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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

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Abstract

In the present work, we have analysed the promoter region of the sunflower nuclear gene *Hahb4*, encoding an homeodomain-leucine zipper protein involved in water stress responses. This region is represented in two different but highly conserved alleles of 1015 and 1221 bp, respectively, in the sunflower hybrid studied. To gain insight into the structure and function of these promoter forms, we have obtained plants stably transformed with different fragments fused to the β-glucuronidase (*gus*) reporter gene. Histochemical staining indicated that both *Hahb4* promoter forms direct expression in roots, cotyledons and leaves during the entire plant life cycle. No expression was observed in reproductive organs. The analysis of progressive upstream deletions of the promoters suggested that a minimal 417/421 bp fragment is required for basal expression. The presence of positive regulatory elements between nucleotides −601/608 and −818/−1024 from the transcription initiation site (depending on the promoter) and a sequence required for specific expression in the root central cylinder between −818/−1024 and −1015/−1221 has been detected. Water stress, ABA or NaCl treatments induced *Hahb4* promoter-dependent β-glucuronidase expression as observed by Northern blot hybridization experiments. Putative regulatory elements involved in the regulation of other genes were detected in the promoter fragment required for expression. These elements, together with experimental evidence, were analysed with the aim of elucidating the molecular mechanisms that participate in the expression of this gene.

Keywords: Gene expression; HD-Zip; Promoter analysis; Regulatory element; Salt inducible; Transcription factor; Water stress

1. Introduction

The homeodomain is a 60-amino-acid motif present in a number of eukaryotic transcription factors involved in developmental processes [1]. Genes containing homeoboxes have been isolated from many eukaryotic organisms including fungi, mammals, and plants [2]. Plant homeoboxes can be divided into several families, according to sequence conservation and structure in and outside the homeodomain [3,4]. 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 [5–8]. It has been suggested, and experimentally demonstrated in some cases, that these proteins are involved in regulating developmental processes associated with the response of plants to environmental conditions ([3,9,10]; Dezar et al., unpublished results).

One of the most common environmental stresses to which plants are exposed is dehydration. Although many seeds tolerate extreme water stress, tolerance is rare in vegetative parts of the plant. Plants respond to water stress with the expression of a specific set of genes, which allows them to adapt to altered environmental conditions [11–13] The hormone abscisic acid (ABA) plays an important role in a subset of these responses [13–15].

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We have previously reported the isolation, characterisation and expression studies of a gene member of the sunflower HD-Zip family, *Hahb4* [16]. The studies performed indicated that *Hahb4* is up-regulated by water stress and ABA in sunflower roots, stems, and leaves. This led us to propose that *Hahb4* may function in the signalling cascade that controls a subset of the ABA-mediated responses of sunflower to water stress. In order to establish if this gene plays a functional role in water stress responses, transgenic *Arabidopsis thaliana* plants that overexpress *Hahb4* under the control of the 35S cauliflower mosaic virus promoter were obtained. Transformed plants show a specific phenotype, presenting improved development, a healthier aspect and higher survival rates than wild-type plants when they are subjected to water stress conditions [Dezar et al., unpublished results].

In this paper, we describe the isolation and characterisation of two promoter regions corresponding to different alleles of the sunflower gene *Hahb4*. We have transformed *Arabidopsis* plants with constructs that bear different portions of these regions fused to the *gus* reporter gene and analysed expression by histochemistry and Northern blots. Our results indicate that regions located between −318 and −417/421 of the transcription initiation site are necessary for the transcriptional activity of *Hahb4* and contain regulatory elements involved in tissue-specific expression. For ABA or water stress inducibility, regions located between −601/608 and −818 (in the shorter allele) or −1024 (in the larger allele) are required, but maximum induction was observed when either of the two entire promoter regions were included. Positive elements required for the expression in the root central cylinder are located between −818/1024 and −1015 (in the shorter allele) or −1221 (in the larger allele).

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). Plants were grown on soil in a growth chamber at 22–24 °C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approximately 200 μE m^{-2} s^{-1}. Plants used for the different treatments were grown in Petri dishes containing Murashige and Skoog (MS) medium, 0.8% agar. The dishes were kept at 4 °C for 2 days and then transferred to growth chamber conditions and kept for variable periods of time as indicated in the figures legends.

