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Research article

Two ABREs, two redundant root-specific and one W-box *cis*-acting elements are functional in the sunflower *HAHB4* promoter

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Abstract

HAHB4 is a sunflower gene encoding a homeodomain–leucine zipper (HD-Zip) transcription factor. It was previously demonstrated that this gene is regulated at the transcriptional level by several abiotic factors and hormones. A previous analysis in the PLACE database revealed the presence of four putative ABREs. In this work these four elements and also one W-box and two root-specific expression elements were characterized as functional. Site-directed mutagenesis on the promoter, stable transformation of Arabidopis plants as well as transient transformation of sunflower leaves, were performed. The analysis of the transformants was carried out by histochemistry and real time RT-PCR. The results indicate that just one ABRE out of the four is responsible for ABA, NaCl and drought regulation. However, NaCl induction occurs also by an additional ABA-independent way involving another two overlapped ABREs. On the other hand, it was determined that the W-box located 5′ upstream is responsive to ethylene and only two root-specific expression elements, among the several detected, are functional but redundant. Conservation of molecular mechanisms between sunflower and Arabidopsis is strongly supported by this experimental work.

Keywords: *HAHB4* promoter; ABRE; Sunflower; Drought; W-box; Root-specific

1. Introduction

Genes containing homeoboxes have been isolated from many eukaryotic organisms including fungi, mammals, and plants [6,16,27]. They encode transcription factors containing a homeodomain, a conserved 60-amino-acid motif. Plant homeobox genes can be classified into several families according to sequence conservation and structure in and outside the homeodomain [2,6,36]. Members of one of these families have a distinct feature: they code for proteins termed homeodomain–leucine zipper (HD-Zip), because they contain a homeodomain associated with a leucine zipper, a coiled-coil structure involved in dimerization [2,18,29,33,35]. These proteins are involved in regulating developmental processes associated with the response of plants to environmental conditions [5,6,37].

Little is known about promoters from other species than the model ones and also about the conservation of the functionality of their *cis*-acting elements. Several *cis*-acting elements are described in different genes as functional [10,19,23,31,38,39,40,42,43,45]. However, some of them are present in other genes but do not show any sign of activity. Even more, genes that are responsive to a certain external stimuli do not exhibit the same *cis*-acting elements [1,14,44]. Therefore, the same sequence may be active in response to alternative factors within different promoters, whereas a similar response may be mediated by different boxes in each promoter [48,49]. ABRE (ABA responsive element) is a *cis*-acting element described in several promoters as responsive to this hormone [39] and W-box is a *cis*-acting element recognized by WRKY transcription factors, involved in pathogen related responses [11,30,50,52]. Some of these W-boxes are also responsive to ethylene [30]. Regarding HD-Zip encoding genes,

Abbreviations: HD, homeodomain; Zip, leucine zipper; LPF, large promoter fragment; ABA, abscisic acid; ABRE, ABA responsive element; W-box, WRKY-binding element.

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little or nothing has been described about *cis*-acting elements directing the response to abiotic stress factors or hormones. On the other hand, regulation of sunflower genes, especially those encoding transcription factors is a poorly explored subject although the agronomic importance of this crop. It is worth noting that recent progress has been done on the knowledge about *cis*-acting elements involved in the abiotic stress response in this plant [3,10].

HAHB4 is a sunflower gene encoding a transcription factor belonging to the subfamily I of HD-Zip proteins. Its expression is regulated by ABA, drought, salt stress and ethylene [15,28]. Transgenic *Arabidopsis* plants either ectopically/constitutively expressing it or expressing this gene after induction by hydric stress exhibit drought tolerance [4,9] and a delay in senescence [28]. In a previous work, the isolation and a partial characterization of two forms of this gene promoter from a sunflower hybrid line were described. The analysis in the PLACE database revealed the presence of four ABREs in this promoter, two of them overlapped in complementary strands. Both promoter forms showed the same behavior as responsive to drought and salt stress as well as to ABA. It was also shown that this promoter is also responsive to ethylene, indicating that regulation of the expression by all these external factors occurs at the transcriptional level [8,28]. Transformation of Arabidopsis plants with constructs in which this promoter was successively shortened allowed to determine that for the expression in the root vascular cylinder the region comprised between 1000 and 1200 is needed and a minimum of 800 bp upstream the transcription initiation site are necessary to detect a response to ABA, high salt concentration or water stress.

In this paper we deeply analyzed the sunflower *HAHB4* promoter. Besides the four putative ABREs, we detected in the PLACE database the existence of one W-box and several different root-specific elements that have already been identified and characterized as functional in promoters of other genes [12,13,25,34,41,46,47]. Then, we demonstrated which ones among these elements were functional. The studies were performed doing mutational deletions within the promoter followed by the obtention of transgenic plants with constructs bearing these different segments fused to the reporter gene *GUS*. Arabidopsis plants were stably transformed while sunflower leaves were transiently transformed. The use of both *Arabidopsis* and sunflower, helped to elucidate whether this response is conserved between species. Alternative techniques allowed us to determine that ABA, salt and drought responses share an ABA-dependent pathway. However, salt response involves an additional alternative pathway. The functionality of the W-box detected in the 5′ extreme was demonstrated as well as that of two redundant root-specific boxes among the several present in this promoter.

2. Methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). *Helianthus annuus* L. (sunflower cv. contiflor 15, from Zeneca) seeds were grown on soil in a culture room at 28 °C. Growth conditions were previously described [28].

