Critical Review

Plant Transcription Factors from the Homeodomain-Leucine Zipper Family I. Role in Development and Stress Responses

María Florencia Perotti
Pamela Anahí Ribone
Raquel Lía Chan *

Instituto de Agrobiotecnología del Litoral Universidad Nacional del Litoral, CONICET, Centro Científico Tecnológico CONICET Santa Fe, Santa Fe, Argentina

Abstract

In front of stressful conditions plants display adaptation mechanisms leading to changes in their morphology, physiology, development and molecular composition. Transcription factors (TFs) play crucial roles in these complex adaptation processes. This work is focused in the homeodomain-leucine zipper I (HD-Zip I) family of TFs, unique to plants. First discovered in 1991, they were identified and isolated from monocotyledonous and dicotyledonous plants showing high structural similarity and diversified functions. These TFs have, besides the homeodomain and leucine zipper, conserved motifs in their carboxy-termini allowing the interaction with the basal machinery and with other regulatory proteins. The model dicotyledonous plant *Arabidopsis thaliana* has 17 HD-Zip I members; most of them regulated by external stimuli and hormones. These TFs are involved in key developmental processes like root and stem elongation, rosette leaves morphology determination, inflorescence stem branching, flowering and pollen hydration. Moreover, they are key players in responses to environmental stresses and illumination conditions. Several HD-Zip I encoding genes from different species were protected in patents because their overexpression or mutation generates improved agronomical phenotypes. Here we discuss many aspects about these TFs including structural features, biological functions and their utilization as biotechnological tools to improve crops. © 2017 IUBMB Life, 69(5):280–289, 2017

Keywords: *transcription factors; homeodomain-leucine zipper; HD-Zip I;* Arabidopsis thaliana*; biotechnological tools.*

General Introduction

Plants are different from animals in many aspects. They did not evolve movement to escape adverse environmental conditions and continuously develop new organs during their life cycle. Why these organisms did not evolve movement? Most likely because they are able to convert light energy in carbohydrates, and light is ubiquitous worldwide. Anyway, although they lack the ability to move, plants adapt themselves to the surrounding environment and, nowadays, more than 300,000 plant species inhabit the world (1). For such adaptation plants display morphological, biochemical, physiological and molecular changes governed by sophisticated regulatory machinery that in most cases responds to the environmental conditions. This machinery acts at different levels and several actors play significant roles. Notably, in plants, the transcriptional level takes a more leading role compared with organisms from other kingdoms (2–4). At this level, both transcription factors (TFs) and *cis*-acting elements present in the regulatory regions of target genes are essential.

Abbreviations: *HD, homeodomain; Zip, leucine zipper; TF, transcription factor; AHA, aromatic large hydrophobic acidic residues; ABA, abscisic acid; PP2C, protein phosphatases type 2C; AUX, auxin; BDL, BONDELOS; IAA, indole-3-acetic acid; GLU, glucanase; WT, wild type; GA, gibberellins; CTR, carboxyl-terminus region; NTR, amino-terminus region*

Volume 69, Number 5, May 2017, Pages 280–289

**Address correspondence to: Raquel Lía Chan, Instituto de Agrobiotecnología del Litoral, CONICET-UNL, Centro Científico Tecnológico CONICET Santa Fe, Colectora Ruta Nac. N° 168 km. 0, Paraje El Pozo, 3000 Santa Fe, Argentina. Tel: +54-342-451-1370, ext: 5018. Fax: +54-342-451-1370.*
E-mail: rchan@fbcb.unl.edu.ar
M.F.P. and P.A.R. contributed equally to this work.

Received 30 December 2016; Accepted 24 February 2017
DOI 10.1002/iub.1619
Published online 24 March 2017 in Wiley Online Library (wileyonlinelibrary.com)

Plant TFs

TFs are modular proteins possessing at least two different types of domains: a DNA-binding domain, which recognizes and binds specific *cis*-acting elements, present in the regulatory regions of their target genes, and a protein–protein interaction domain (5). TFs are able to change the cell transcriptome through a very intricate network, which includes activation and repression of specific targets that, in place, activate or repress other targets.

Besides the differences in their DNA target sequence, the regulatory possibilities of TFs are extended by several factors. For instance, many TFs can be post-translationally modified by phosphorylation, sumoylation or ubiquitination (6). Furthermore, among TFs, an important group is also able to form homo- or heterodimers, which diversifies even more their regulatory capabilities.

TFs are classified in families and subfamilies mostly according to their DNA-binding domains (2). However, other structural and functional features are also important for such classification.

As it was mentioned before, transcriptional regulation plays a leading role in plants and 3%–6% of genes encode TFs, a significantly higher proportion than in animals. Around 1,500 TFs have been identified in the *Arabidopsis* (*Arabidopsis thaliana*) genome (2) and 1,600 in that of rice (*Oryza sativa*) (3). Although around 45% plant TFs belong to families also existing in other kingdoms, notably, the number of members of such families is significantly enlarged in plants. This can suggest the existence of specific regulatory pathways in these organisms (7). As plants are continuously exposed to environmental changes, they alter their growth and architecture in response to such alterations. Many TFs have crucial roles both in development and in adaptation responses, whereas others act only when an external factor stimulates them.

In this review, we will focus on one of the TF groups, unique to plants, the homeodomain (HD)-leucine zipper I (Zip I; HD-Zip I) family. The choice of this group was based on the fact that these TFs play essential roles in development in response to environmental conditions, and among them, a few became biotechnological tools to improve crops. It is important to note that most of the knowledge about biological roles of these TFs originated in studies performed in model plants such as *Arabidopsis* and rice. However, some studies about HD-Zip TFs from other species will be commented.

HD-Zip Family

HDs as DNA-binding domains were first discovered in animals as closely related to developmental events. Moreover, these domains were named HDs because their mutation or ectopic expression causes the change in body segment by another, producing a homeotic effect (8). The HD is a conserved 60 amino-acid motif that folds into a bundle of three α helices (named I to III), connected by a loop and a turn. HDs are highly conserved between proteins from different kingdoms, indicating that their structure is crucial to maintain functionality.

In 1991 it was reported the identification of the first plant TF containing a HD, the maize KNOTTED1, mainly involved in meristem maintenance (9). Its name was given because its ectopic expression induces areas of irregular cell division along secondary veins of the lamina producing “knotted” leaves. One year later, AtHB-1, a HD-Zip TF, was identified in *Arabidopsis* by Ruberti et al. (10) and since then, many HD-containing genes have been identified and isolated from a wide variety of monocotyledonous and dicotyledonous plant species (11). These TFs were proposed to be key players in plant specific developmental processes, such as those associated to external stimuli and stresses. However, plant TFs containing HDs did not exhibit canonical homeotic effects when they were over- or ectopically expressed like those from animals.

Particular kind of HD-containing genes are the HD-Zip TFs. These proteins have a unique association of a HD, which is the binding domain and a Zip located downstream the HD, which acts as a dimerization motif (12,13). Although both HD and Zip can be found alone or associated to other motifs in TFs from other kingdoms, this association is unique to plants.

