



Plant transcription factors from the Homeodomain-Leucine Zipper family I. Role in development and stress responses

Journal:	<i>IUBMB Life</i>
Manuscript ID	TBMB-16-0316-WJW.R2
Wiley - Manuscript type:	Critical Review
Date Submitted by the Author:	n/a
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Keywords:	transcription factors, homeodomain-leucine zipper, HD-Zip I, Arabidopsis thaliana, biotechnological tools

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4 **Plant transcription factors from the Homeodomain-Leucine Zipper family I. Role**
5 **in development and stress responses**
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Abstract

In front of stressful conditions plants display adaptation mechanisms leading to changes in their morphology, physiology, development and molecular composition. Transcription factors play crucial roles in these complex adaptation processes. This work is focused in the Homeodomain-Leucine Zipper I (HD-Zip I) family of transcription factors, 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 transcription factors 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 transcription factors 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 transcription factors including structural features, biological functions and their utilization as biotechnological tools to improve crops.

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 300000 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.

Plant Transcription Factors

Transcription factors (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 hetero- dimmers, 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 1500 TFs have been identified in the *Arabidopsis* (*Arabidopsis thaliana*) genome (2) and 1600 in that of rice (*Oryza sativa*) (3). Although around 45 % plant TFs belong to

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3 families also existing in other kingdoms; notably, the number of members of such
4 families is significantly enlarged in plants. This can suggest the existence of specific
5 regulatory pathways in these organisms (7). Since plants are continuously exposed to
6 environmental changes, they alter their growth and architecture in response to such
7 alterations. Many TFs have crucial roles both in development and in adaptation
8 responses whereas others act only when an external factor stimulates them.
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11 In this review, we will focus in one of the TF groups, unique to plants, the
12 Homeodomain-Leucine Zipper I (HD-Zip I) family. The choice of this group was based
13 on the fact that these TFs play essential roles in development in response to
14 environmental conditions and among them, a few became biotechnological tools to
15 improve crops. It is important to note that most of the knowledge about biological roles
16 of these TFs originated in studies performed in model plants such as Arabidopsis and
17 rice. However, some studies about HD-Zip TFs from other species will be commented.
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25 26 **The Homeodomain-Leucine Zipper family**

27 Homeodomains (HD) as DNA-binding domains were first discovered in animals as
28 closely related to developmental events. Moreover, these domains were named HDs
29 because their mutation or ectopic expression causes the change of a body segment by
30 another, producing a homeotic effect (8). The HD is a conserved 60 amino-acid motif
31 that folds into a bundle of three α helices (named I to III), connected by a loop and a
32 turn. HDs are highly conserved between proteins from different kingdoms, indicating
33 that their structure is crucial to maintain functionality.
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39 In 1991 it was reported the identification of the first plant TF containing a HD, the
40 maize KNOTTED1, mainly involved in meristem maintenance (9). Its name was given
41 because its ectopic expression induces areas of irregular cell division along secondary
42 veins of the lamina producing “knotted” leaves. One year later, AtHB-1, a HD-Zip TF,
43 was identified in Arabidopsis by Ruberti and coworkers (10) and since then, many HD-
44 containing genes have been identified and isolated from a wide variety of
45 monocotyledonous and dicotyledonous plant species (11). These TFs were proposed to
46 be key players in plant specific developmental processes, such as those associated to
47 external stimuli and stresses. However, plant TFs containing HDs did not exhibit
48 canonical homeotic effects when they were over- or ectopically expressed like those
49 from animals.
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3 Particular kind of HD-containing genes are the Homeodomain-Leucine Zipper (HD-
4 Zip) TFs. These proteins have a unique association of a HD, which is the binding
5 domain and a leucine zipper (ZIP) located downstream the HD, which acts as a
6 dimerization motif (12, 13). Although both HD and ZIP can be found alone or
7 associated to other motifs in TFs from other kingdoms, this association is unique to
8 plants.
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12 The ZIP folds into an α -helix having a leucine in each seventh position on the same side
13 of the helix. This three-dimensional structure allows the formation of dimmers through
14 hydrophobic interactions (12, 14). The efficient recognition and DNA binding depends
15 on the relative orientation of the monomers (15).
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19 After the identification of the first plant HD-Zip TF, further studies conducted
20 worldwide by several research groups lead to the discovery of TFs presenting this
21 association of domains in all plant species in which they were searched for. Their
22 structural and functional characterization lead to a classification in four subfamilies
23 named I-IV (15). This review will be focused on subfamily I because the knowledge
24 about the functions of these TFs significantly grew in the last years revealing essential
25 roles in plant development and stress responses.
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32 33 **The subfamily HD-Zip I**