2.2. Reporter gene constructs and Arabidopsis plants transformation

The construct used as control bearing the whole *HAHB4* promoter fused to the *GUS* reporter gene cDNA in the pBI 101.3 vector (*LPF:GUS)* as well as constructs −318 and −416 were previously obtained as described [8]. Mutant and chimerical constructs in which one box or an entire segment are deleted were performed using *LPF:GUS* as template following the technique described in Ho et al. [22]. Essentially, in every case, two flanking DNA segments surrounding the element to be deleted were amplified by PCR in separate reactions using for the upstream segment (segment A) PROTT26 as 5′ primer and a specific reverse primer and for the downstream segment (segment B) IPCR8 as 3′ primer and a specific forward primer (see primer sequences in Table 1). The two specific primers have an overlapped region of 18 bp. The resulting products were mixed in a Taq polymerase buffer and 0.5 mM of each dNTP, 2.5 mM $MgCl_2$ and 5 units of the Taq DNA polymerase. Hybridization and extension of the overlapping segments were carried out following this program: 10 cycles of 30 s at 94 °C, 90 s at 62 °C and 2 min at 72 °C. After that a normal PCR amplification was performed using IPCR8 (5′-CGCGGATCCGAGGGTTTGATAAGTGAT-3′) and PROTT26 (5′-GCGGTCGACACCTGGCACATCG-TATCTT-3′) as primers.

Double and triple mutant segments were obtained using simple mutants as probes. Mutated PCR products were cloned

Table 1
Primers used in the construction of mutants and chimeras

Construct	Specific forward primers used	Specific reverse primers used
Mut ABRE-5′	5′-CACCTACAATCAATTCACACTTCACCA-3′	5′-GTGAATTGATTGTAGGTGTGTTGTGGT-3′
Mut ABRE-m	5′-GTCTGGATCAAACATCAGGTCTCTCCC-3′	5′-CTGATGTTTGATCCAGACAAAGGCGGA-3′
Mut ABRE-3′	5′-ATAACCAAATAAACGTACAACTGACCA-3′	5′-GTACGTTTATTTGGTTATGTCGATTCT-3′
Mut W-box	5′-TTTCCTTTTTCATATTAAAAGTAGTAGCCC-3′	5′-TTAATATGAAAAAGGAAATGAAATTGGTGA-3′
Mut root A	5′-GTATCTTATTTGTCGTTTCCAACACACC-3′	5′-AAAGCACAAATAAGATACGATGTGCAG-3′
Mut root B	5′-CATACTTTTGTGCGATCGGAAATTTTA-3′	5′-CGATCGCACAAAAGTATGGTTAAACCA-3′
LPF Δ6:1	5′-TCGGGATACCAACGCGTACACCTGTGC-3′	5′-TACGCGTTGGTATCCCGATGTGGTGAA-3′
LPF Δ2:1	5′-TTTGTTTGCCAACGCGTACACCTGTGC-3′	5′-TACGCGTTGGCAAACAAAAGTACAAGT-3′
LPF Δ2:4/3:1	5′-TTTGTTTGCGATGCGAACGAGTGGTTT-3′	5′-GTTCGCATCGCAAACAAAAGTACAAGT-3′
	5′-CCGCCTTTGCAACGCGTACACCTGTGC-3′	5′-TACGCGTTGCAAAGGCGGACTTAGGTT-3′

directing *GUS* expression in pBI 101.3 restricted with *Sal* and *BamH*. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404, and transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the floral dip procedure [7]. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way and used as negative and positive controls respectively.

2.3. Transient transformation of sunflower leaves

Sunflower leaf disks (eleven mm in diameter) were submerged in *Agrobacterium tumefaciens* (strain LBA4404 grown overnight and then incubated during 4 h in 100 μM acetosyringone, 10 mM $MgCl_2$) suspension (at a 0.5 DO density) supplemented with 15 μl/l of Silwet L77 and subjected to vacuum during 1 h. After washing in order to eliminate remaining cells, the disks were placed in fresh liquid MS (24 h later supplemented with 250 mg/l cefotaxime) for a period of 3 days. After washing with PBS, the samples were freezed with liquid nitrogen and total RNA isolated for analysis. For each construct, two disks originated from different plants were analyzed and the experiment repeated at least twice. As a control of the infiltration test, *GUS* reporter gene expression in these experiments was measured by histochemical assays as previously described [8].

2.4. Plant treatments

For water stress treatments, 14- to 20-day-old plants grown in Petri dishes were placed on filter paper during 2–3 h until water stress was clearly observed. Then, total RNA was extracted as described below. Control plants were kept in MS-dishes. To analyze induction by ABA, the plants (14- to 20-day-old) were placed in 100 μM ABA for 4–6 h and then harvested for RNA isolation. Treatments with NaCl (150 mM) were carried out in the way described above.

For ethylene treatments, 21-day-old Arabidopsis plants, grown in Petri dishes were changed to a new dish where the MS media was supplemented with 20 or 40 μM ACC, and maintained during 1 h until they were harvested for RNA isolation and analysis. Sunflower transiently transformed leaf disks were treated in the same way applying 15 min of vacuum to facilitate the contact.

2.5. RNA isolation and real time RT-PCR measurements

RNA samples for real-time RT-PCR were prepared with Trizol® reagent (Invitrogen™) and qRT-PCR analysis was performed as previously described [28].