The Zip folds into an α-helix having a leucine in each seventh position on the same side of the helix. This three-dimensional structure allows the formation of dimers through hydrophobic interactions (12,14). The efficient recognition and DNA binding depends on the relative orientation of the monomers (15).

After the identification of the first plant HD-Zip TF, further studies conducted worldwide by several research groups lead to the discovery of TFs presenting this association of domains in all plant species in which they were searched for. Their structural and functional characterization lead to a classification in four subfamilies named I–IV (15). This review will be focused on subfamily I because the knowledge about the functions of these TFs significantly grew in the last few years revealing essential roles in plant development and stress responses.

Subfamily HD-Zip I

Members of this subfamily encode approximately 35 KDa proteins. In these TFs the HD is highly conserved whereas the Zip is more diversified (16). *In vitro* assays allowed to determine that these proteins preferably bind the pseudopalindromic sequence CAAT(A/T)ATTG and that the dimerization through the Zip is a prerequisite for DNA binding (12,17,18). The affinity of the protein for a certain DNA sequence, but not the specificity, is affected by the amino acids of the HD N-terminal arm (19). More recently, using a novel technique to determine TFs binding sites in a whole genome, O’Malley et al. identified the pseudopalindrome AAT(N)ATT as the target sequence of HD-Zip I TFs, which shows a perfect coincidence with the core of the previously determined sequence (20).

A recent survey performed with the complete sequences of HD-Zip I proteins from several species allowed the

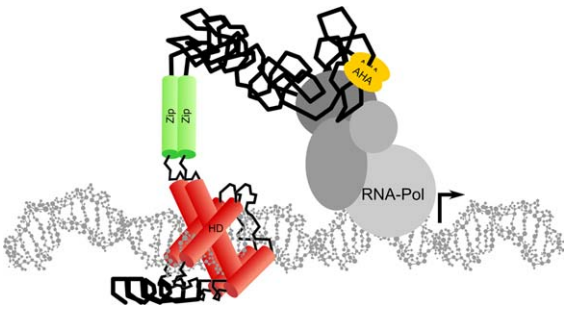

FIG 1 *Schematic representation of the structure of HD-Zip I TFs. HD-Zip I proteins recognize DNA by the HD helix III (red), forming homo- or heterodimers through the ZIP (green). Transcription of the targets is activated by the interaction of the AHA motif (yellow) with the basal transcription machinery (grey).*

identification of other conserved motifs. Particularly, an aromatic large hydrophobic acidic residues (AHA) transactivation motif was found in the C-terminal region of these proteins (Fig. 1) (21). AHA motifs were first described as activation motifs present in tomato heat stress factors TFs (22). These motifs form an amphipathic and negatively charged helix to contact components of the basal transcription complex (23).

Arabidopsis HD-Zip I Members

Why *Arabidopsis*? *Arabidopsis* was chosen as model plant due to several reasons: its rather small genome was the first one to be completely sequenced, it can be routinely transformed, it is a small plant allowing multiple experiments in reduced spaces and there are many available tools that facilitate molecular and physiological studies such as ample databases and mutant depositories. Given these characteristics, HD-Zip TFs from this species were the best studied. In *Arabidopsis*, HD-Zip subfamily I is composed of 17 members named ATHB1/HAT5, ATHB3/HAT7, ATHB5–ATHB7, ATHB12, ATHB13, ATHB16, ATHB20–ATHB23, ATHB40 and ATHB51–ATHB54, which have been classified in six groups according to phylogenetic relationships and intron/exon patterns (24).

Biological Functions of HD-Zip I TFs

After the discovery of HD-Zip proteins in 1991 by Ruberti et al. (10), many HD-Zip encoding genes had been identified and isolated from *A. thaliana* and other species such as sunflower (25), tomato (*Lycopersicum esculentum*) (26,27), carrot (*Daucus carota*) (28), cucumber (*Cucumis sativus*) (29), cotton (*Gossypium* sp) (30) and rice (*O. sativa*) (31), among others (32–34).

The initial investigations were mainly focused on the elucidation of some genetic and biochemical properties of these newly identified TFs (12,35). The first functional study about AtHB-1 (called now AtHB1 according to international nomenclature rules) was conducted by Aoyama and coworkers in 1995 and since then, several works were devoted to describe the function of this kind of proteins (36–41).

Although at first of these TFs were mainly involved in the responses to abiotic stress conditions, further studies indicated that HD-Zip I TFs play important roles also in other plant processes, such as development or biotic stress responses (11).

Functions in Hormone Signalling Pathways

Several reports linked HD-Zip I TFs with hormone signalling pathways, especially with abscisic acid (ABA) sensing and transduction (24). *AtHB7* and *AtHB12* are paralogous genes with a high sequence similarity and this pair of members was the most deeply characterized. Both genes are upregulated in plants subjected to drought and after ABA treatments (38,42,43). Moreover, when *AtHB7* and *AtHB12* were overexpressed in *Arabidopsis*, they conferred a hypersensitive response to ABA in root elongation assays, a delay in inflorescence stem elongation, rounder rosette leaves, shorter petioles and enhanced branching in the inflorescence stem (38). Later it was demonstrated that ATHB7 and ATHB12 positively regulate protein phosphatases type 2C (*PP2C*) and repress PYL5 and PYL8, two ABA receptors, in response to ABA stimulus (44) (Fig. 2).

Other HD-Zip I encoding genes participating in ABA responses are *AtHB5*, *AtHB6* and *AtHB20*. Seedlings overexpressing *AtHB5* displayed an increased sensitivity to ABA which allowed proposing this TF as a positive regulator of ABA response (45) (Fig. 2). In a more recent work *AtHB5* was also involved in auxin (AUX) responses, as it was reported that *BDL* (BONDELOS) expression was negatively regulated by this TF (46). BDL is an AUX/indole-3-acetic acid (IAA) inhibitor. Notably, ABA was the only hormone which was shown as able to regulate *AtHB5* expression (45). With an opposite function in ABA responses, *athb20* mutant plants displayed lower germination rates and increased sensitivity to this hormone during seed germination. Hence, *AtHB20* has been proposed as an ABA sensor and as a positive regulator of the break of seed dormancy (47) (Fig. 2).

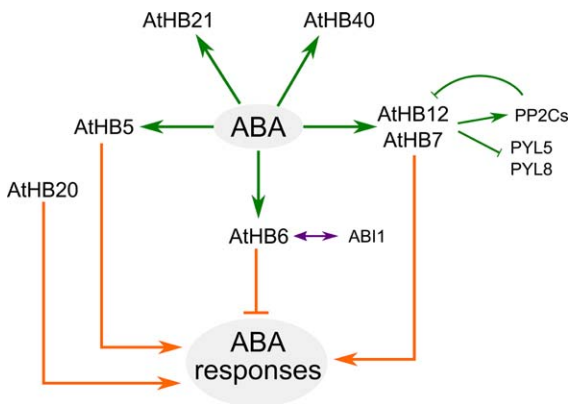

FIG 2 *Several* Arabidopsis *HD-Zip I TFs participate in ABA signalling pathways. Green lines indicate transcriptional regulation by ABA; orange lines indicate regulatory pathways triggered by the signalled HD-Zip TFs involving ABA (data obtained by mutant/overexpressor analyses) (*38, 39, 42-45, 47-49*); purple lines indicate protein–protein interactions.*

On the other hand, *AtHB6* has been described as a negative regulator of ABA signalling pathways because plants overexpressing *AtHB6* showed a clear ABA insensitivity. Moreover, it was demonstrated that AtHB6 is able to interact with ABI1, a key component of ABA signal transduction pathway (48).