2.2. Isolation of genomic clones

Two regions of 1015 and 1221 bp, respectively, upstream of the transcription initiation site of *Hahb4* were isolated in overlapping segments by inverse PCR as described previously [16] and sequenced. Then, they were amplified as entire fragments by PCR with oligonucleotides PROM1 (5′-GCGGTCGACACCTGGCACATCGTATCTT-3′) and PROM2 (5′-CGCGGATCCGAGGGTTTGATAAGTGAT-3′) using genomic sunflower DNA as template. These DNA fragments were cloned directly in the pGEM-Teasy plasmid (Promega Corp.) and sequenced. The corresponding *Sal*I/*Bam*HI fragments were then cloned in pBI101.3 previously digested with the same enzymes. In this way, the promoter regions corresponding to the two alleles of the hybrid contiflor 15 (Zeneca) of *Hahb4* were available for further constructions.

2.3. RNA isolation and analysis

Total RNA was isolated as described by Carpenter and Simon [17]. For Northern blot analysis, specific amounts of RNA were electrophoresed through 1.5% (w/v) agarose/6% formaldehyde gels. The integrity of the RNA and equality of RNA loading were verified by ethidium bromide staining. RNA was transferred to Hybond-N nylon membranes (Amersham Corp.) and hybridised overnight at 65 °C to ^{32}P-labelled probes in buffer containing 6× SSC, 0.1% (w/v) polyvinylpirrolidone, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.2% (w/v) SDS, and 10% (w/v) polyethylene glycol 8000. Filters were washed with 2× SSC plus 0.1% (w/v) SDS at 65 °C (4 times, 15 min each), 0.1× SSC plus 0.1% (w/v) SDS at 37 °C during 15 min, dried and exposed to Kodak BioMax MS films. To check the amount of total RNA loaded and transferred in each lane, filters were then re-probed with a 25S rDNA from Vicia faba under similar conditions as those described above, except that hybridization was performed at 62 °C and the wash with 0.1× SSC was omitted. For gus detection, a full-length cDNA probe was obtained by restriction of pBI101.3 with *Bam*HI/*Sac*I. Hybridization was performed at 68 °C to avoid unspecific reactions.

2.4. Reporter gene constructs and plant transformation

Constructs containing upstream deletions of the promoters were obtained by PCR amplification with oligonucleotides Prom200 (5′-CCCAAGCTTTTATCTCAACCGAAAGTGAC-3′), Prom300 (5′-CCCAAGCTTAACCTAAGTCCGCCTTTG-3′), Prom400 (5′-GCGAAGCTTGATGCGAACGAGTGGTTTA-3′), Prom500 (5′-GGCAAGCTTCCTTGTCTCCCATCATCA-3′), Prom600 5′-GGCAAGCTTATTCACCACATCGGGATA-3′), Prom700 (5′-CCCAAGCTTCCCGTCTCTACTCGTCTA-3′), Prom800 (5′-GCGAAGCTTACTTGTACTTTTGTTTGC-3′), Prom900 (5′-CCCAAGCTTCACCAATTTCATTTCC-3′) and Prom2, digested with *Hind*III/*Bam*HI and cloned in pBI101.3. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV2260, and transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the

floral dip procedure [18]. Transformed plants were selected on the basis of kanamycin resistance and positive PCR carried out on genomic DNA with a specific primer for *Hahb4* and a *gus*-specific primer (5′-TTGGGGTTTCTA-CAGGAC-3′). Five to ten independent lines for each construct were further reproduced and homozygous T3 and T4 plants were used to analyse *gus* expression. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way and used as negative and positive controls respectively.