3. Results

3.1. Analysis of HAHB4 promoter in the PLACE database

The analysis of both forms of the *HAHB4* promoter with the PLACE database (http://www.dna.affrc.go.jp/PLACE) allowed the identification of putative responsive elements in accordance to previously reported expression studies. No remarkable differences turned out between these two forms. Besides the presence of four putative ABREs (two of them, almost in the same position of the DNA complementary strands, position −286/−292, from now on called site M), another one located in position −161/−167 (now called site 3′) and the last one in position −1143/−1149 (named site 5′) from the transcription initiation site, the informatic analysis revealed the existence of a W-box element located at position −1103/−1109, and many different root-specific expression boxes along the whole sequence. Based on the results obtained with deleted constructs, some of them were chosen for further analysis as described below. Other elements were also detected by sequence analysis but they were not taken into consideration because they bear no relation with the actual knowledge arisen from the expression studies performed. A schematic representation of the localization and the corresponding sequence of each identified functional element in the whole promoter is showed in the figure corresponding to each analysis.

3.2. Only one of the four ABREs showed to be responsive to ABA

Since *HAHB4* expression is regulated by ABA [8,15], we decided to analyze the four ABREs in order to determine whether they were functional in this sunflower promoter. The analysis was carried out by doing deletion mutation of each one separately. Considering that the two forms of this promoter showed the same ABREs located at equivalent positions, we decided to perform the full analysis only with one of them, the large form, called *LPF*. Each construct, bearing a promoter mutation and directing the reporter gene *GUS*, was used to transform Arabidopsis plants and to transiently transform sunflower leaves. In both cases, the plants were treated with 100 μM ABA and *GUS* transcript levels were measured by quantitative RT-PCR. The results showed that Arabidopsis plants transformed with the control construct as well as with the 5′-ABRE or M-ABRE mutants present higher levels of *GUS* transcript after ABA treatments compared with RNA levels measured in untreated plants (Fig. 1B) while mutants in the 3′-ABRE showed a loss of inducibility by the hormone. Double and triple mutants were obtained showing that only the double mutant 5′ + M still responded to ABA while the mutants including the 3′-ABRE were no longer inducible, indicating that the 3′-ABRE is the only functional *cis*-acting element, at least out of these four detected in this promoter by the use of the PLACE database (Fig. 1B).

Sunflower leaves were transiently transformed with the same constructs and their analysis corroborated the results obtained with stable transformed Arabidopsis plants. Similar induction ratios were observed with control, 5′-site or M site simple or double mutated constructs and no induction at all when 3′-mutants were tested (Fig. 1C), what indicated the

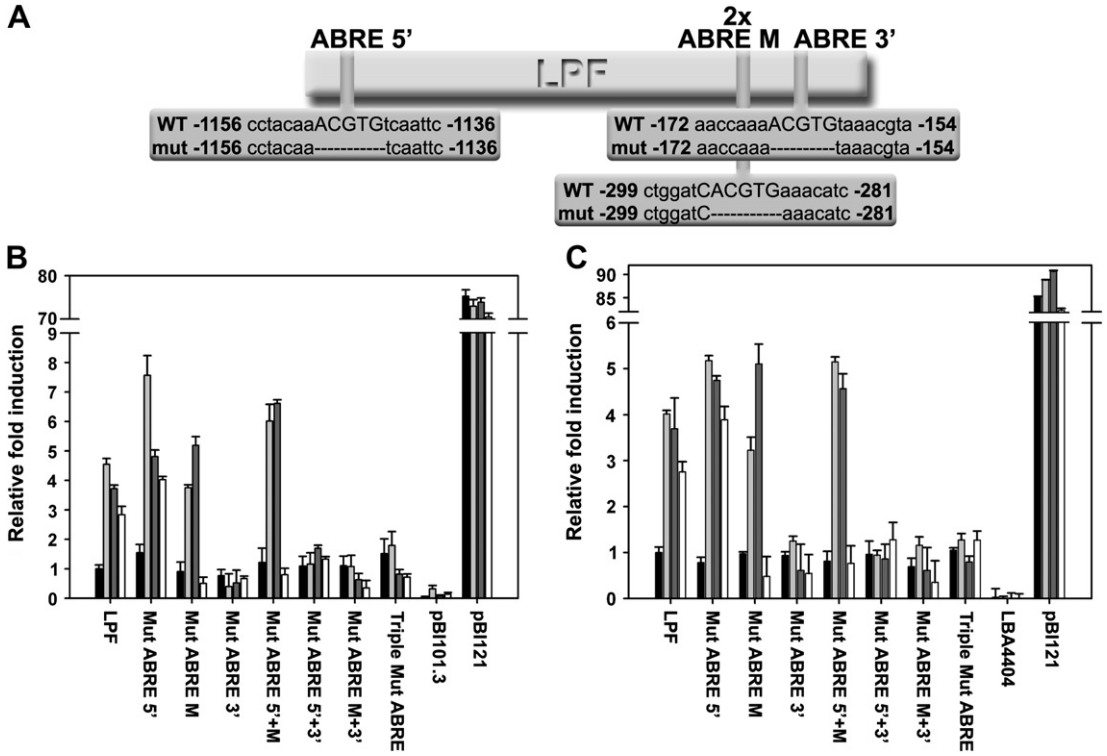

Fig. 1. ABA, drought and salt responses of the *HAHB4* promoter involve different *cis*-acting elements. A: Schematic representation of the ABREs localization in the *HAHB4* promoter (*LPF*). The indicated positions are related to the transcription initiation site. ABREs sequences are marked in upper cases, the surrounding sequences in lower cases and the deleted region signaled as ''————————''. B: *GUS* RNA transcript level in plants transformed with the indicated constructs subjected to treatments with 100 μM ABA (light gray bars), hydric stress (dark gray bars) or NaCl (white bars) compared with the level measured in control conditions for the WT construct (black bars). Plants transformed with pBI 101.3 and pBI 121 were used as negative and positive controls respectively. C: *GUS* RNA transcript level in sunflower leaves transiently transformed with the indicated constructs subjected to treatments with 100 μM ABA (light gray bars), hydric stress (dark gray bars) or NaCl (white bars) compared with the level measured in control conditions for the WT construct (black bars). Transient transformations with the host cells (*Agrobacterium tumefaciens* LBA 4404) or with the same cells carrying pBI121 were carried out as negative and positive controls respectively. Mut 5′, Mut 3′ and Mut M indicate a construct where the entire promoter (LPF) fused to *GUS* was mutated in each of the sites.

existence of a high conservation of this element's functionality between both species.