Functions in Plant Responses to Environmental Conditions

Most reports characterizing HD-Zip I functions related these TFs with abiotic stress responses and a few ones with illumination conditions sensing (11,15).

Considering the role of HD-Zip I TFs in the response to abiotic stresses, besides *AtHB7* and *AtHB12* already described, it was reported that the over- and ectopic expression of *AtHB13* conferred to *Arabidopsis* transgenic plants tolerance to freezing temperatures, severe drought and salinity, both during vegetative and reproductive stages (25). This tolerant behaviour is achieved by stabilizing cell membranes through the induction of glucanase (*GLU*) and the genes encoding the pathogenesis related proteins *PR2* and *PR4* (*25*). On the other hand, *AtHB6* also mediates drought stress responses acting downstream of ABI1 and ABI2. This gene is induced in seedlings subjected to water deficit and osmotic stress conditions (49).

Considering light perception and its effect on development, four studies related HD-Zip I TFs to such response. AtHB1 was described as acting downstream of PIF1 to promote hypocotyl elongation, especially in response to short-day photoperiod (40). Meanwhile, AtHB16 has been proposed as a suppressor of the flowering time sensitivity to photoperiod. This is because plants having high *AtHB16* transcript levels entered the reproductive phase later than wild-type (WT) plants in a long-day photoperiod, but earlier in short-day conditions. The opposite effect was observed in transgenic plants with reduced levels of *AtHB16*. Furthermore, hypocotyl length assays performed with these transgenic plants provided evidence that *AtHB16* could function as a mediator of blue light response (37). AtHB23, another member of this family, has been proved to interact with PhyB. *Athb23* mutant plants grown under red light showed altered hypocotyl length and defects in germination and cotyledon expansion (50). The fourth member associated to light responses so far is *AtHB3,* which is rapidly downregulated after seedling de-etiolation under far red light (51). Unfortunately, no further studies have been reported until now, but it cannot be ruled out that additional members of the family were involved in light responses.

Functions in Plant Development

In addition to the assigned roles in response to environmental conditions described above, HD-Zip I TFs also have important functions in development even when plants are grown in optimal conditions (Fig. 3). For instance, leaf development is influenced by the action of AtHB1, AtHB7, AtHB12, AtHB13 and AtHB16. For example, AtHB16 was reported as a negative regulator of cell expansion because plants overexpressing *AtHB16* exhibited smaller leaves than plants defective in *AtHB16* expression or WT controls (37). *AtHB13* was shown to be a

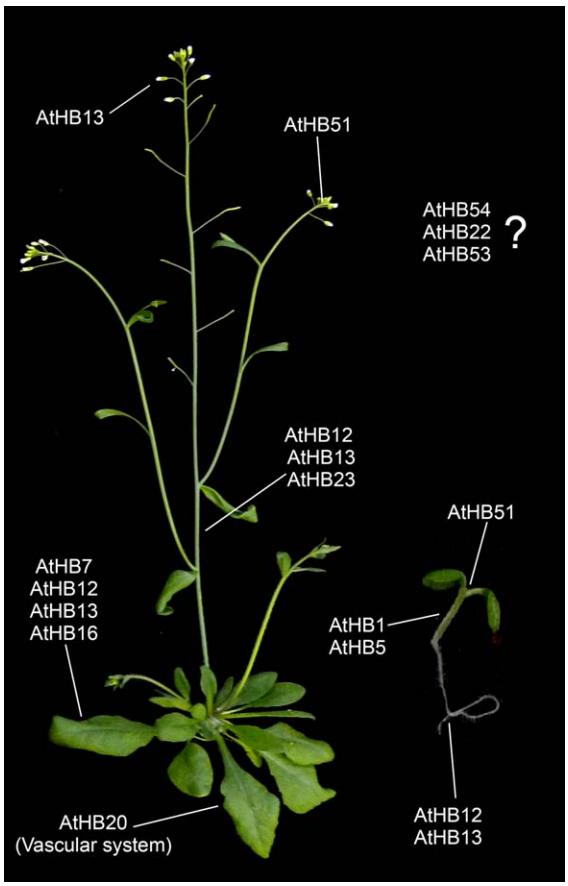

FIG 3 *Illustrative photographs of an* Arabidopsis *plant showing HD-Zip I functions in plant development. HD-Zip I members names are signalled in the plant based on literature data.*

regulator of cotyledon and leaf development in response to carbon availability in early developmental stages (52). Furthermore both *AtHB12* and *AtHB7*, described previously as involved in ABA and stress responses, were reported as inducers of leaf expansion in young and mature plants (53). *AtHB12* was also reported as a negative regulator of inflorescence stem elongation by repressing genes participating in the synthesis of gibberellins (54), whereas *AtHB13* and *AtHB23* negatively regulate the same event by controlling cell proliferation (41). In addition, *AtHB13* takes part of pollen hydration process by inducing the expression of critical pollen coat proteins (41). Considering HD-Zip I TFs participating in development and environmental responses, Aoyama et al. obtained transgenic tobacco plants overexpressing *AtHB1* and, after characterization, concluded that this TF is a regulator of light responses and leaf development (36).

In the same sense, *AtHB20* has been proposed as a regulator of vascular development because histochemical assays performed with plants transformed with its promoter fused to the *GUS* reporter gene showed staining around the emerging veins and a rapid upregulation by IAA treatment (55).

AtHB51, also named LMI1, was described as a positive regulator of *CAL*, *AGL24* and *SVP* acting downstream of LFY to

regulate meristem-identity (39,56). This TF has been assigned a second role, independent of LFY, in leaf and bract development (39).

Besides the above-mentioned functions for *AtHB5*, Stamm et al. assigned this TF a role as a regulator of *EXPANSIN3* in Gibberellins (GAs)-mediated hypocotyl expansion (57). Finally, and regarding root development, *AtHB12*, but not its paralogue *AtHB7*, was reported to be involved in root growth during early stages of plant development (53). A similar role was assigned to *AtHB13* (58).

Are HD-Zip I TFs Redundant or do they have Clearly Differentiated Functions?

The existence of 17 TFs presenting a highly conserved HD-Zip domain and the same binding affinity and specificity (18,24) suggested essential functions for these proteins. It is worth noting that *Arabidopsis* genome shows recent duplications of many of these TFs and functions' redundancy is very possible. However, in some cases, expression patterns of *Arabidopsis* HD-Zip I paralogous genes suggest differentiated functions (53).