34 Members of this subfamily encode ~35 KDa proteins. In these TFs the HD is highly
35 conserved whereas the ZIP is more diversified (16). *In vitro* assays allowed to
36 determine that these proteins preferably bind the pseudopalindromic sequence
37 CAAT(A/T)ATTG and that the dimerization through the ZIP is a prerequisite for DNA
38 binding (12, 17, 18). The affinity of the protein for a certain DNA sequence, but not the
39 specificity, is affected by the amino acids of the HD N-terminal arm (19). More
40 recently, using a novel technique to determine TFs binding sites in a whole genome,
41 O'Malley and coworkers identified the pseudopalindrome AAT(N)ATT as the target
42 sequence of HD-Zip I TFs, which shows a perfect coincidence with the core of the
43 previously determined sequence (20).
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47 A recent survey performed with the complete sequences of HD-Zip I proteins from
48 several species allowed the identification of other conserved motifs. Particularly, an
49 AHA transactivation motif was found in the C-terminal region of these proteins (Figure
50 1; 21). AHA (Aromatic large Hydrophobic Acidic residues) motifs were first described
51 as activation motifs present in tomato HSF (Heat Stress Factors) TFs (22). These motifs
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3 form an amphipathic and negatively charged helix to contact components of the basal
4 transcription complex (23).

7 Arabidopsis HD-Zip I members

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

29 After the discovery of HD-Zip proteins in 1991 by Ruberti and coworkers (10), many
30 HD-Zip encoding genes had been identified and isolated from *Arabidopsis thaliana* and
31 other species such as sunflower (25), tomato (*Lycopersicon esculentum*; 26, 27), carrot
32 (*Daucus carota*; 28), cucumber (*Cucumis sativus*; 29), cotton (*Gossypium sp*; 30) and
33 rice (*Oryza sativa*; 31), among others (32–34).
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38 The initial investigations were mainly focused on the elucidation of some genetic and
39 biochemical properties of these newly identified TFs (12, 35). The first functional study
40 about AtHB-1 (called now AtHB1 according to international nomenclature rules) was
41 conducted by Aoyama and coworkers in 1995 and since then, several works were
42 devoted to describe the function of this kind of proteins (36–41).
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46 Although at first of these TFs were mainly involved in the responses to abiotic stress
47 conditions, further studies indicated that HD-Zip I TFs play important roles also in other
48 plant processes, such as development or biotic stress responses (11).
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52 Functions in hormone signaling pathways

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

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

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

28 Functions in plant responses to environmental conditions

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

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

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

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36 In addition to the assigned roles in response to environmental conditions described
37 above, HD-Zip I TFs also have important functions in development even when plants
38 are grown in optimal conditions (Figure 3). For instance, leaf development is influenced
39 by the action of AtHB1, AtHB7, AtHB12, AtHB13 and AtHB16. For example, AtHB16
40 was reported as a negative regulator of cell expansion because plants overexpressing
41 *AtHB16* exhibited smaller leaves than plants defective in *AtHB16* expression or WT
42 controls (37). *AtHB13* was shown to be a regulator of cotyledon and leaf development
43 in response to carbon availability in early developmental stages (52). Furthermore both
44 *AtHB12* and *AtHB7*, previously described as involved in ABA and stress responses,
45 were reported as inducers of leaf expansion in young and mature plants (53). *AtHB12*
46 was also reported as a negative regulator of inflorescence stem elongation by repressing
47 genes participating in the synthesis of gibberellins (54) whereas *AtHB13* and *AtHB23*
48 negatively regulate the same event by controlling cell proliferation (41). In addition,
49 *AtHB13* takes part of pollen hydration process by inducing the expression of critical
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3 pollen coat proteins (41). Considering HD-Zip I TFs participating in development and
4 environmental responses, Aoyama and coworkers obtained transgenic tobacco plants
5 overexpressing *AtHB1* and, after characterization concluded that this TF is a regulator
6 of light responses and leaf development (36).
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10 In the same sense, *AtHB20* has been proposed as a regulator of vascular development
11 because histochemical assays performed with plants transformed with its promoter
12 fused to the *GUS* reporter gene showed staining around the emerging veins and a rapid
13 up-regulation by IAA treatment (55).
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16 *AtHB51*, also named LMI1, was described as a positive regulator of *CAL*, *AGL24* and
17 *SVP* acting downstream of *LFY* to regulate meristem-identity (39, 56). This TF has
18 been assigned a second role, independent of *LFY*, in leaf and bract development (39).
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20 Besides the above mentioned functions for *AtHB5*, Stamm and coworkers assigned this
21 TF a role as a regulator of *EXPANSIN3* in GA-mediated hypocotyl expansion (57).
22 Finally, and regarding root development, *AtHB12*, but not its paralogue *AtHB7*, was
23 reported to be involved in root growth during early stages of plant development (53). A
24 similar role was assigned to *AtHB13* (58).
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31 **Are HD-Zip I transcription factors redundant or do they have clearly** 32 **differentiated functions?** 33