3.3. Drought response seems to occur via an ABA dependent way while NaCl response involves an additional alternative ABA-independent pathway

Arabidopsis plants transformed with the constructs described above were subjected to severe water stress and subsequently *GUS* transcript levels were measured by quantitative RT-PCR. The results, shown in Fig. 1B indicate that the same *cis*-element located 3′ in this promoter is responsible for drought response while the mutations of sites 5′ and M, as well as double mutations not involving the 3′-site, did not alter *GUS* transcript levels in stress conditions. Accordingly, similar results were observed when sunflower leaves were transiently transformed with the same constructs (Fig. 1C).

On the other hand, the response to salt stress turned out to involve a different mechanism. In this case, the 3′-ABRE seemed to be essential for this response: its mutation caused a significant decrease in *GUS* transcript levels, both in *Arabidopsis* and sunflower. However, mutation in the M-ABRE, until now apparently irrelevant, caused insensitiveness to the salt treatment in transgenic plants, indicating that also a second ABA-independent mechanism is taking place in the regulation of *HAHB4*.

3.4. The putative W-box located in position −1103/−1109 is responsible for the ethylene-mediated response

Regulation of *HAHB4* by ethylene at the transcriptional level was recently reported [28]. The analysis of this gene promoter revealed the presence of a W-box (see above). In order to elucidate whether this was a functional *cis*-acting element, a mutant construct was obtained where the six-nucleotide-box was deleted. Arabidopsis plants and sunflower leaves were transformed with this construct and they were subjected to treatments with ACC 20 or 40 μM, an intermediate compound in ethylene biosynthesis. *GUS* transcript levels were measured in treated and untreated transformed plants as well as in the plants transformed with the non-mutated segment used as control, and the results are shown in Fig. 2. Transcript levels of *GUS* measured in these samples indicated that the mutation of this site abolished ethylene responsiveness and therefore that this W-box is responsive to ACC.

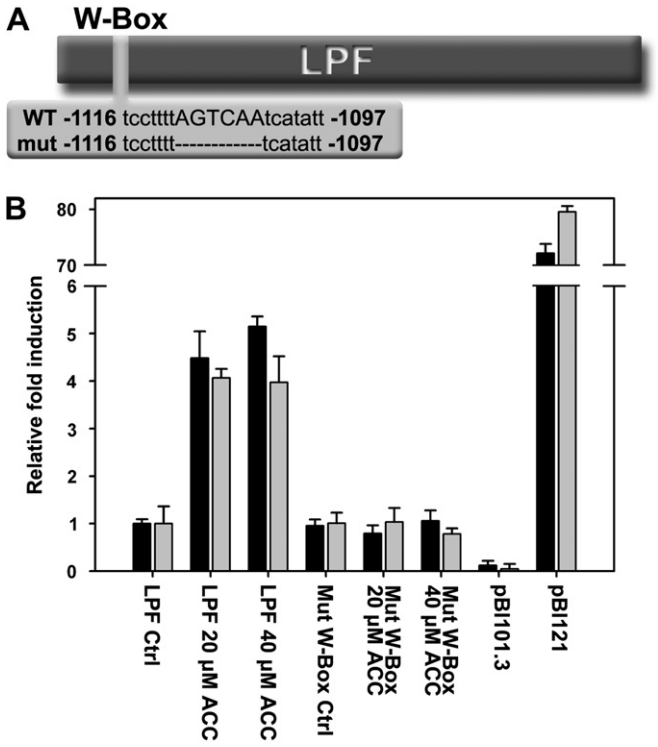

Fig. 2. The W-box located in the 5′ extreme of the *HAHB4* promoter is functional and responsive to ACC. A: Schematic representation of the location of the W-box detected by bioinformatics analysis in the promoter region of the *HAHB4* promoter. The box sequence is marked in upper cases, the surrounding sequences in lower cases and the deleted region signaled as "——————". B: *GUS* transcript levels in Arabidopsis transgenic plants (black bars) or in transiently transformed sunflower leaves (gray bars) subjected to a treatment with ACC (20 or 40 μM) compared with the level of the same transcripts measured in control conditions for the WT construct. Plants transformed with pBI 101.3 and pBI 121 were used as negative and positive controls respectively. Mut indicates a construct where the W-box was deleted in the entire promoter (LPF) fused to *GUS*.