The current availability of a multiplicity of sequences, thanks to next generation sequencing, allowed identifying HD-Zip I TFs in varied monocots and dicots. This knowledge helped to elucidate the existence of conserved motifs in the carboxy- and aminotermini of these proteins (CTR and NTR, respectively) (21). Besides allowing a new classification of HD-Zip I TFs taking into account such motifs, this discovery provided further support for differentiated functions more than redundancy. Why? These uncharacterized motifs could be interacting with different specific partner proteins and in this way, regulating different pathways although all HD-Zip I bind the same pseudopalindrome. Moreover, transactivation activity has been experimentally demonstrated for several HD-Zip I TFs (24,59,60). However, the role of the CTRs in such transactivation was confirmed only for AtHB12 (61) and later for AtHB1 (21) but without a fine identification of the motifs inside the CTR, responsible for such activity. Only 2 years later, AHA-like motifs were shown to be active for *Arabidopsis* AtHB1, AtHB7, AtHB12 and AtHB13 in both, plants and yeasts (41,62).

Experimental data revealing the existence of active AHA motifs in the CTR of HD-Zip I TFs helped to support the hypothesis of differentiated functions. Notably, the four tested AHA motifs of HD-Zip I members exhibited different behaviours in their interactions as well as different protein partners (62).

Other evidences supporting differential functions instead of redundancy were obtained by overexpression and ectopic expression studies. HD-Zip I proteins, able to bind the same DNA sequence, provoked clearly different phenotypes when overexpressed, supporting the existence of specific interactions with other proteins. However, and besides differentiated functions, redundancy for these TFs could not be ruled out. For example, it was recently reported that *AtHB21, AtHB40* and *AtHB53,* all members of clade VI, have redundant functions inhibiting branching under limited illumination (63).

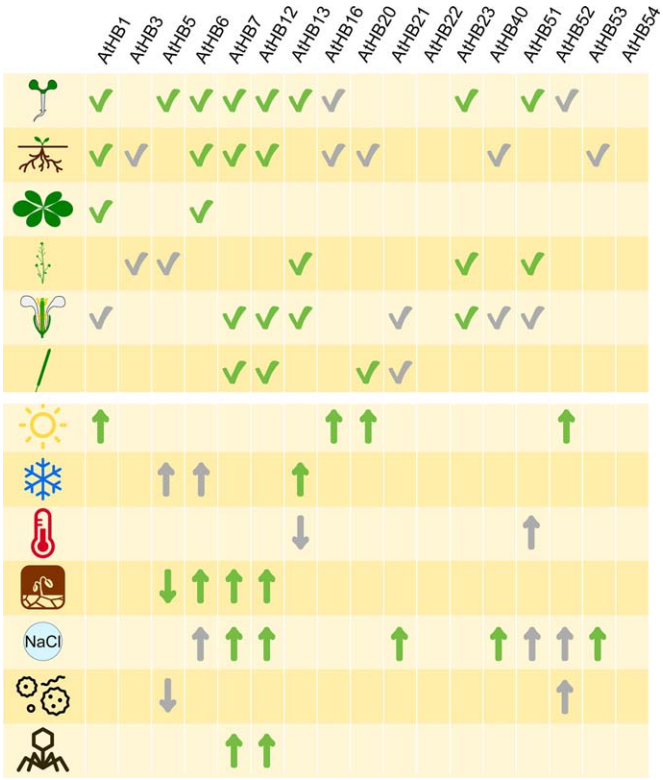

FIG 4

Arabidopsis *HD-Zip I genes are expressed in different plant tissues and organs. The figure was constructed based on published referenced manuscripts and available databases (http://bar.utoronto.ca/), indicating expression of HD-Zip encoding genes in tissues, organs and in response to external treatments. Upper panel: ticks indicate evidence of expression (from a cited paper in green; from a database, in grey). Lower panel: arrows indicate repression (↓) or induction (↑) of the expression by external factors. When data were obtained from the literature, arrows are green; whereas from databases in grey.*

Why HD-Zip I TFs are so Finely Regulated?

The expression of a gene is regulated by many factors, both internal and external, that define a precise spatiotemporal pattern. In this sense, genes encoding members of the HD-Zip I family show different expression patterns (24). In general, the expression of most members is limited to particular sets of cells of a certain organ/organs or tissue/tissues (40,41,52,63). However, this narrow expression pattern is highly influenced by external stimuli such as light or stressful conditions during limited and precise periods of time. Figure 4 aims to sum up the available knowledge about HD-Zip I TFs expression patterns. Part of this knowledge was taken from the available

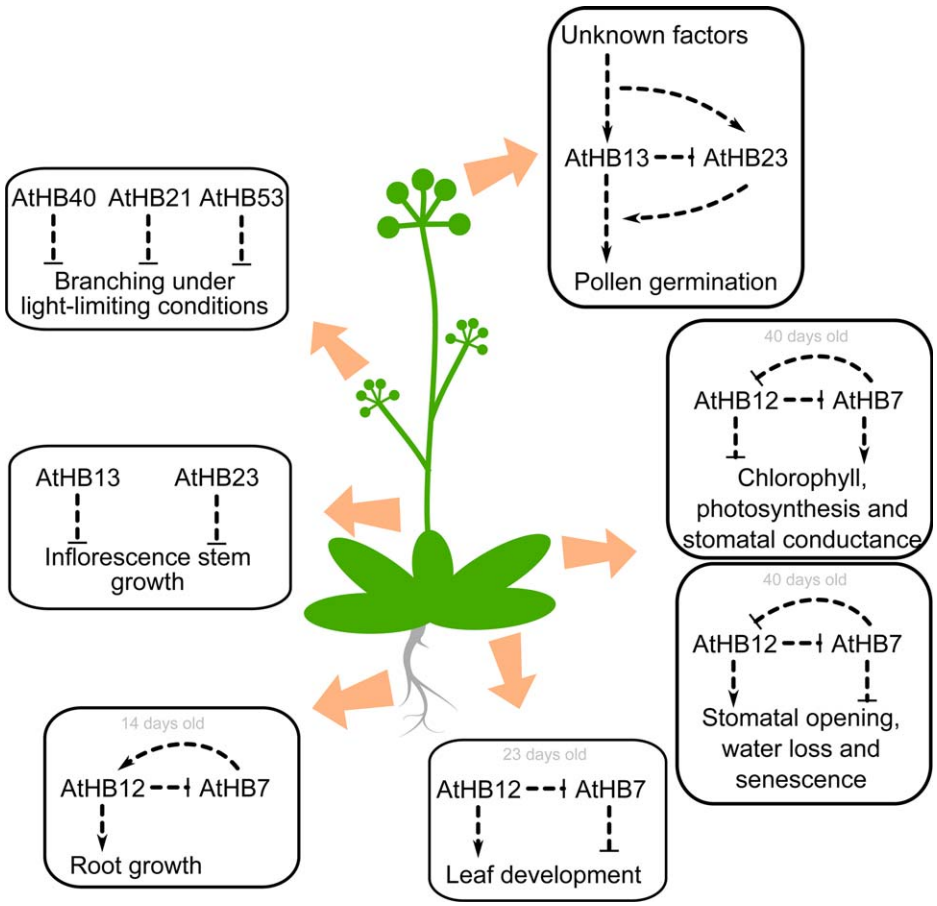

FIG 5 *Schematic representation of the roles of HD-Zip I paralogous genes in specific regulatory pathways of* Arabidopsis *development. The regulation exerted between paralogous genes is shown in each square according to the literature (*41, 53, 63*).*

literature and another part from databases constructed with big public data (http://bar.utoronto.ca/).