34 The existence of 17 TFs presenting a highly conserved HD-Zip domain and the same
35 binding affinity and specificity (18, 24) suggested essential functions for these proteins.
36 It is worth noting that Arabidopsis genome shows recent duplications of many of these
37 TFs and functions' redundancy is very possible. However, in some cases, expression
38 patterns of Arabidopsis HD-Zip I paralogous genes suggest differentiated functions
39 (53).
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44 The current availability of a multiplicity of sequences, thanks to next generation
45 sequencing, allowed identifying HD-Zip I TFs in varied monocots and dicots. This
46 knowledge helped to elucidate the existence of conserved motifs in the carboxy- and
47 amino- termini of these proteins (CTR and NTR, respectively; 21). Besides allowing a
48 new classification of HD-Zip I TFs taking into account such motifs, this discovery
49 provided further support for differentiated functions more than redundancy. Why?
50 These uncharacterized motifs could be interacting with different specific partner
51 proteins and in this way, regulating different pathways although all HD-Zip I bind the
52 same pseudopalindrome. Moreover, transactivation activity has been experimentally
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3 demonstrated for several HD-Zip I TFs (24, 59, 60). However, the role of the CTRs in
4 such transactivation was confirmed only for AtHB12 (61) and later for AtHB1 (21) but
5 without a fine identification of the motifs inside the CTR, responsible for such activity.
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7 Only two years later, AHA-like motifs were shown to be active for Arabidopsis AtHB1,
8 AtHB7, AtHB12, and AtHB13 in both, plants and yeasts (41, 62).
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11 Experimental data revealing the existence of active AHA motifs in the CTR of HD-Zip I
12 TFs helped to support the hypothesis of differentiated functions. Notably, the four tested
13 AHA motifs of HD-Zip I members exhibited different behaviors in their interactions as
14 well as different protein partners (62).
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18 Other evidences supporting differential functions instead of redundancy were obtained
19 by overexpression and ectopic expression studies. HD-Zip I proteins, able to bind the
20 same DNA sequence, provoked clearly different phenotypes when overexpressed,
21 supporting the existence of specific interactions with other proteins. However, and
22 besides differentiated functions, redundancy for these TFs could not be ruled out. For
23 example, it was recently reported that *AtHB21*, *AtHB40* and *AtHB53*, all members of
24 clade VI, have redundant functions inhibiting branching under limited illumination (63).
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31 **Why HD-Zip I TFs are so finely regulated?**

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33 The expression of a gene is regulated by many factors, both internal and external, that
34 define a precise spatiotemporal pattern. In this sense, genes encoding members of the
35 HD-Zip I family show different expression patterns (24). In general, the expression of
36 most members is limited to particular sets of cells of a certain organ/s or tissue/s (40,
37 41, 52, 63). However, this narrow expression pattern is highly influenced by external
38 stimuli such as light or stressful conditions during limited and precise periods of time.
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43 **Figure 4** aims to sum up the available knowledge about HD-Zip I TFs expression
44 patterns. Part of this knowledge was taken from the available literature and another part
45 from databases constructed with big public data (<http://bar.utoronto.ca/>).
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49 As it was mentioned above, after the completion of the Arabidopsis Genome Initiative,
50 a deep analysis of HD-Zip I sequences allowed to establish that the encoding genes
51 evolved by a series of gene duplications: *AtHB13* and *-23*, *AtHB7* and *-12*, *AtHB6* and *-*
52 *16*, *AtHB21* and *-40* and *AtHB3* and *-20* (24). Notably, two of the more recent
53 publications about HD-Zip I TFs reported the relationship between paralogous genes. In
54 those works the existence of a fine regulation between paralogues was demonstrated and
55 such regulation depends on plant developmental stage and tissue/organ (41, 53). *AtHB7*
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3 and *AtHB12* exhibit specific and not overlapping expression patterns and roles. These
4 genes achieve their functions by affecting the expression of each other when plants are
5 grown in standard conditions. Studying mutant and overexpressor plants, it was found
6 that *AtHB7* induces the expression of *AtHB12* in early stages, but represses it in
7 senescent plants (53). Another case is that of the pair *AtHB13/AtHB23*. *AtHB13*
8 participates in pollen hydration, but in plants with very low levels of *AtHB13* expression
9 (achieved by silencing), *AtHB23* is induced by an unknown mechanism and replaces
10 *AtHB13*. Meanwhile, in inflorescence stem elongation process, both *AtHB13* and
11 *AtHB23* are needed for a proper development and no cross-regulation was observed
12 (41). Figure 5 sums up the current knowledge about functions of paralogous genes as
13 well as the cross-regulation exerted between members of each pair.

