the HD-Zip I genes are responsive to one of the external conditions applied including ABA, water deficit stress, salt, cold and light. Expression analysis together with plant transformation with this type of genes offered experimental support to the initial theory introduced by Schena and Davis (1992), which suggested that HD-Zip I and II proteins regulate plant development in response to environmental conditions.

HD-ZIP I PROTEINS AND STRESS TOLERANCE

In other species like sunflower (*Helianthus annuus*), rice (*Oryza sativa*), hot pepper (*Capsicum annum*) and barley (*Hordeum vulgare*), several HD-Zip encoding genes were isolated and turned out to be regulated by abiotic stress, mediated or not by ABA (Meijer *et al.* 2000; Gago *et al.* 2002; Sawa *et al.* 2002; Yi *et al.* 2004). However, over-expression experiments have been carried out with a reduced number of HD-Zip genes responding to abiotic stress. For instance, *ATHB7* and *12* were over-expressed in *Arabidopsis* resulting in phenotypically altered plants that did not show any enhanced stress tolerance (Olsson *et al.* 2004). HAHB4 is the sunflower HD-Zip protein most related to ATHB7 and 12, being the three of them, together with OSHOX6 from rice (Meijer *et al.* 2000) and HPLZP from *Prunus americana* (Acc Nº AF139497) in the same branch of the phylogenetic tree (Gago *et al.* 2002). However, over-expression of sunflower *HAHB4* led authors to obtain drought-tolerant *Arabidopsis* plants which only share some morphological characteristics with *ATHB7* and *12* over-expressing transgenic plants, as well as a marked delay in development (Dezar *et al.* 2005a). In spite of these undesired characteristics observed, once stress tolerance was achieved, these characteristics could be avoided changing the constitutive promoter by another inducible under stress conditions, saving in this form in the metabolic costs due to the constitutive expression and thus improving the biotechnological system. In the case of *HAHB4*, changing the constitutive promoter 35S CaMV used in preliminary essays by a stress-inducible one resulted in transgenic plants with relatively enhanced stress tolerance, but phenotypically indistinguishable from wild type ones (Manavella *et al.* 2006). The best genic construction turned out to be a chimera bearing the *HAHB4* promoter, followed by the intron of the *Arabidopsis* gene *Cox5c*, which enhanced the basal expression of the gene. This resulted in phenotypically normal plants with the highest water stress tolerance (Cabello *et al.* 2007). The tolerance conferred by *HAHB4* was explained by its participation in the synthesis and response to 10-20 μM ethylene. Overexpressing plants are less sensitive to external ethylene, enter the senescence pathway later and do not show the typical triple response. Transcriptome analysis of these plants revealed that HAHB4 has a repressive effect on genes related to ethylene synthesis and signaling. This gene is up regulated by ethylene or its precursor ACC in its genomic environment slowing the ethylene-mediated senescence entry (Manavella *et al.* 2006).

All the experimental results obtained up to now indicate that HD-Zip coding genes may be excellent biotechnological tools under the control of inducible promoters instead of constitutive ones.

FUTURE PERSPECTIVES

The knowledge about gene expression in response to abiotic stress, and the way they participate in each mechanism keeps enlarging, and it would be of great consequences if more efforts were invested in both, breeding and genetic manipulation programs. Identification of transcription factors, able to switch defense responses, will contribute to obtain potential biotechnological tools. In this sense, HD-Zip proteins appear to be good candidates to confer tolerance to a combination of abiotic stresses, as they actually have place in the natural environment (Mittler *et al.* 2006). Research on the genes that encode such proteins ought to be taken further, including also different species from the usual models. Moreover, it keeps unclear what effect may have the expression of a heterologous gene in each plant species. A complete characterization of promoters and the analysis of their activities in transgenic plants will be vital to achieve success. Physiological studies combined with molecular research will aid to a better comprehension of the system as a whole. Besides, regarding the use of transgenic crops, it is of great importance to understand which additional mechanisms the transgene may unchain, so as to guarantee the quality of the product. Proper research will enable humanity to satisfy the increasing food demand by the achievement of highly productive and safe crops.

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