As it was mentioned above, after the completion of the Arabidopsis Genome Initiative, a deep analysis of HD-Zip I sequences allowed to establish that the encoding genes evolved by a series of gene duplications: *AtHB13* and *−23*, *AtHB7* and *−12*, *AtHB6* and *−16*, *AtHB21* and *−40* and *AtHB3* and *−20* (*24*). Notably, two of the more recent publications about HD-Zip I TFs reported the relationship between paralogous genes. In those works the existence of a fine regulation between paralogues was demonstrated and such regulation depends on plant developmental stage and tissue/organ (41,53). *AtHB7* and *AtHB12* exhibit specific and not overlapping expression patterns and roles. These genes achieve their functions by affecting the expression of each other when plants are grown in standard conditions. Studying mutant and overexpressor plants, it was found that AtHB7 induces the expression of *AtHB12* in early stages, but represses it in senescent plants (53). Another case is that of the pair *AtHB13/AtHB23*. AtHB13 participates in pollen hydration, but in plants with very low levels of *AtHB13* expression (achieved by silencing), *AtHB23* is induced by an unknown mechanism and replaces *AtHB13*. Meanwhile, in inflorescence stem elongation process, both *AtHB13* and *AtHB23* are needed for a proper development and no cross-regulation was observed (41). Figure 5 sums up the current knowledge about functions of paralogous genes as well as the cross-regulation exerted between members of each pair.

This kind of complex regulation between members of the same TF family was reported for other families. Such are the cases of WRKY and bHLH superfamilies (64,65). In the case of HD-Zip I TFs this fine cross-regulation between paralogues seems to be necessary for fine-tune plant development. It would be interesting to know if other paralogous pairs within the HD-Zip I TF family (*i.e.*, *AtHB20* and *−3*, *AtHB6* and *−16*) also display such a fine cross-regulation; unfortunately, such studies are not available yet.

Considerations about the Tools Applied to Study HD-Zip I TFs. Overexpressor or Mutant Plants?

The current knowledge about HD-Zip I TFs was acquired by different techniques including sequencing, next generation sequencing, expression patterns and characterization of

overexpressing and/or mutant plants, among others (12,40,52,53,63). This knowledge helped to understand the regulation of gene expression in plants as well as to discover signal transduction pathways. Moreover, this understanding conducted to develop biotechnological tools to improve crops.

Among the used techniques, it is worth mentioning that many studies were carried out by ectopic and/or constitutive expression of HD-Zip I genes which conducted to controversies. Why is the acquired knowledge controversial? Why scientists continue to use over- and ectopic expression as tool?

Non-model plants are frequently difficult to transform, and genes from non-model plants do not always exhibit conserved functions with those of model plants. Overexpression approaches significantly helped in obtaining approximations of genes function, both in model and non-model plants (27,36–38,66,67). However, ectopic and/or constitutive expression of a certain gene, particularly a TF encoding gene, can cause artefactual effects because the proteins can ectopically regulate gene expression via binding natural and/or unnatural target gene promoters or interacting with proteins that are normally not coexpressed with them. The resulting phenotypes could or could not be associated with the natural function of the gene within the source plant; so it is necessary to keep in mind that conclusions arising from such experiments are limited (68). These facts emphasize the importance and necessity of using different approaches to elucidate biological functions of a given gene.

Alternative strategies include the use of mutant or silenced plants (69). These experiments are limited to the study of genes from plant species capable of being transformed or those for which mutant repositories are available (70). In general, this is not the case of genes from crops like sunflower, maize or wheat. Silenced or mutant plants allow the scientist to infer gene functions based on differential phenotypes. It is possible to find a mutant plant for almost any *Arabidopsis* gene since many years ago. However, T DNA-Seq, a next generation sequencing methodology, revealed that many of the available lines have more than one insertion, sowing doubts about some conclusions arisen from mutants' characterization if complementation studies were not performed.

On the other hand, especially when the function of a TF is assessed, it is important to consider the existence of functional redundancy between genes of the same family. Redundancy makes difficult to find differences between the mutant and the control plant, making the generation of double, triple or even multiple mutants mandatory. Artificial microARNs might be a helpful tool both when trying to silence multiple genes in a single plant or when there is not a proper T-DNA insertion mutant for the gene of interest. Moreover, novel genome editing techniques, like CRISPR/Cas9 technology, promise to be powerful to silence specific genes in species able to be transformed.

Another point to consider is that the silencing or mutation of a TF encoding gene can also produce side effects. The plant can display alternate pathways to solve the situation that do not naturally occur.

Besides these approaches, it is important to establish the expression pattern of certain genes. Free available databases like that of the University of Toronto for *Arabidopsis*, rice or other species (http://bar.utoronto.ca/) are very helpful, but the data obtained must be corroborated by independent techniques. Analyses of transformed plants with promoters fused to reporter genes like *GUS* or *GFP* can be very useful. These techniques allow determining spatial and temporal expression patterns in plants grown in different conditions and/or after various treatments.

How HD-Zip I Became Biotechnological Tools?

Although the ectopic or/and constitutive expression of TFs has been shown to be a limited strategy for determining the function of a given gene, this experimental approach has led to unexpected biotechnological uses. When overexpressed many TFs, belonging to this and other families, generated differential phenotypes which include desirable agronomic traits such as improved yield, better water use efficiency and tolerance to biotic or abiotic stress factors, among others.

In particular, HD-Zip I TFs are included in several patents as a core part of the inventions. A recent search in patent databases (Patent Lens, https://www.lens.org/lens/) resulted in 12 patent applications (or granted patents) involving HD-Zip I TFs. Among them most have claims based on transgenic expression whereas only two are based on the silencing of a HD-Zip I gene. Claims include better development and tolerance to a variety of abiotic stresses (71,72).

Of the above mentioned 12 patents 2 involve *Arabidopsis* genes, whereas the rest protect genes from other species like wheat, rice, sunflower, coffee and maize.