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

28 29 30 31 32 33 34 35 **Considerations about the tools applied to study HD-Zip I TFs. Overexpressor or** 36 **mutant plants?**

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38 The current knowledge about HD-Zip I TFs was acquired by different techniques
39 including sequencing, next generation sequencing, expression patterns, and
40 characterization of overexpressing and/or mutant plants, among others (12, 40, 52, 53,
41 63). This knowledge helped to understand the regulation of gene expression in plants as
42 well as to discover signal transduction pathways. Moreover, this understanding
43 conducted to develop biotechnological tools to improve crops.

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

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60 Non-model plants are frequently difficult to transform and genes from non-model plants
not always exhibit conserved functions with those of model plants. Overexpression
approaches significantly helped in obtaining approximations of genes function, both in

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3 model and non-model plants (27, 36–38, 66, 67). However, ectopic and/or constitutive
4 expression of a certain gene, particularly a TF encoding gene, can cause artifactual
5 effects because the proteins can ectopically regulate gene expression via binding natural
6 and/or non-natural target gene promoters or interacting with proteins that are normally
7 not co-expressed with them. The resulting phenotypes could or could not be associated
8 with the natural function of the gene within the source plant; so it is necessary to keep in
9 mind that conclusions arising from such experiments are limited (68). These facts
10 emphasize the importance and necessity of using different approaches to elucidate
11 biological functions of a given gene.

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

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

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

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37 Besides these approaches, it is important to establish the expression pattern of certain
38 genes. Free available databases like that of the University of Toronto for Arabidopsis,
39 rice or other species (<http://bar.utoronto.ca/>) are very helpful, but the data obtained must
40 be corroborated by independent techniques. Analyses of transformed plants with
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3 promoters fused to reporter genes like *GUS* or *GFP* can be very useful. These
4 techniques allow determining spatial and temporal expression patterns in plants grown
5 in different conditions and/or after various treatments.
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8 9 10 **How HD-Zip I became biotechnological tools?**

11 Although the ectopic or/and constitutive expression of TFs has been shown to be a
12 limited strategy for determining the function of a given gene, this experimental
13 approach has led to unexpected biotechnological uses. When overexpressed many TFs,
14 belonging to this and other families, generated differential phenotypes which include
15 desirable agronomic traits such as improved yield, better water use efficiency and
16 tolerance to biotic or abiotic stress factors, among others.
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18 In particular, HD-Zip I TFs are included in several patents as a core part of the
19 inventions. A recent search in patent databases (Patent Lens, <https://www.lens.org/lens/>)
20 resulted in twelve patent applications (or granted patents) involving HD-Zip I TFs.
21 Among them most have claims based on transgenic expression whereas only two are
22 based on the silencing of a HD-Zip I gene. Claims include better development and
23 tolerance to a variety of abiotic stresses (71, 72).
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25 Two out of the above mentioned twelve patents involve Arabidopsis genes whereas the
26 rest protect genes from other species like wheat, rice, sunflower, coffee and maize.
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28 The first HD-Zip I included in a patent application was the Arabidopsis AtHB12, which
29 was protected as a tool to enhance tolerance to drought, salinity, or general abiotic
30 stresses (73). Later, *AtHB16* was protected as part of a genetic tool to improve grass
31 quality of bahiagrass and related species (74). The overexpression of *AtHB16*
32 suppresses or reduces the formation of seedheads, increases the number of vegetative
33 tillers per plant and also improves tolerance to abiotic stress (73). Besides the
34 Arabidopsis members, HD-Zip I TFs from other species conferring beneficial traits
35 when overexpressed that can be mentioned are the rice *HOX5*, the sunflower *HaHB1*,
36 *HaHB4* and *HaHB11*, the coffee *CaHB12* and the *Triticum aestivum* *TaHDZipI-2*.
37 *HaHB4*, a sunflower gene which expression, driven by its own promoter or a
38 constitutive one, confers tolerance to drought and improves yield both in control and
39 stress conditions (75). ThHDZIP-1 from *Thellungiella halophila* improved tolerance to
40 salinity and drought (76). For a more detailed description of patents involving HD-Zip I
41 TFs see references 71 and 72.
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3 All the above referred patents consider ectopic or/and constitutive expression of HD-Zip
4 I TFs. Furthermore, two patents involve the knock-down of a HD-Zip I encoding gene.
5 These are the cases of *Vrs1* and *ZmME293*. When *Vrs1* was silenced in barley, or its
6 homolog in wheat, these crops yield significantly increased (77, 78). In a similar way,
7 the silencing of *ZmME293* improved plant performance (79).
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11 The relative low presence in patent applications of overexpressed HD-Zip I TFs from
12 Arabidopsis, the most used plant for such experiments, is probably due to the fact that
13 overexpression of its own genes causes natural silencing by the small RNA machinery
14 (80) making the appreciation of differential phenotypes difficult. Silencing by the small
15 RNA machinery probably occurs in all plant species; however, it was particularly
16 demonstrated that the expression of Arabidopsis HD-Zip I encoding genes was silenced
17 in Arabidopsis whereas, HD-Zip I genes from other plant species were not silenced in
18 Arabidopsis (81).
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26 **What is it currently unknown about HD-Zip I TFs? Why is it important?**