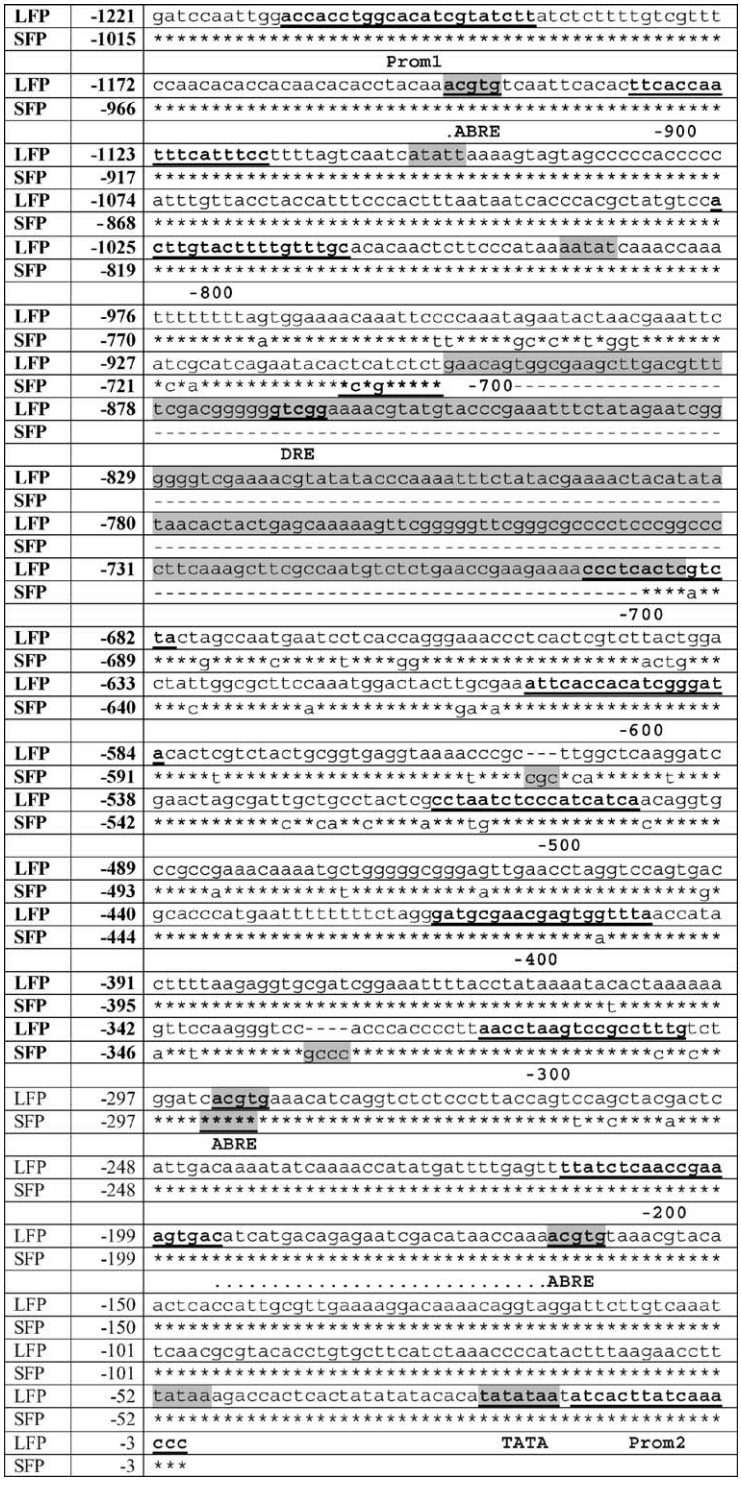

Fig. 1. Comparison between genomic sequences of sunflower *Hahb4* promoters. The transcription initiation site is indicated as +1. The TATA-box and four putative ABREs are shown in bold, shaded and underlined. Oligonucleotides used in successive cloning are in bold and underlined. The shaded sequence indicates the insertion observed in the LPF with respect to the SPF. Stars indicate absolute conservation of nucleotides.

2.5. Histochemical GUS staining

In situ assays of GUS activity were performed as described by Jefferson et al. [19]. Whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1% Triton X-100 and, after applying vacuum for 5 min; they were incubated at 37 °C overnight. Chlorophyll was cleared from green plant tissues by immersing them in 70% ethanol.

2.6. 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 tubes with water. To analyse induction by ABA, the plants were placed in 100 μM ABA for 4–6 h and then harvested for RNA isolation. Treatments with NaCl or mannitol were carried out in the same way as with ABA, but different salt concentrations were used as indicated in the figure legends.

3. Results

3.1. Two different alleles encode Hahb4 in sunflower

We have previously described the isolation by inverse PCR of a 720 nucleotide region located upstream of the transcription initiation site of *Hahb4* [16]. Considering the large size of the sunflower genome, we have decided to extend this segment to perform the analysis of the promoter. Surprisingly, we have obtained two divergent sequences mainly due to an insertion upstream of position −689. To verify this observation, two oligonucleotides that hybridise at the borders of the characterised fragments were used in a new PCR reaction with total genomic DNA from the sunflower hybrid Contiflor 15 as template. In this