The first HD-Zip I included in a patent application was the *Arabidopsis AtHB12*, which was protected as a tool to enhance tolerance to drought, salinity or general abiotic stresses (73). Later *AtHB16* was protected as part of a genetic tool to improve grass quality of bahiagrass and related species (74). The overexpression of *AtHB16* suppresses or reduces the formation of seed heads, increases the number of vegetative tillers per plant and also improves tolerance to abiotic stress (73). Besides the *Arabidopsis* members, HD-Zip I TFs from other species conferring beneficial traits when overexpressed that can be mentioned are the rice *HOX5*, the sunflower *HaHB1*, *HaHB4* and *HaHB11*, the coffee *CaHB12* and the *Triticum aestivum TaHD-ZipI-2*. *HaHB4,* a sunflower gene which expression, driven by its own promoter or a constitutive one, confers tolerance to drought and improves yield both in control and stress conditions (75). ThHDZIP-1 from *Thellungiella halophila* improved tolerance to salinity and drought (76). For a more detailed description of patents involving HD-Zip I TFs see ref. 71 and 72.

All the above referred patents consider ectopic or/and constitutive expression of HD-Zip I TFs. Furthermore two patents involve the knock-down of a HD-Zip I encoding gene. These

are the cases of *Vrs1* and *ZmME293*. When *Vrs1* was silenced in barley, or its homolog in wheat, these crops yield significantly increased (77,78). Similarly the silencing of *ZmME293* improved plant performance (79).

The relative low presence in patent applications of overexpressed HD-Zip I TFs from *Arabidopsis*, the most used plant for such experiments, is probably due to the fact that overexpression of its own genes causes natural silencing by the small RNA machinery (80) making the appreciation of differential phenotypes difficult. Silencing by the small RNA machinery probably occurs in all plant species; however, it was particularly demonstrated that the expression of *Arabidopsis* HD-Zip I encoding genes was silenced in *Arabidopsis* whereas, HD-Zip I genes from other plant species were not silenced in *Arabidopsis* (81).

What is it Currently Unknown about HD-Zip I TFs? Why is it Important?

Although several members of the HD-Zip I family have been deeply characterized, most of them, from model and non-model plants are poorly described. The current knowledge about sequence conservation was significantly increased during the last decade. However, functional studies, even for *Arabidopsis* members, are still requiring further investigations. The few well-characterized TFs from this family have been shown to have essential functions for plant development and plant responses to environmental conditions. Further knowledge regarding the unstudied members will help understand key events of the plant life and, moreover, to create new biotechnological tools to improve plant yield, especially crops.

Acknowledgements

The authors thank Dr. Agustín Arce for critical reading of the manuscript and helpful suggestions. This work was supported by CONICET, Agencia Nacional de Promoción Científica y Tecnológica (PICT 2014 3779 and PICT 2014 3300) and Universidad Nacional del Litoral (UNL). M.F.P. and P.A.R. are CONICET Ph.D. Fellows and R.L.C. is a career member of the same institution.

References

[1] Chapman, A. D. (2009) *Numbers of Living Species in Australia and the World*. 2nd edn. Australian Biological Resources Study, Canberra.

[2] Riechmann, J. L. (2002) Transcriptional regulation: a genomic overview. In C. R. Somerville y E. M. Meyerowitz ed. *The Arabidopsis Book*. p. e0085, American Society of Plant Biologists, Rockville, MD.

[3] Xiong, Y., Liu, T., Tian, C., Sun, S., Li, J., et al. (2005) Transcription factors in rice: a genome-wide comparative analysis between monocots and eudicots. Plant Mol. Biol. 59, 191–203.

[4] Mitsuda, N. and Ohme-Takagi, M. (2009) Functional analysis of transcription factors in *Arabidopsis*. Plant Cell Physiol. 50, 1232–1248. [PMC][10.1093/pcp/pcp075] [19478073].

[5] Wray, G. A. (2003) Transcriptional regulation and the evolution of development. Int. J. Dev. Biol. 47, 675–684.

[6] Singh, K., Foley, R. C., and Oñate-Sánchez, L. (2002) Transcription factors in plant defense and stress responses. Curr. Opin. Plant Biol. 5, 430–436.

[7] Shiu, S. -H., Shih, M. -C., and Li, W. -H. (2005) Transcription factor families have much higher expansion rates in plants than in animals. Plant Physiol. 139, 18–26.

[8] Gehring, W. (1987) Homeo boxes in the study of development. Science 236, 1245–1252.

[9] Vollbrecht, E., Veit, B., Sinha, N., and Hake, S. (1991) The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350, 241–243.

[10] Ruberti, I., Sessa, G., Lucchetti, S., and Morelli, G. (1991) A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. EMBO J. 10, 1787–1791.

[11] Harris, J. C., Hrmova, M., Lopato, S., and Langridge, P. (2011) Modulation of plant growth by HD-Zip class I and II transcription factors in response to environmental stimuli. New Phytol. 190, 823–837.

[12] Sessa, G., Morelli, G., and Ruberti, I. (1993) The Athb-1 and −2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. EMBO J. 12, 3507–3517.

[13] Schena, M. and Davis, R. W. (1992) HD-Zip proteins: members of an *Arabidopsis* homeodomain protein superfamily. Proc. Natl. Acad. Sci. USA 89, 3894–3898.

[14] Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240, 1759–1764.

[15] Ariel, F. D., Manavella, P. A., Dezar, C. A., and Chan, R. L. (2007) The true story of the HD-Zip family. Trends Plant Sci. 12, 419–426.

[16] Chan, R. L., Gago, G. M., Palena, C. M., and Gonzalez, D. H. (1998) Homeoboxes in plant development. Biochim. Biophys. Acta 1442, 1–19.

[17] Palena, C. M., Gonzalez, D. H., and Chan, R. L. (1999) A monomer-dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA. Biochem. J. 341, 81–87.

[18] Johannesson, H., Wang, Y., and Engström, P. (2001) DNA-binding and dimerisation preferences of *Arabidopsis* homeodomain- leucine zipper transcription factors in vitro. Plant Mol. Biol. 45, 63–73.

[19] Palena, C. M., Tron, A. E., Bertoncini, C. W., Gonzalez, D. H., and Chan, R. L. (2001) Positively charged residues at the N-terminal arm of the homeodomain are required for efficient DNA binding by homeodomain-leucine zipper proteins. J. Mol. Biol. 308, 39–47.

[20] O'Malley, R. C., Huang, S. C., Song, L., Lewsey, M. G., Bartlett, A., et al. (2016) Cistrome and epicistrome features shape the regulatory DNA landscape. Cell 165, 1280–1292.

[21] Arce, A. L., Raineri, J., Capella, M., Cabello, J. V., and Chan, R. L. (2011) Uncharacterized conserved motifs outside the HD-Zip domain in HD-Zip subfamily I transcription factors; a potential source of functional diversity. BMC Plant Biol. 11, 42.

[22] Treuter, E., Nover, L., Ohme, K., and Scharf, K. -D. (1993) Promoter specificity and deletion analysis of three heat stress transcription factors of tomato. Mol. Gen. Genet. 240, 113–125.

[23] Döring, P., Treuter, E., Kistner, C., Lyck, R., Chen, A., et al. (2000) The role of AHA motifs in the activator function of tomato heat stress transcription factors HsfA1 and HsfA2. Plant Cell 12, 265–278.