3.5. Two root-specific-expression boxes act independently and exhibit redundant functions

Histochemical staining shown in Fig. 3 indicates that the entire promoter fragment, *LPF*, directs *GUS* expression in the roots central cylinder and in the growing lateral roots as well as in the vascular system of leaves (Fig. 3A). In order to determine which of the identified *cis*-elements were active, plants were transformed with serial deletions of the promoter, previously obtained [8], and histochemical analysis was performed. This analysis revealed that when the first 300 bp of the promoter were tested, no activity was detected (Fig. 3B). A minimum of 400 bp upstream the transcription initiation site was necessary and sufficient to conserve the organ-specific activity showed by the entire promoter (Fig. 3C). Five putative *cis*-acting elements (one CTCTT, −386; two ATATT, −235 and −14; one CACCTG, −85 and ACTTTA, −60) reported as directing root-specific expression in other promoters are located between −400 and the transcription initiation site. Only one of these boxes is located between −300 and −400 (CTCTT). The fact that the construct *−300:GUS* did not exhibit any activity suggested that this element (located in −381/−386) may be responsible for root-specific expression. On the other hand and in order to further analyze this promoter, chimerical constructs with different segments of this promoter were used to direct *GUS* expression in *Arabidopsis*. Two of these chimerical constructs, containing the 100 bp upstream the transcription initiation site plus the regions of 600 bp or 200 bp located in the 5′ upstream terminus of this promoter bear six putative boxes (one ACTTTA, −1047; one ATATT, −1096; one CTCTT, −1181; one CACCTG, −1202 and two CAACA, −1166 and −1156). Plants transformed with these constructs showed a similar histochemical pattern compared with the construct *−400:GUS* regarding the expression in the central cylinder of the roots and the vascular system (Fig. 3D,F). These observations indicated the existence of an extra active element located upstream −1000 since the segment comprised between −416 and −301 (see above) is not included in these chimeras.

Simple mutant constructs in which each putative root-directing box was deleted (ACTTTA, −1052/−1047; ATATT, −1100/−1096; CTCTT, −1184/−1181; CACCTG, −1207/−1202; CAACA, −1170/−1166; CAACA −1160/−1156; CTCTT, −390/−386; ATATT, −239/−235; ATATT −18/−14; CACCTG, −90/−85 and ACTTTA, −65/−60) or double mutants in which two repeated boxes were deleted (ACTTTA, −1052/−1047 plus ACTTTA, −65/−60; CTCTT,−1184/−1181 plus CTCTT, −390/−386; ATATT, −1100/−1096 plus ATATT, −239/−235; CACCTG, −1207/−1202 plus CACCTG, −90/−85 and CAACA, −1170/−1166 plus CAACA −1160/−1156), were obtained and analyzed in *Arabidopsis* transgenic plants. None of them, apart from the two mutants described below, in which the boxes located in positions −381/−386 and −1182/−1187 were deleted (not shown and Fig. 3 J), showed a distinctive expression pattern compared with the one exhibited by the control construct by histochemical analysis (data not shown), indicating that none of them are functional as tissue-specific *cis*-acting elements in the *HAHB4* promoter.

As it can be observed in Fig. 3H,I, none of both simple mutants, in sites −381/−386 and −1182/−1187, caused any effect in root expression. However, a third construct, carrying the double mutant, A plus B, showed a great loss of function (Fig. 3E), indicating that both elements are redundant. Therefore, the presence of at least one of them is essential to direct gene expression in the central cylinder of roots and leaves vascular system.

Comparing the tissue-specific GUS expression directed by the chimeras Δ6:1 and Δ2:1 with that of the construct Δ2:4/3:1, it could be observed a difference in the lateral root initiation staining. The two first ones showed no expression in this tissue while the third did so, suggesting the existence of one or more *cis*-acting elements responsible for this tissue-specific expression localized in the segment comprised between −416 and −301.

4. Discussion

The analysis performed with the PLACE database of the *HAHB4* promoter revealed that it exhibits numerous *cis*-acting