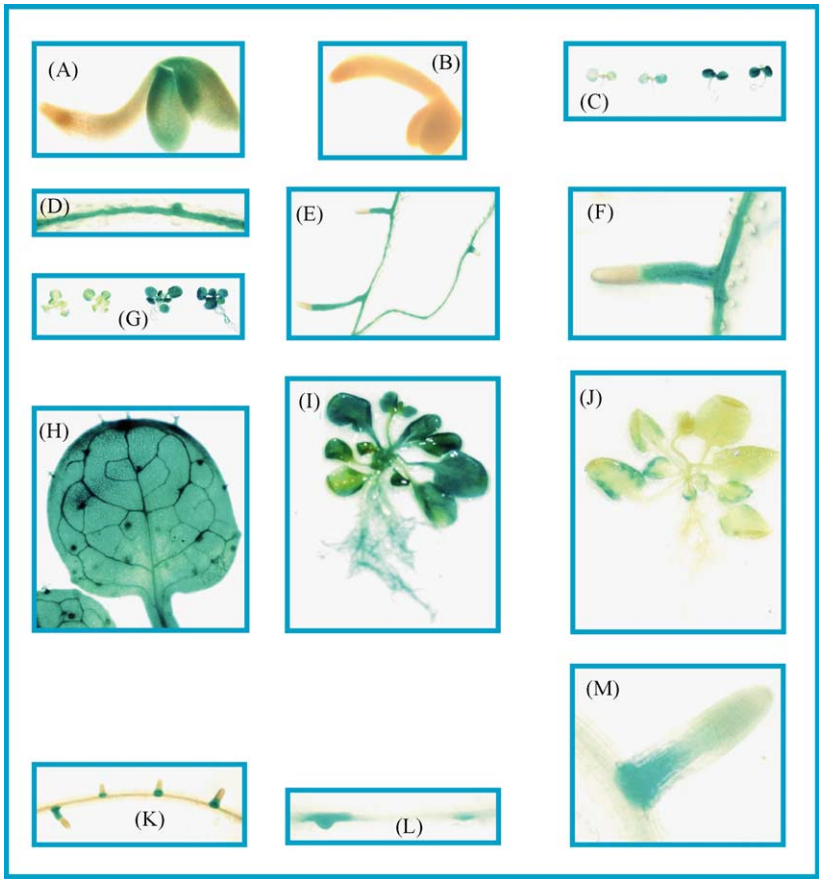

Fig. 2. Histochemical localisation of GUS activity in *Arabidopsis* plants transformed with the *Hahb4* promoter fused to the *gus* reporter gene. (A) seedling transformed with the entire promoter fragment 24 h after germination; (B) a similar seedling transformed with the −417/421 construction. (C) Right panel, 4-day-old seedlings transformed with LPF and in the left, seedlings transformed with the −417/421 construction. (D) and (E) 20-day-old plant roots transformed with SPF. (F) a detail of part E. (G) comparison between 20-day-old plants transformed with the −417/421 (left) construct or the entire promoter fragment (right); (H) leaf of a transformed plant with SPF. (I) 30-day-old plants transformed with the LPF; (J) the same experiment as in part I but the plants were transformed with the −417/421 construction. (K) 20-day-old roots transformed with the −601/608 construct; (L) a detail of part K but with plants transformed with the −514/518 construct. (M) secondary roots of plants transformed with the −417/421.

reaction, two different products differing in size that specifically hybridised with partial segments of *Hahb4* were obtained. Sequencing of these products confirmed that they represent two different genomic fragments, with a low proportion of nucleotide differences in the first 689 bp upstream of the transcription initiation site, a 213 bp insertion in one of the fragments, and almost identical sequences upstream of this insertion (Fig. 1). Oligonucleotides used in further constructions are indicated in the same figure for easier understanding. We speculate that the two promoter fragments may represent two different alleles since a hybrid sunflower variety was used as source of DNA. However, we cannot rule out that they arise from a recently duplicated region of the sunflower genome. The respective fragments were named LPF (large promoter fragment) and SPF (short promoter fragment) in order to simplify the interpretation in future experiments.