[24] Henriksson, E., Olsson, A. S. B., Johannesson, H., Johansson, H., Hanson, J., et al. (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. Plant Physiol. 139, 509–518.

[25] Cabello, J. V., Arce, A. L., and Chan, R. L. (2012) The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. Plant J. 69, 141–153.

[26] Meissner, R. and Theres, K. (1995) Isolation and characterization of the tomato homeobox gene THOM1. Planta 195, 541–547.

[27] Hu, T., Ye, J., Tao, P., Li, H., Zhang, J., et al. (2016) The tomato HD-Zip I transcription factor SlHZ24 modulates ascorbate accumulation through positive regulation of the d -mannose/l -galactose pathway. Plant J. 85, 16–29.

[28] Kawahara, R., Komamine, A., and Fukuda, H. (1995) Isolation and characterization of homeobox-containing genes of carrot. Plant Mol. Biol. 27, 155–164.

[29] Liu, W., Fu, R., Li, Q., Li, J., Wang, L., et al. (2013) Genome-wide identification and expression profile of homeodomain-leucine zipper Class I gene family in *Cucumis sativus*. Gene 531, 279–287.

[30] Zhang, J., Zhu, Q. -H., Moncuquet, P., Llewellyn, D., and Wilson, I. (2016) Genome-wide identification and characterization of the homeodomain-leucine zipper I family of genes in cotton (*Gossypium* spp.). Plant Gene 7, 50–61.

[31] Meijer, A. H., Scarpella, E., Van Dijk, E. L., Qin, L., Taal, A. J. C., et al. (1997) Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice. Plant J. 11, 263–276.

[32] Chen, X., Chen, Z., Zhao, H., Zhao, Y., Cheng, B., et al. (2014) Genome-wide analysis of soybean HD-Zip gene Family and expression profiling under salinity and drought treatments. PLoS ONE 9, e87156.

[33] Ge, X. -X., Liu, Z., Wu, X. -M., Chai, L. -J., and Guo, W. -W. (2015) Genome-wide identification, classification and analysis of HD-ZIP gene family in citrus, and its potential roles in somatic embryogenesis regulation. Gene 574, 61–68.

[34] Cao, L., Yu, Y., DuanMu, H., Chen, C., Duan, X., et al. (2016) A novel Glycine soja homeodomain-leucine zipper (HD-Zip) I gene, Gshdz4, positively regulates bicarbonate tolerance and responds to osmotic stress in *Arabidopsis*. BMC Plant Biol. 16, 184.

[35] Schena, M. and Davis, R. W. (1994) Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. Proc. Natl. Acad. Sci. USA 91, 8393–8397.

[36] Aoyama, T., Dong, C. H., Wu, Y., Carabelli, M., Sessa, G., et al. (1995) Ectopic expression of the *Arabidopsis* transcriptional activator Athb-1 alters leaf cell fate in tobacco. Plant Cell 7, 1773–1785.

[37] Wang, Y., Henriksson, E., Söderman, E., Henriksson, K. N., Sundberg, E., et al. (2003) The *Arabidopsis* homeobox gene, ATHB16, regulates leaf development and the sensitivity to photoperiod in *Arabidopsis*. Dev. Biol. 264, 228–239.

[38] Olsson, A., Engström, P., and Söderman, E. (2004) The homeobox genes ATHB12 and ATHB7encode potential regulators of growth in response to water deficit in *Arabidopsis*. Plant Mol. Biol. 55, 663–677.

[39] Saddic, L. A., Huvermann, B., Bezhani, S., Su, Y., Winter, C. M., et al. (2006) The leafy target LMI1 is a meristem identity regulator and acts together with LEAFY to regulate expression of cauliflower. Development 133, 1673–1682.

[40] Capella, M., Ribone, P. A., Arce, A. L., and Chan, R. L. (2015) *Arabidopsis thaliana* HomeoBox 1 (AtHB1), a homedomain-leucine zipper I (HD-Zip I) transcription factor, is regulated by phytochrome-interacting factor 1 to promote hypocotyl elongation. New Phytol. 207, 669–682.

[41] Ribone, P. A., Capella, M., and Chan, R. L. (2015) Functional characterization of the homeodomain leucine zipper I transcription factor AtHB13 reveals a crucial role in *Arabidopsis* development. J. Exp. Bot. 66, 5929–5943.

[42] Söderman, E., Mattsson, J., and Engström, P. (1996) The *Arabidopsis* homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. Plant J. 10, 375–381.

[43] Hjellström, M., Olsson, A. S. B., Engström, P., Söderman, E. M., Hjellström, M., et al. (2003) Constitutive expression of the water deficit-inducible homeobox gene ATHB7 in transgenic *Arabidopsis* causes a suppression of stem elongation growth. Plant Cell Environ. 26, 1127–1136.

[44] Valdés, A. E., Övernäs, E., Johansson, H., Rada-Iglesias, A., and Engström, P. (2012) The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities. Plant Mol. Biol. 80, 405–418.

[45] Johannesson, H., Wang, Y., Hanson, J., and Engström, P. (2003) The *Arabidopsis thaliana* homeobox gene ATHB5 is a potential regulator of abscisic acid responsiveness in developing seedlings. Plant Mol. Biol. 51, 719–729.

[46] De Smet, I., Lau, S., Ehrismann, J. S., Axiotis, I., Kolb, M., et al. (2013) Transcriptional repression of bodenlos by HD-ZIP transcription factor HB5 in Arabid. J. Exp. Bot. 64, 3009–3019.

[47] Barrero, J. M., Millar, A. A., Griffiths, J., Czechowski, T., Scheible, W. R., Udvardi, M., et al. (2010) Gene expression profiling identifies two regulatory genes controlling dormancy and ABA sensitivity in *Arabidopsis* seeds. Plant J. 61, 611–622.

[48] Himmelbach, A., Hoffmann, T., Leube, M., Höhener, B., and Grill, E. (2002) Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in *Arabidopsis*. EMBO J. 21, 3029–3038.

[49] Söderman, E., Hjellström, M., Fahleson, J., and Engström, P. (1999) The HD-Zip gene ATHB6 in *Arabidopsis* is expressed in developing leaves, roots and carpels and up-regulated by water deficit conditions. Plant Mol. Biol. 40, 1073–1083.

[50] Choi, H., Jeong, S., Kim, D. S., Na, H. J., Ryu, J. S., et al. (2014) The homeodomain-leucine zipper ATHB23, a phytochrome B-interacting protein, is important for phytochrome B-mediated red light signaling. Physiol. Plant 150, 308–320.

[51] Roig-Villanova, I., Bou, J., Sorin, C., Devlin, P. F., and Martínez-García, J. F. (2006) Identification of primary target genes of phytochrome signaling. Early transcriptional control during shade avoidance responses in *Arabidopsis*. Plant Physiol. 141, 85–96.

[52] Hanson, J., Regan, S., and Engström, P. (2002) The expression pattern of the homeobox gene ATHB13 reveals a conservation of transcriptional regulatory mechanisms between *Arabidopsis* and hybrid aspen. Plant Cell Rep. 21, 81–89.