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

46 The authors would thank Dr. Agustín Arce for critical reading of the manuscript and
47 helpful suggestions.
48

49 This work was supported by CONICET, Agencia Nacional de Promoción Científica y
50 Tecnológica (PICT 2014 3779 and PICT 2014 3300) and Universidad Nacional del
51 Litoral (UNL). MFP and PAR are CONICET Ph. D. Fellows and RLC is a career
52 member of the same institution.
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Legends to Figures

Figure 1: Schematic representation of the structure of HD-Zip I transcription factors. 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).

Figure 2: Several Arabidopsis HD-Zip I transcription factors participate in ABA signaling pathways. Green lines indicate transcriptional regulation by ABA; orange lines indicate regulatory pathways triggered by the signaled HD-Zip TFs involving ABA (data obtained by mutant/overexpressor analyses, references 38, 39, 42-45, 47-49); purple lines indicate protein-protein interactions.

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

Figure 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.

Figure 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, and 63).

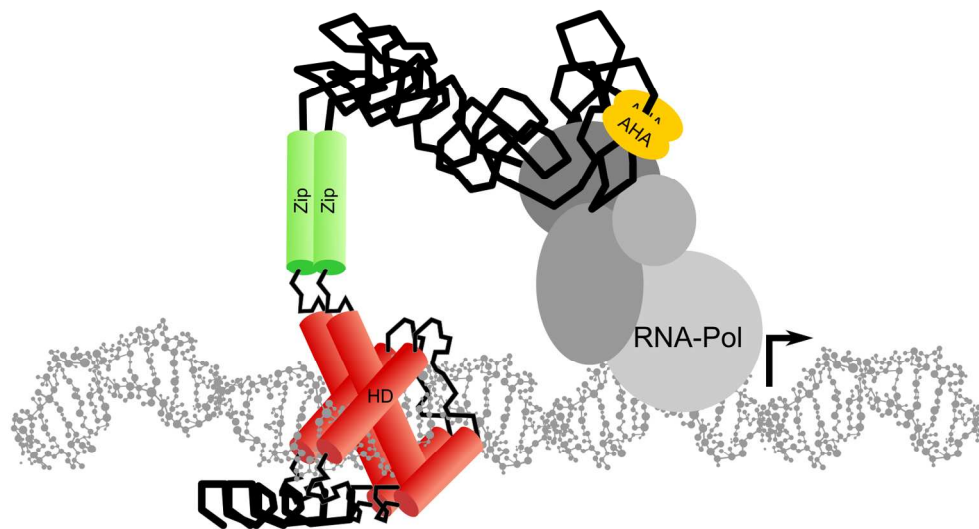


Figure 1: Schematic representation of the structure of HD-Zip I transcription factors. HD-Zip I proteins recognize DNA by the HD helix III (red), forming homo- or heterodimers through the ZIP (green). Transcription of the targets is activated by the interaction of the AHA motif (yellow) with the basal transcription machinery (grey).
Particularly, an AHA transacti
154x84mm (299 x 299 DPI)