3.2. The Hahb4 promoters direct tissue-specific GUS expression

To characterise the expression patterns conferred by these promoter regions, we subcloned the entire fragments in front of the *gus* gene coding sequence contained in the binary vector pBI101.3 and used these clones to obtain *Arabidopsis* transgenic plants by *Agrobacterium*-mediated transformation. Five to ten independent transgenic lines bearing each construction were analysed by histochemical staining of GUS activity using X-gluc as substrate. At early stages of development, GUS activity was detected mainly in cotyledons (Fig. 2A and C) and the root central

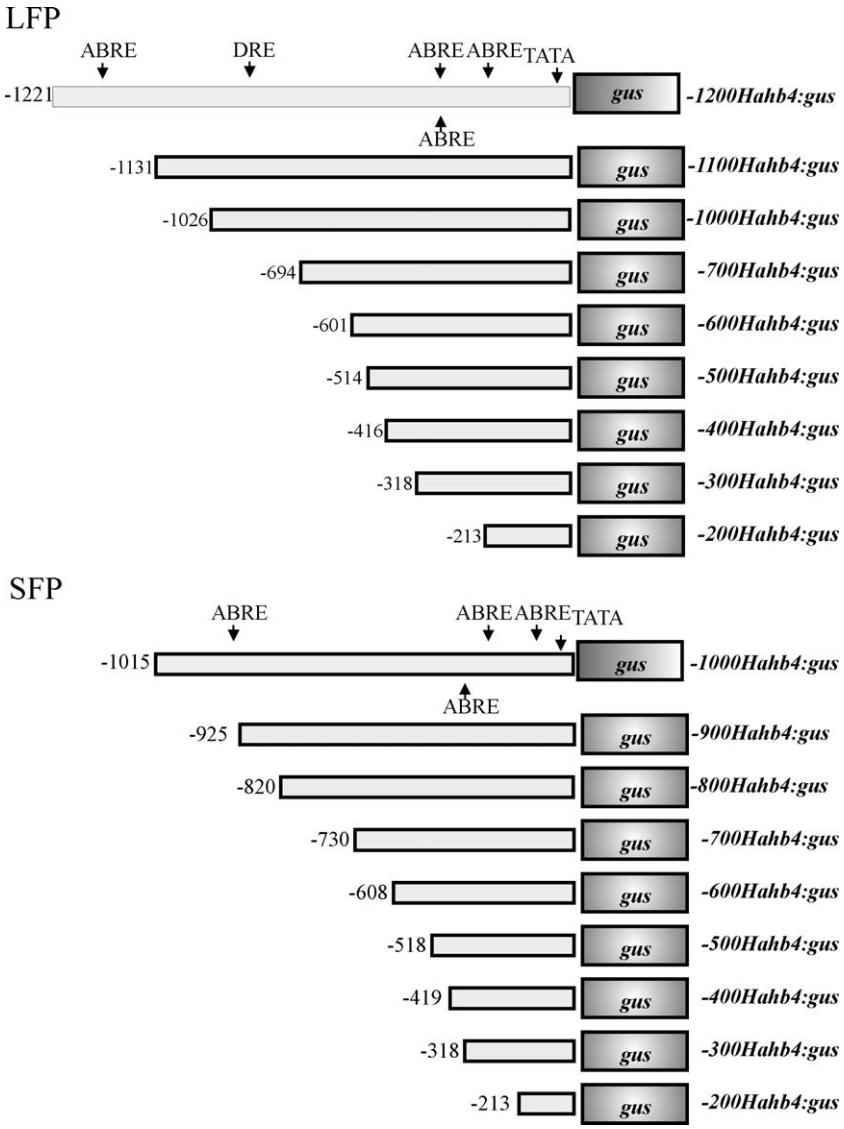

Fig. 3. Schematic representation of the different constructs used to transform *Arabidopsis* plants. The numbers to the left of the bars (−1221, −1015, etc.) indicate the upstream end of the promoter fragment present in each construct, respective to the transcription start site; the downstream end was at −1 for all constructs. On the right, the name used for each construct as cited in the text is shown. (A) Constructs derived from the LPF; (B) constructs derived from the SPF.

cylinder as well as in the lateral roots growing (not shown). Upon seedling growth, staining was progressively localised to the vascular cylinder of the root (Fig. 2D and E). GUS activity was still detected in the lateral roots growing (Fig. 2F). Leaves were stained during all the stages of development (Fig. 2G and H), but reproductive organs did not display GUS activity (not shown). In adult plants, expression was detected preferentially in the vascular cylinder of primary and secondary roots (Fig. 2E) as well as in leaves (Fig. 2I).