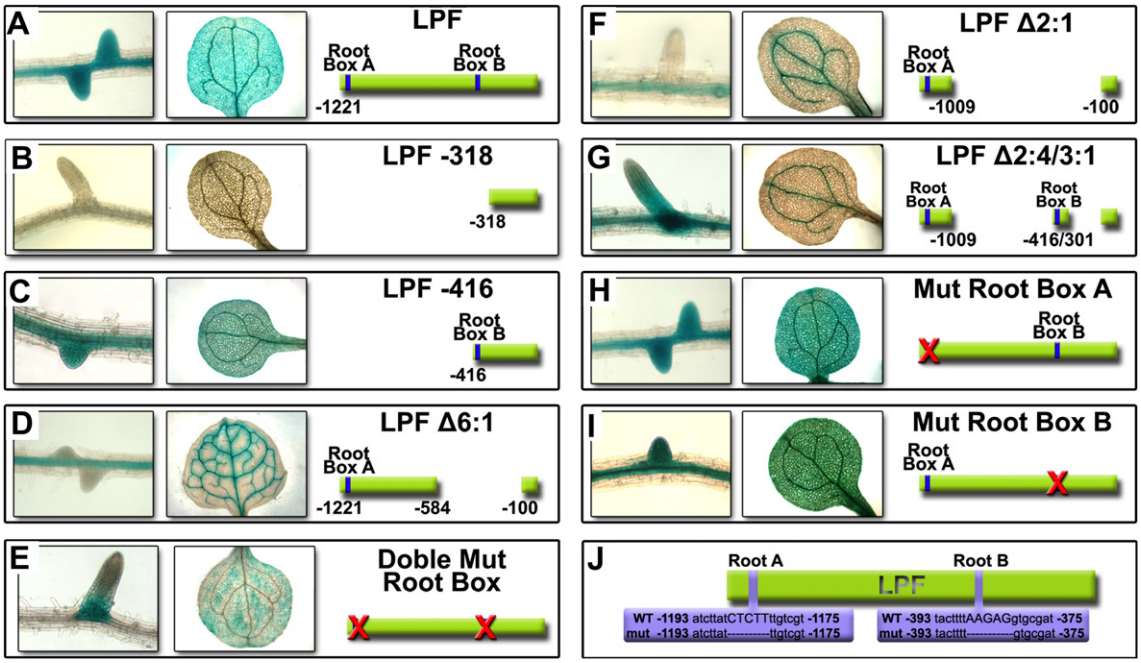

Fig. 3. The two putative root-specific expression *cis*-acting elements are functional but redundant. Histochemistry of GUS in Arabidopsis plants transformed with: A: *LPF:GUS* (entire *HAHB4* promoter region); B: deleted construct *−318:GUS*; C: deleted construct *−416:GUS*; D: chimerical construct delta *6:1:GUS*; E: double mutant in root-specific sites; F: chimerical construct D*2:1*; G: chimerical construct D*2:4/3:1*; H: *LPF:GUS* mutated in the root site A; I: *LPF:GUS* mutated in the root site B. J: Schematic representation of the location of the mutated roots-specific motifs detected by bioinformatics analysis in the promoter region of the *HAHB4* promoter. The box sequence is marked in upper cases, the surrounding sequences in lower cases and the deleted region signaled as "————————". For each construct, schematized in the right panel, expression was analyzed in roots (left panel) and in leaves (middle panel). Root-specific expression *cis*-acting elements are in blue, whereas those mutated are shown in red.

elements, described as responsive to a wide range of abiotic and biotic factors as well as to a set of hormones. The only remarkable difference between the two forms of this promoter is a DRE (drought responsive element) only present in the large form (*LPF*) at position −864. DREs are described as reactive to osmotic stress in several drought responsive genes, and sometimes interact with ABREs [32,49]. Since the same behavior was observed in plants transformed with each of the two forms when they were subjected to water stress, ABA or high salinity, it seems unlikely that this element exhibited functionality in this promoter.

Among the four putative ABREs, only one, located nearest the transcription initiation site, showed a response to ABA while the other three are not responsive to the hormone treatments. A peculiar behavior was observed in plants transformed with the 5′ mutant when they were treated with ABA (an enhanced expression compared with non-mutated plants) but not when they were subjected to high salinity or drought (Fig. 1B,C). This observation led us to conclude that this element plays the role of a negative regulator. It is difficult to explain such phenomenon in a different way. However, no bibliographic reports describe ABREs playing such a role. Experimental artifacts are discarded since the experiment was repeated several times both in Arabidopsis and sunflower.

As it has been reported by other authors [17,51], ABREs may act together with coupling elements. In this promoter one of such elements (ACGCGT) is localized at position −99/−93 near the 3′-ABRE and probably conforming a ABRE-CE module [23,24,52]. Considering that they were not deleted or mutated in this work and that they are present in the wild type constructs, it is possible that they interact with the 3′ ABRE. It would be necessary to mutate them in order to corroborate this hypothesis. However, it is clear that the deletion of the 3′ ABRE by itself is enough to observe a loss of function. On the other hand, salt induction involves an additional ABRE that did not respond to the hormone action, indicating the existence of two separate pathways, one ABA-dependent and one ABA-independent. A short sequence, similar to a DRE minimal core (C**C**GAC) where the second position is changed (C**G**GAC), is present near the M ABRE (−306 in *LPF*). Although such a sequence was not identified as functional [49], it cannot be ruled out the possibility that it interacts with the M ABRE in order to regulate the response to salinity. It is clear from the obtained results that both salt-responsive ABREs exhibit non-redundant functions since the mutation of any one of them caused the loss of responsiveness to salinity, even if the other one remains untouched. Moreover, these observations suggested that a cooperative mechanism involves these two elements. Besides, it was possible to establish the relationship between drought and ABA in the regulation of this gene and the existence of an additional independent signal transduction pathway involved in the response to high salt concentrations. Previous reports supported the idea that such elements act in alternative ways depending on the gene promoter in which they are present [49].

The W-box present in this promoter showed to be involved in the response to ethylene. Its deletion abolished of the response to the treatments with this hormone, indicating that this W-box is responsible for the ethylene regulation of *HAHB4*, probably acting together with *trans*-acting elements such WRKY transcription factors.

Regarding organ-specific expression, a considerable number of putative boxes were identified by the analysis in the PLACE database. Only two out of them could be characterized as functional in the vascular system, which actually exhibit a redundant activity, what suggests how significant the expression of *HAHB4* may be in roots. Staining in the leaves vascular system also disappeared when the two boxes were deleted, probably due to the loss of expression in the root central cylinder and the concomitant loss of transported dye. This hypothesis was confirmed when leaves and roots from the same plants were subjected to histochemistry in separate tubes (not shown). Interestingly, this *cis*-acting element (CTCTT) was described as participating in the activation of the leghemoglobin encoding gene in infected legume root nodules and not specifically directing central cylinder expression [13,46] indicating that this box is implicated also in an alternative activity, at least in this promoter. Regarding the expression in lateral roots initiations, we were able to determine that elements directing such expression may be present in the segment comprised between −416 and −301. However, none of the mutants of the known root-specific sites present in this segment showed a differential expression pattern, what suggests that other unknown *cis*-acting elements are responsible for such expression. Little is known about expression in roots of homologous genes. The Arabidopsis genes that bear closest resemblance to *HAHB4*, *ATHB7* and *ATHB12*, are responsive to ABA, salt or drought. However, no further information is available about functional *cis*-acting elements in these genes promoters [20,21,26,33]. On the other hand, it is well known that genes encoding transcription factors from other families are regulated by ABA as a result of cooperative mechanisms, different from the one described here [48,49,52].