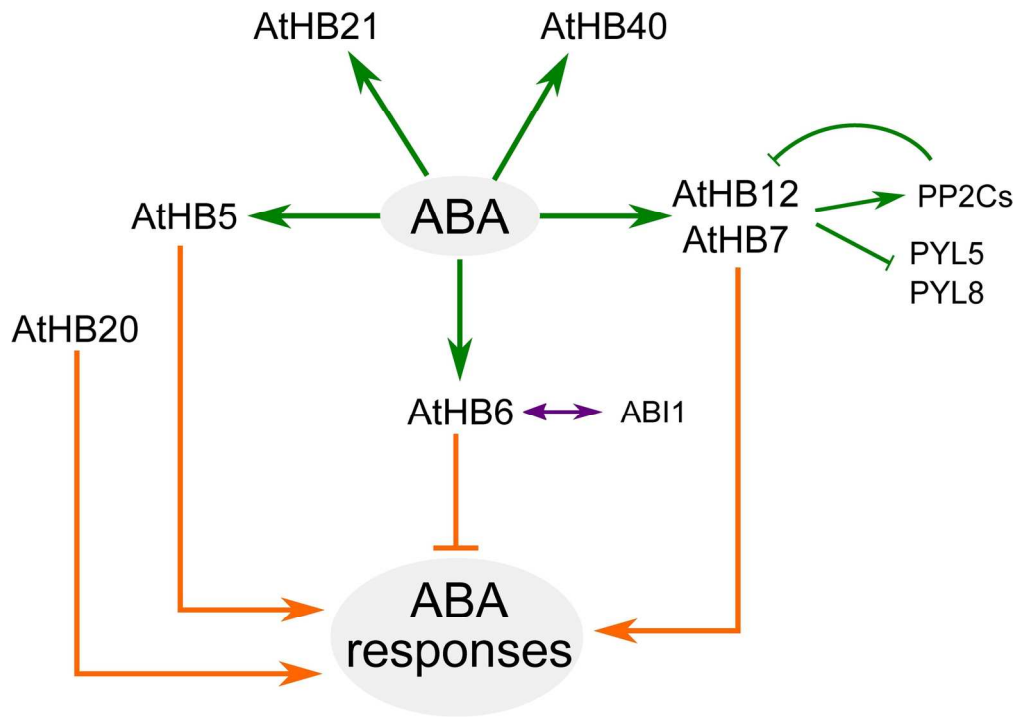
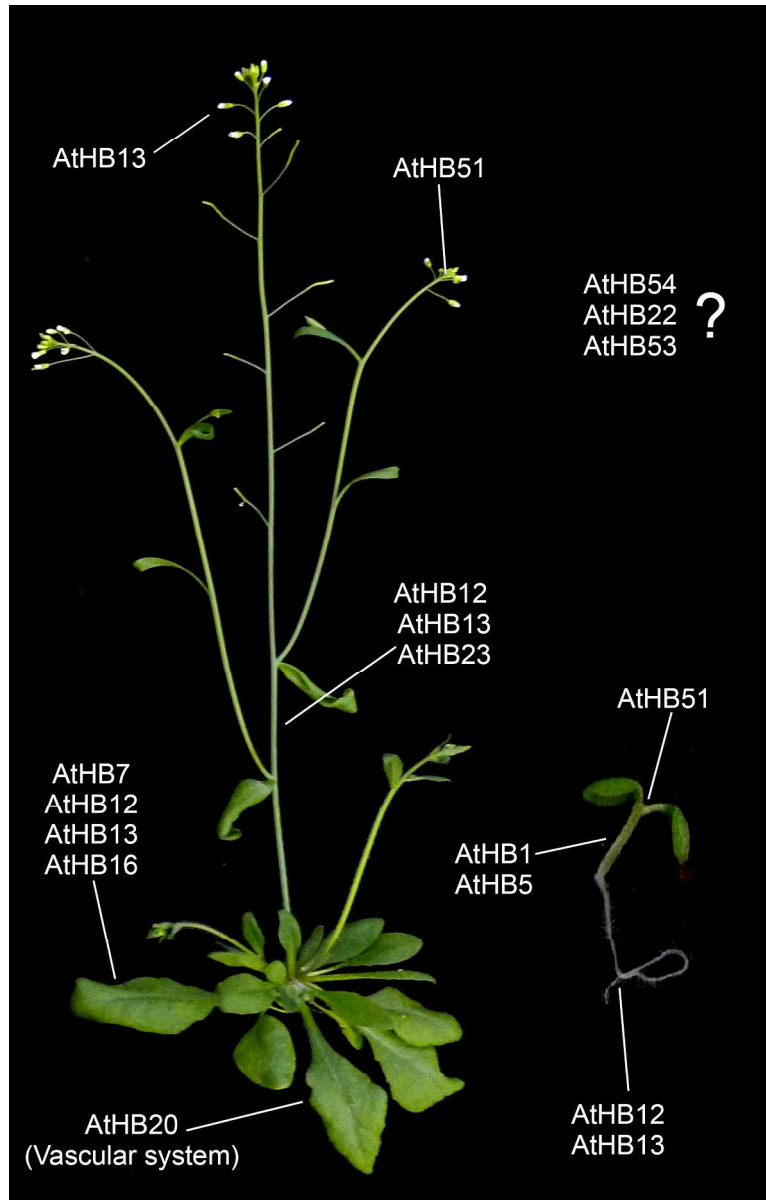