3.3. A minimal segment of 417/421 bp is required for expression

To define the minimal promoter region required for the observed expression patterns, we performed series of nested deletions from the upstream portion of the entire promoter (Fig. 3). The deletions were done approximately each 100 bp. The different constructs were introduced into *Arabidopsis* and analysed in transformed plants by GUS histochemistry. Plants bearing constructs where the expression was directed by the −318 or −214 segments did not show any expression (data not shown). Plants transformed with −417/−421:*gus* showed a weaker expression than the entire promoter in leaves and cotyledons during the same developmental stages (Fig. 2B, C, G and J). In roots, the situation was different: expression was visualised only in lateral roots growing but not in the central cylinder, indicating that the sequence that directs expression in these cell types is located upstream of 417/421 bp from the transcription initiation site (Fig. 2K, L and M). Plants bearing −514/518:*gus*, −601/608:*gus*, −694/704:*gus* and −818/1024:*gus* showed a similar behaviour with respect to cell specific localisation than −417/421:*gus*, but histochemical staining increased with the size of the promoter fragment, indicating that a combination of different elements present along this region is necessary to enhance expression. In fact, it is difficult to differentiate between plants transformed with −417/421:*gus* and the ones transformed with −601/608:*gus* or −818/1024:*gus*. They are almost indisguishable between them and show slightly stronger reactions than −417/421:*gus* in histochemistry. When transgenic plants bearing the −818/−1024:*gus* construct were analysed, expression was almost identical, though slightly weaker, than the one observed when transgenic plants bearing each of the entire promoter segments were analysed, with the sole exception of the root central cylinder that was not stained. These results indicate that almost all the important motifs directing organ specific expression are located downstream of position −417/421. The fact that plants with the −318:*gus* construct did not show GUS activity indicates that essential elements are located between −318 and −417/421, although their presence downstream of −318 cannot be ruled out. Upstream of position −818/−1024 (depending on the promoter), a sequence conserved between both alleles directs expression in the root central cylinder. Several positive regulatory elements might be located between −417/421 and −1015/1221.

3.4. Hahb4 promoter activity is induced by water stress, NaCl and ABA

We have previously shown that *Hahb4* transcript levels are regulated by the incubation of sunflower plants in solutions containing ABA or when the plants are subjected to water stress [16]. To investigate if this regulation takes place at the transcriptional level, we have grown plants transformed with the different promoter fragments in Petri dishes containing MS medium. The plants were treated with either ABA or NaCl, or subjected to a severe water stress. Transcript levels of *gus* were measured by Northern blot hybridization in extracts prepared from independent transgenic lines. Transcript levels were higher and easily visualised after ABA or dehydration treatments (Fig. 4), suggesting the existence of transcriptional regulation by these external factors. We have also tested the effect of giberellines (GA_3) and IAA on *gus* transcript levels. No changes were observed with these treatments (data not shown). Notably, transcript levels in transgenic plants subjected to water stress or ABA 100 μM were slightly higher than the positive control used (i.e., plants transformed with pBI121), indicating that the *Hahb4* promoter directs high expression levels upon induction. Levels reached with the constructs bearing each of the promoter forms were essentially similar and responded identically to inductive treatments (Fig. 4).

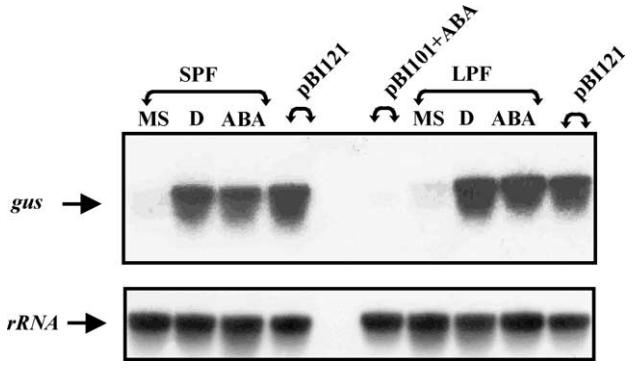

Fig. 4. Inducibility of the *Hahb4* promoter forms. Plants transformed with the small (SPF) or large (LPF) promoter fragments fused to *gus* were grown in MS, subject to drought (D) or treated with 100 μM ABA. Plants transformed with pBI121 were used as positive control. Plants transformed with pBI101.3 and treated with 100 μM ABA were used as negative control. For each plant type and treatment, total RNA (10 μg) was extracted from 3-week-old transformed plants. RNA was electrophoresed, blotted onto nylon membranes, and probed with a ^{32}P-labelled *gus* cDNA as detailed in Section 2. The same filter was hybridised with an rRNA probe as a control of RNA loading and transfer (lower panel).