One important conclusion arisen from the experimental data is that recognition of *cis*-acting elements is strongly conserved between Arabidopsis and sunflower, especially those responsive to external factors, such as ABA, ethylene, salinity or drought. Almost identical results were obtained analyzing stable transformed Arabidopsis or transiently transformed sunflower leaves. In the case of tissue-specific expression we are not able to test such expression since stable sunflower transgenic plants would be needed and up to now, no ordinary protocols to obtain them are available. However, the fact that external factors regulation seems to be conserved between species suggests that it would be worth trying to use chimerical promoters as biotechnological tools combining sequences in order to achieve a desired expression pattern of a certain gene. Regarding *HAHB4* regulation, it was possible to identify the boxes responsible for ABA, drought, salt and ethylene responses as well as the two boxes that direct expression in roots.

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References

[1] H. Abe, K. Yamaguchi-Shinozaki, T. Urao, T. Iwasaki, D. Hosokawa, K. Shinozaki, Role of Arabidopsis MYC and MYB homologs in drought-and abscisic acid-regulated gene expression, Plant Cell 9 (1997) 1859–1868.

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

[3] M. Barcala, A. García, P. Cubas, C. Almoguera, J. Jordano, C. Fenoll, C. Escobar, Distinct heat-shock element arrangements that mediate the heat shock, but not the late-embryogenesis induction of small heat-shock proteins, correlate with promoter activation in root-knot nematode feeding cells, Plant Mol. Biol. 66 (2008) 151–164.

[4] J.V. Cabello, C.A. Dezar, P.A. Manavella, R.L. Chan, The intron of the Arabidopsis thaliana COX5c gene is able to improve the drought tolerance conferred by the sunflower *Hahb*-4 transcription factor, Planta 226 (2007) 1143–1154.

[5] M. Carabelli, G. Sessa, S. Baima, G. Morelli, I. Ruberti, The *Arabidopsis Athb-2* and *-4* genes are strongly induced by far-red-rich light, Plant J. 4 (1993) 469–479.

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

[7] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.

[8] C.A. Dezar, G.V. Fedrigo, R.L. Chan, The promoter of the sunflower HD-Zip protein gene Hahb4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA, Plant Sci. 169 (2005) 447–459.

[9] C.A. Dezar, G.M. Gago, D.H. Gonzalez, R.L. Chan, *HAHB4*, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants, Transgenic Res. 14 (2005) 429–440.

[10] J. Díaz-Martín, C. Almoguera, P. Prieto-Dapena, J.M. Espinoza, J. Jordano, Functional interaction between two transcription factors involved in the developmental regulation of a small heat stress protein gene promoter, Plant Physiol. 139 (2005) 1483–1494.

[11] T. Eulgem, P. Rushton, E. Schmelzer, K. Hahlbrock, I. Somssich, Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors, EMBO J. 18 (1999) 4689–4699.

[12] Y. Fang, A.M. Hirsch, Studying early nodulin gene *enod40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa, Plant Physiol. 116 (1998) 53–68.

[13] V. Fehlberg, M.F. Vieweg, E.M. Dohmann, N. Hohnjec, A. Puhler, A.M. Perlick, H. Kuster, The promoter of the leghaemoglobin gene VfLb29: functional analysis and identification of modules necessary for its activation in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots, J Exp. Bot. 56 (2005) 799–806.

[14] M. Fujita, Y. Fujita, K. Maruyama, M. Seki, K. Hiratsu, M. Ohme-Takagi, L. Tran, K. Yamaguchi-Shinozaki, K. Shinozaki, A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway, Plant J. 39 (2004) 863–876.

[15] G.M. Gago, C. Almoguera, J. Jordano, D.H. Gonzalez, R.L. Chan, *HAHB4*, a homeobox-leucine zipper gene potentially involved in abscisic acid-dependent responses to water stress in sunflower, Plant Cell Environ. 25 (2002) 633–640.