Figure 2: Several Arabidopsis HD-Zip I transcription factors participate in ABA signaling pathways. Green lines indicate transcriptional regulation by ABA; orange lines indicate regulatory pathways triggered by the signaled HD-Zip TFs involving ABA (data obtained by mutant/overexpressor analyses, references 38, 39, 42-45, 47-49); purple lines indicate protein-protein interactions.

Later it was demonstrated that
161x114mm (300 x 300 DPI)



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

In addition to the assigned ro
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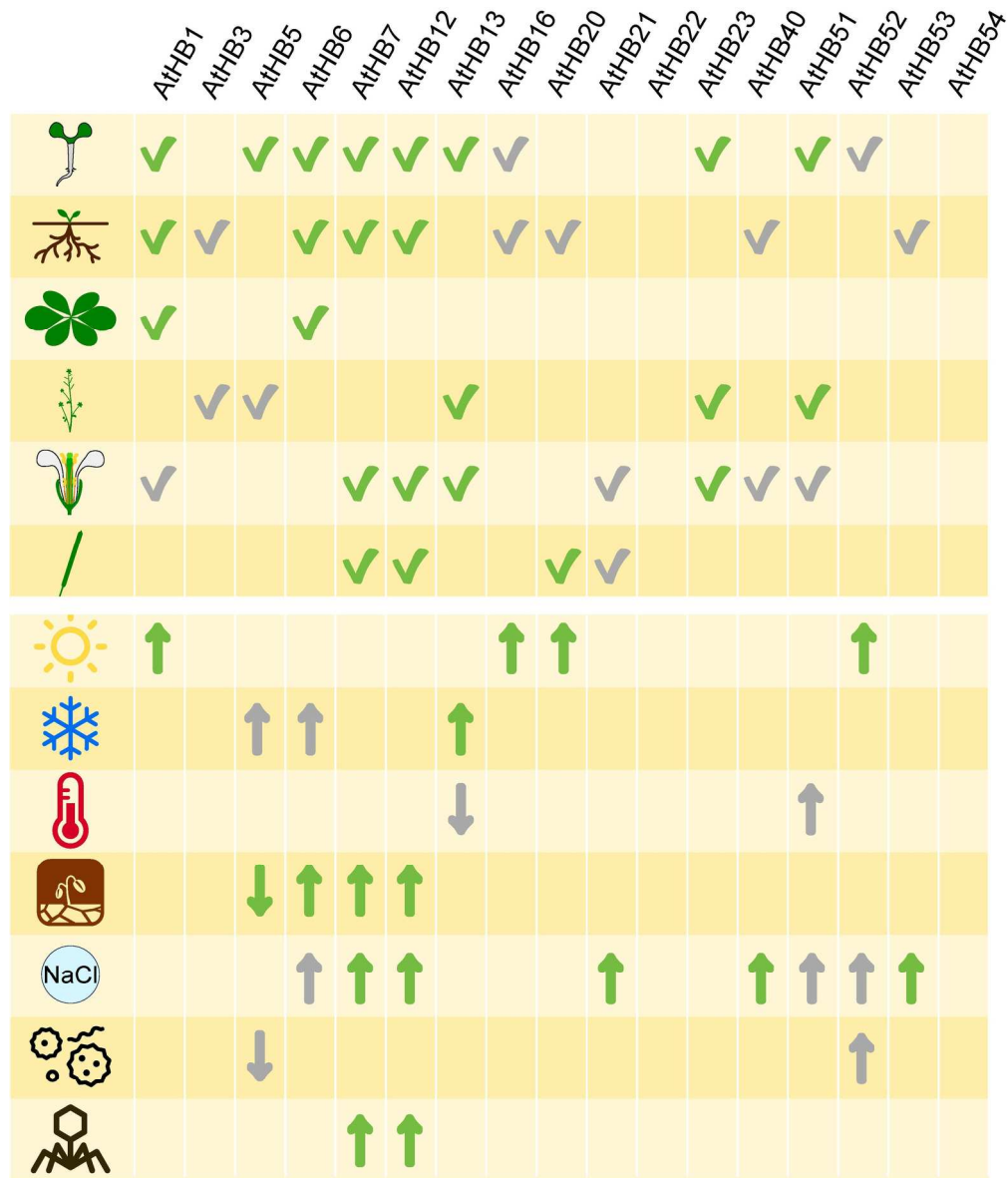