3.5. The Hahb4 promoter contains positive regulatory elements upstream of position −417/421

The same deletion mutant constructs described above were used to analyse stress inducibility. Transgenic plants bearing different promoter segments were analysed by Northern blot hybridization using *gus* as probe. Treatments with ABA, NaCl or dehydration stress was done. When plants bearing the −818/1024 construct were analysed, an inducible expression was detected but it was clearly weaker than in plants bearing either of the two entire promoter regions, indicating that an enhancer sequence must be located upstream this position. On the other hand, no *gus* signal could be detected in plants carrying the proximal 417/421 bp or shorter promoter fragments under the different stress treatments (Fig. 5). Intermediate constructs were also analysed with the same negative result (not shown). These experiments indicated that a minimal fragment of 818/1024 bp is required to visualise ABA or stress inducible activity of the *Hahb4* promoter. Salt induction of the promoter activity was also tested. Treatments with 100, 200 and 300 mM NaCl were performed and *gus* transcript levels were analysed in the same way. As it can be appreciated in Fig. 6, NaCl induced promoter activity when the constructs present in *Arabidopsis* plants were either SPF or LPF. Constructs with the −601/608 segment, and smaller fragments did not respond to salt stress. Induction by NaCl

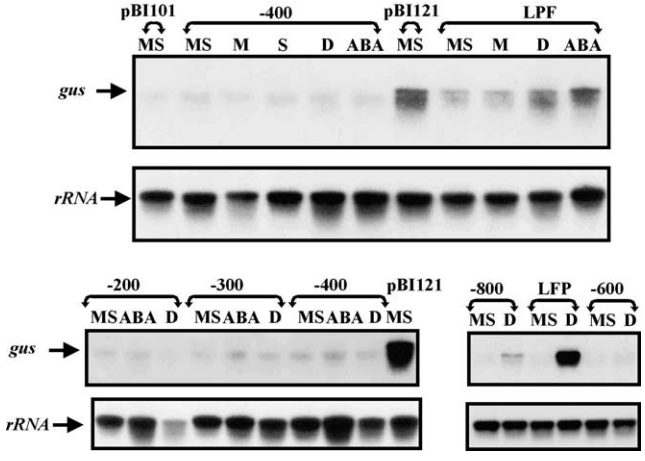

Fig. 5. Deletion constructs of the *Hahb4* promoter are not inducible by ABA up to −818/1024 from the transcription initiation site. A: *Arabidopsis* plants transformed with the large promoter fragment (LPF) or the −417*:gus* construct were grown in MS, or supplemented with 100 mM mannitol (M) or 200 mM NaCl (S), subjected to drought (D) or treated with 100 μM ABA. Plants transformed with pBI121 were used as positive control. Plants transformed with pBI101.3 were used as negative control. B: Plants transformed with either the −417*:gus*, −300*:gus* or −200*:gus* construct were grown in MS, subjected to drought (D) or treated with 100 μM ABA. Plants transformed with pBI121 were used as positive control. For each plant type and treatment, total RNA (10 μg) was extracted from 3-week-old transformed plants. RNA was electrophoresed, blotted onto nylon membranes, and probed with a ^{32}P-labelled *gus* cDNA as detailed in Section 2. The same filter was hybridised with an rRNA probe as a control of RNA loading and transfer (corresponding lower panel).

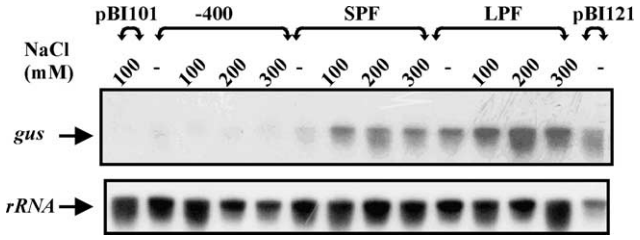

Fig. 6. The *Hahb4* promoter is inducible by NaCl. Two-week-old plants transformed with LPF, SPF, or deletion mutants were treated with 100, 200 or 300 mM NaCl in Petri dishes. Plants transformed with pBI121 were used as positive control. Plants transformed with pBI101.3 were used as negative control. For each plant type and treatment, total RNA (15 μg) was extracted, electrophoresed, blotted onto nylon membranes, and probed with a ^{32}P-labelled *gus* cDNA as detailed in Section 2. The same filter was hybridised with an rRNA probe as a control of RNA loading and transfer (lower panel).

was almost as high as that produced by ABA or water deficit stress. Once again both alleles behaved similarly and showed maximal induction with 200 mM NaCl (Fig. 6).