[53] Ré, D. A., Capella, M., Bonaventure, G., and Chan, R. L. (2014) *Arabidopsis* AtHB7 and AtHB12 evolved divergently to fine tune processes associated with growth and responses to water stress. BMC Plant Biol. 14, 150.

[54] Son, O., Hur, Y. -S., Kim, Y. -K., Lee, H. -J., Kim, S., et al. (2010) ATHB12, an ABA-inducible homeodomain-leucine zipper (HD-Zip) protein of *arabidopsis*, negatively regulates the growth of the inflorescence stem by decreasing the expression of a gibberellin 20-oxidase gene. Plant Cell Physiol. 51, 1537–1547.

[55] Mattsson, J., Ckurshumova, W., and Berleth, T. (2003) Auxin signaling in *Arabidopsis* leaf vascular development. Plant Physiol. 131, 1327–1339.

[56] Grandi, V., Gregis, V., and Kater, M. M. (2012) Uncovering genetic and molecular interactions among floral meristem identity genes in *Arabidopsis thaliana*. Plant J. 69, 881–893.

[57] Stamm, P., Topham, A. T., Mukhtar, N. K., Jackson, M., Tome, D., et al. (2016) The transcription factor ATHB5 affects GA-mediated plasticity in hypocotyl cell growth during seed germination. Plant Physiol. 173, 307–325.

[58] Silva, A. T., Ribone, P. A., Chan, R. L., Ligterink, W., and Hilhorst, H. W. M. (2016) A predictive coexpression network identifies novel genes controlling the seed-to-seedling phase transition in *Arabidopsis thaliana*. Plant Physiol. 170, 2218–2231.

[59] Meijer, A. H., de Kam, R. J., d'Erfurth, I., Shen, W., and Hoge, J. H. (2000) HD-Zip proteins of families I and II from rice: interactions and functional properties. Mol. Gen. Genet. 263, 12–21.

[60] Wang, Y. J., Li, Y. D., Luo, G. Z., Tian, A. G., Wang, H. W., et al. (2005) Cloning and characterization of an HDZip I gene GmHZ1 from soybean. Planta 221, 831–843.

[61] Lee, Y. -H., Oh, H. -S., Cheon, C. -I., Hwang, I. -T., Kim, Y. -J., et al. (2001) Structure and expression of the *Arabidopsis thaliana* homeobox gene Athb-12. Biochem. Biophys. Res. Commun. 284, 133–141.

[62] Capella, M., Ré, D. A., Arce, A. L., and Chan, R. L. (2014) Plant homeodomain-leucine zipper I transcription factors exhibit different functional AHA motifs that selectively interact with TBP or/and TFIIB. Plant Cell Rep. 33, 955–967.

[63] González-Grandío, E., Pajoro, A., Franco-Zorrilla, J. M., Tarancón, C., Immink, R. G. H., and Cubas, P. (2016) Abscisic acid signaling is controlled by a BRANCHED1/HD-ZIP I cascade in *Arabidopsis* axillary buds. Proc. Natl. Acad. Sci. USA 114, 245–254.

[64] Zhu, E., You, C., Wang, S., Cui, J., Niu, B., et al. (2015) The DYT1-interacting proteins bHLH010, bHLH089 and bHLH091 are redundantly required for *Arabidopsis* anther development and transcriptome. Plant J. 83, 976–990.

[65] Cheng, H., Liu, H., Deng, Y., Xiao, J., Li, X., et al. (2015) The WRKY45-2 WRKY13 WRKY42 transcriptional regulatory cascade is required for rice resistance to fungal pathogen. Plant Physiol. 167, 1087–1099.

[66] Cabello, J. V. and Chan, R. L. (2012) The homologous homeodomain-leucine zipper transcription factors HaHB1 and AtHB13 confer tolerance to drought and salinity stresses via the induction of proteins that stabilize membranes. Plant Biotechnol. J. 10, 815–825.

[67] Cabello, J. V., Giacomelli, J. I., Piattoni, C. V., Iglesias, A. A., and Chan, R. L. (2016) The sunflower transcription factor HaHB11 improves yield, biomass and tolerance to flooding in transgenic *Arabidopsis* plants. J. Biotechnol. 222, 73–83.

[68] Lloyd, A. (2003) Vector construction for gene overexpression as a tool to elucidate gene function. In *Plant Functional Genomics*. (Grotewold, E., ed.). pp. 329–344, Humana Press, New Jersey.
[69] O'Malley, R. C., Barragan, C. C., and Ecker, J. R. (2015) A user's guide to the *Arabidopsis* T-DNA insertion mutant collections. In *Methods in Molecular Biology* (Alonso, J. M., and Stepanova, A. N., ed.). pp. 323–342, Springer Science+Bussines Media, New York.
[70] O'Malley, R. C. and Ecker, J. R. (2010) Linking genotype to phenotype using the *Arabidopsis* unimutant collection. Plant J. 61, 928–940.
[71] Arce, A., Cabello, J., and Chan, R. (2008) Patents on Plant Transcription Factors. Recent Pat. Biotechnol. 2, 209–217.
[72] Ribone, P. A., Capella, M., Arce, A. L. and Chan, R. L. (2015) What do we know about homeodomain-leucine zipper I transcription factors? Functional and biotechnological considerations. In *Plant Transcription Factors* (Gonzalez, D. H., ed.). pp. 343–356, Elsevier, Amsterdam.
[73] Jong-Yoon Chun, K. and Yong-Hun Lee, S. (1999) Transcription factor gene induced by water deficit and abscisic acid isolated from *Arabidopsis thaliana*. US patent 5,981,729.65.
[74] Altpeter, F. and Zhang, H. (2008) Materials and methods for improving quality and characteristics of grasses. WO2008021397A1.
[75] Chan, R. L., Cabello, J. V., and Giacomelli, J. I. (2013) HaHB11 provides improved plant yield and tolerance to abiotic stress. WO2013116750A1.
[76] Wang, J., Cui, H., He, Y., and Tian, D. (2013) Transcription factor hdzip-1 from thellungiella halophila, and coding gene and application thereof. WO 2014172829 A1
[77] Komatsuda, T., Yano, M., and Matsumoto, T. (2007b) Barley row type gene and use thereof. WO2007069677A1.
[78] Komatsuda, T., Matsumoto, T., Sakuma, S., and Ogawa, T. (2014) Wheat with increased grain-bearing number and method for producing same, and chemical for increasing grain-bearing number of wheat. WO2014007396A1
[79] Guo, M., Niu, X., Rupe, M., and Schussler, J., (2012) Down-regulation of a homeodomain–leucine zipper I-class homeobox gene for improved plant performance. WO2012148835A1.
[80] Schubert, D. (2004) Silencing in *Arabidopsis* T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16, 2561–2572.
[81] Romani, F. A., Ribone, P. A., Capella, M., Miguel, V. N., and Chan, R. L. (2016) A matter of quantity: common features in the drought response of transgenic plants overexpressing HD-Zip I transcription factors. Plant Sci. 251, 139–154.