Figure 4: Arabidopsis HD-Zip I genes are expressed in different plant tissues and organs. The figure was constructed based on published referenced manuscripts and available databases (<http://bar.utoronto.ca/>), indicating expression of HD-Zip encoding genes in tissues, organs and in response to external 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.

Figure 4 aims to sum up the av
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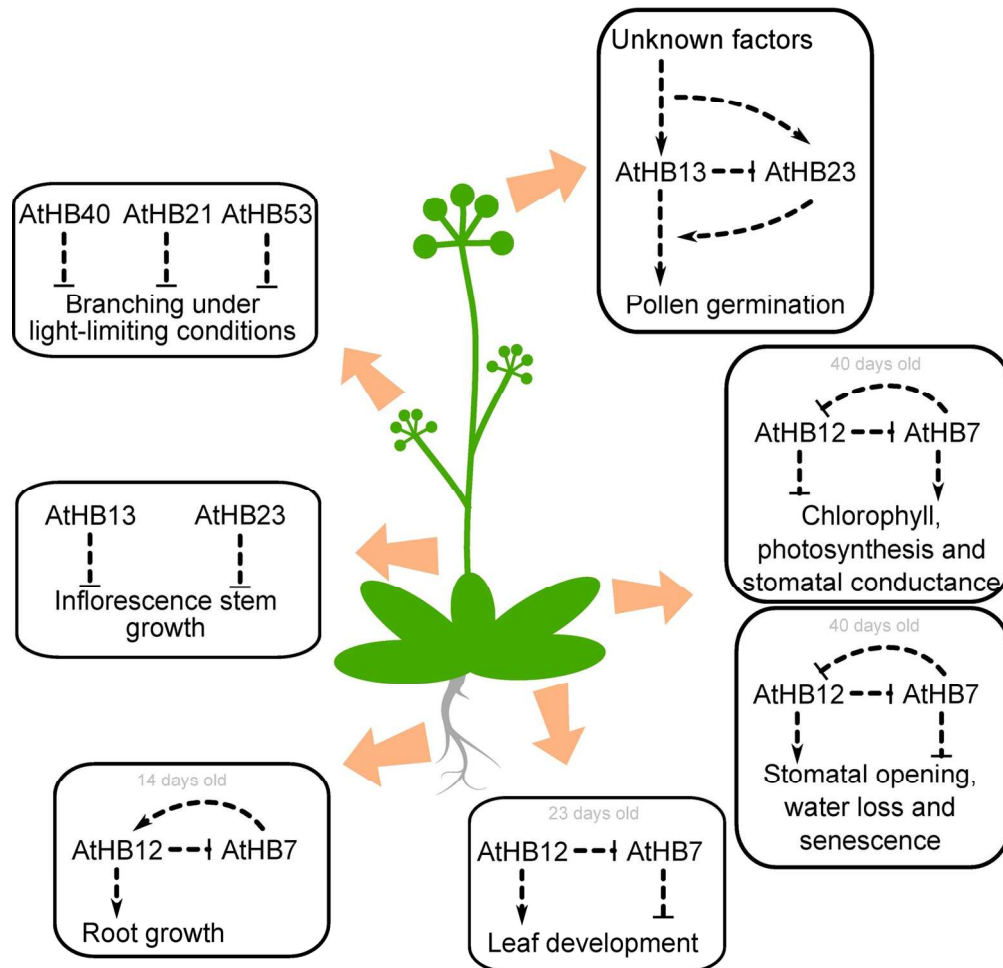


Figure 5: Schematic representation of the roles of HD-Zip I paralogue genes in specific regulatory pathways of Arabidopsis development. The regulation exerted between paralogue genes is shown in each square according to the literature (41, 53, and 63).

Figure 5 sums up the current k
145x140mm (300 x 300 DPI)