[16] W.J. Gehring, M. Affolter, T. Bürglin, Homeodomain proteins, Annu. Rev. Biochem. 63 (1994) 487–526.
[17] J.L. Gomez-Porras, D.M. Riano-Pachon, I. Dreyer, J.E. Mayer, B. Mueller-Roeber, Genome-wide analysis of ABA-responsive elements ABRE and CE3 reveals divergent patterns in Arabidopsis and rice, BMC Genomics 8 (2007) 260.
[18] D.H. Gonzalez, E.M. Valle, G.M. Gago, R.L. Chan, Interaction between proteins containing homeodomains associated to leucine zippers from sunflower, Biochim. Biophys. Acta 1351 (1997) 137–149.
[19] M. Guiltinan, W. Marcotte Jr., R. Quatrano, A plant leucine zipper protein that recognizes an abscisic acid response element, Science 250 (1990) 267–271.
[20] E. Henriksson, A.S. Olsson, H. Johannesson, H. Johansson, J. Hanson, P. Engström, E. Söderman, Homeodomain leucine zipper class I genes in Arabidopsis. Expression patterns and phylogenetic relationships, Plant Physiol. 139 (2005) 509–518.
[21] M. Hjellström, A.S.B. Olsson, P. Engström, E.M. Söderman, Constitutive expression of the water deficit-inducible homeobox gene *ATHB7* in transgenic Arabidopsis causes a suppression of stem elongation growth, Plant Cell Environ. 26 (2003) 1127–1134.
[22] S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77 (1989) 51–59.
[23] T. Hobo, M. Asada, Y. Kowyama, T. Hattori, ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent, Plant J. 19 (1999) 679–689.
[24] B. Kaplan, O. Davydov, H. Knight, Y. Galon, M.R. Knight, R. Fluhr, H. Fromm, Rapid transcriptome changes induced by cytosolic Ca^{2+} transients reveal ABRE-related sequences as Ca^{2+}-responsive *cis* elements in *Arabidopsis*, Plant Cell 18 (2006) 2733–2748.
[25] T. Kobayashi, Y. Nakayama, R. Itai, H. Nakanishi, T. Yoshihara, S. Mori, N. Nishizawa, Identification of novel cis-acting elements, IDE1 and IDE2, of the barley IDS2 gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants, Plant J. 36 (2003) 780–793.
[26] Y.H. Lee, J.Y. Chun, A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment, Plant Mol. Biol. 37 (1998) 377–384.
[27] L. Liu, M.J. White, T.H. MacRae, Transcription factors and their genes in higher plants, Eur. J. Biochem. 262 (1999) 247–257.
[28] P.A. Manavella, A.L. Arce, C.A. Dezar, F. Bitton, J.P. Renou, M. Crespi, R.L. Chan, Cross-talk between ethylene and drought signaling pathways is mediated by the sunflower *HAHB4* transcription factor, Plant J. 48 (2006) 125–137.
[29] J. Mattsson, E. Söderman, M. Svenson, C. Borkird, P. Engström, A new homeobox-leucine zipper gene from *Arabidopsis thaliana*, Plant Mol. Biol. 18 (1992) 1019–1022.
[30] Y. Miao, T. Laun, P. Zimmermann, U. Zentgraf, Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis, Plant Mol. Biol. 55 (2004) 853–867.
[31] J. Mundy, K. Yamaguchi-Shinozaki, N. Chua, Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *RAB* gene, Proc. Natl. Acad. Sci. USA 87 (1990) 1406–1410.
[32] Y. Narusaka, K. Nakashima, Z.K. Shinwari, Y. Sakuma, T. Furihata, H. Abe, M. Narusaka, K. Shinozaki, K. Yamaguchi-Shinozaki, Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses, Plant J. 34 (2003) 137–148.
[33] A.S.B. Olsson, P. Engström, E. Söderman, The homeobox genes *ATHB7* and *ATHB12* encode potential regulators of growth in response to water deficit in *Arabidopsis*, Plant Mol. Biol. 55 (2004) 663–677.
[34] L. Rodriguez-Uribe, O'Connell MA A root-specific bZIP transcription factor is responsive to water deficit stress in tepary bean (*Phaseolus acutifolius*) and common bean (*P. vulgaris*), J. Exp. Bot. 57 (2006) 1391–1398.
[35] I. Ruberti, G. Sessa, S. Lucchetti, G. Morelli, A novel class of proteins containing a homeodomain with a closely linked leucine zipper motif, EMBO J. 10 (1991) 1787–1791.
[36] M. Schena, R.W. Davis, HD-Zip protein members of *Arabidopsis* homeodomain protein superfamily, Proc. Nat. Acad. Sci. USA 89 (1992) 3894–3898.
[37] M. Schena, A.M. Lloyd, R.W. Davis, The HAT4 gene of *Arabidopsis* encodes a developmental regulator, Genes Dev. 7 (1993) 367–379.
[38] Q. Shen, T. Ho, Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a g-box and a novel *cis*-acting element, Plant Cell 7 (1995) 295–307.
[39] Q. Shen, P. Zhang, T. Ho, Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley, Plant Cell 8 (1996) 1107–1119.
[40] S. Simpson, K. Nakashima, Y. Narusaka, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Two different novel *cis*-acting elements of erd1, a clpA homologous Arabidopsis gene function in induction by dehydration stress and dark-induced senescence, Plant J. 33 (2003) 259–270.
[41] C. Sivanandan, T. Sujatha, A. Prasad, R. Resminath, D. Thakare, S. Bhat, Srinivasan, T-DNA tagging and characterization of a cryptic root-specific promoter in Arabidopsis, Biochim. Biophys. Acta 1731 (2005) 202–208.
[42] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proc. Natl. Acad. Sci. USA 94 (1997) 1035–1040.
[43] M. Thomashow, Plant cold acclimatation: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 571–599.
[44] L.-S.P. Tran, K. Nakashima, Y. Sakuma, S.D. Simpson, Y. Fujita, K. Maruyama, M. Fujita, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter, Plant Cell 16 (2004) 2481–2498.
[45] Y. Uno, T. Furihata, H. Abe, R. Yoshida, K. Shinozaki, K. Yamaguchi-Shinozaki, Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions, Proc. Natl. Acad. Sci. 97 (2000) 11632–11637.
[46] M.F. Vieweg, M. Fruhling, H.J. Quandt, U. Heim, H. Baumlein, A. Puhler, H. Kuster, M.P. Andreas, The promoter of the *Vicia faba* L. leghemoglobin gene VfLb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants, Mol. Plant Microbe Interact. 17 (2004) 62–69.
[47] I. Winicov, B. Valliyodan, L. Xue, J. Hoober, The MsPRP2 promoter enables strong heterologous gene expression in a root-specific manner and is enhanced by overexpression of Alfin1, Planta 219 (2004) 925–935.
[48] K. Yamaguchi-Shinozaki, K. Shinozaki, A novel *cis*-acting element in an arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress, Plant Cell 6 (1994) 251–264.
[49] K. Yamaguchi-Shinozaki, K. Shinozaki, Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters, Trends Plant Sci. 10 (2005) 88–94.
[50] D. Yu, C. Chen, X. Chen, Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression, Plant Cell 13 (2001) 1527–1539.
[51] W. Zhang, J. Ruan, T.D. Ho, Y. You, T. Yu, R.S. Quatrano, *Cis*-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*, Bioinformatics 21 (2005) 3074–3081.
[52] X. Zou, J.R. Seemann, D. Neuman, Q.J. Shen, A WRKY gene from creosote bush encodes an activator of the abscisic acid signaling pathway, J. Biol. Chem. 279 (2004) 55770–55779.