3.6. Putative regulatory elements present in the Hahb4 promoter

The region from −1221/1015 to −1 was analysed for the presence of known plant regulatory elements that could be related to the expression patterns observed in transgenic plants using the PLACE database [20]. We have identified a canonical TATA-box near the transcription start site (−17 to −24). Four putative ABREs (abscisic acid responsive elements, [11,13,14,21,22]) have been identified: the first one located at positions −1146 to −1141, two superimposed at positions −291 to −287 in the positive strand and −292 to −288 in the negative strand and one near the transcription initiation site at position −164 to −159 (Fig. 1). A core motif (DRE) described as cold, dehydration and ABA regulating expression element was identified at position −867 to −861 [23,24] only in the LPF. Twenty-nine consensus sequences for other transcription factors involved in responses to water stress, namely Myb and Myc proteins [13] were also identified. Some of these consensus sequences are present in promoters of dehydration-responsive genes like *rd22* or *rd29A* [25,26] and have been well characterised. Other consensus motifs were found, but no correlation between them and *gus* directed expression by this promoter could be established. We cannot rule out the possibility of the existence of other external or hormonal factors that regulate this gene expression but more studies must be performed to elucidate this question.

4. Discussion

HD-Zip proteins are unique to plants. Some years ago it has been suggested that this fact make them good candidates to regulate plant specific developmental processes like adaptation to environmental conditions. Today, accumulat-

ing evidence supports this previous idea, i.e. several genes encoding members of the HD-Zip family are involved in adaptive responses to the environment. Transcript levels for a set of HD-Zip genes are considerably higher when plants are subjected to water stress or treated with ABA [16,27–29]. In at least one case, it has also been shown that a gene of this family has an important role in plant dehydration stress tolerance [Dezar et al., unpublished results]. Another gene, *HAT4*/*Athb2* is involved in light signal transduction pathway(s) [9,10]. However, little is known about the elements that govern the transcriptional regulation of these genes. Studies were performed on these genes, related to organ specific expression. In this sense, Tornero et al. [30] have reported studies that indicate that phloem specific expression during secondary phases of vascular development of the tomato VAHOX1 gene is governed by transcriptional regulation. Söderman et al. [29] reported organ specific transcriptional regulation of *Athb6*, an ABA regulated HD-Zip gene. Organ specific expression of the promoter of *ZHB13*, a plant homeobox HD-Zip III gene, was also studied with promoter-*gus* chimerical fusions [31].

Hahb4 is a sunflower member of the subfamily I of HD-Zip transcription factors isolated in our laboratory. In order to establish if ABA and water stress regulations occur at the transcriptional level we have isolated the promoter region of this gene. In this step, we have found that two different genomic fragments, probably representing allelic forms, are present in the sunflower hybrid we used as source of DNA. In this fragments we have identified several consensus motifs described as ABA and water stress responsive elements in other plant genes [32,33]. These motifs have been localised in proximal and distal regions with respect to the transcription initiation site. These motifs may act separately, in combination, or not function at all in ABA or dehydration stress responsiveness, and may be stimulated by other environmental factors as reported in the literature [34,35]. Occasionally, they are present in promoter regions of genes that are not regulated by these external factors [36,37]. With the aim of establishing if the putative responsive elements are functional or not in *Hahb4*, the two segments and successive deletions were cloned directing *gus* reporter gene expression in transformed plants. The first observation was that the two alleles directed *gus* expression in a similar form in different tissues and organs of the plant. No significant differences could be appreciated between them, indicating that the insertion/deletion at position −696 has not disrupted any functional element. These promoter regions were enough to direct *gus* expression during plant development and in response to external factors like drought, ABA or high salt concentrations. This fact indicates a conservation of regulatory mechanisms between sunflower and *Arabidopsis*. The unique DRE motif we have found in this promoter is located in the insertion segment that is not present in the shorter form. Since both segments are indistinguishable in their response to severe dehydration stress, we can conclude that this motif has not an important role in the response of this gene. In the same way, three of the ABREs are not functional or act in concert with other elements, since they are located in a segment that did not respond to water stress or ABA treatment.

Deletion constructs (Fig. 3) allowed us to conclude that sequences located between −417/421 and the transcription initiation site are required for expression in tissues and organs and that the region between −318 and −417/421 is essential for activity. Sequences located between −818/1024 and −1015/−1221 are required for expression in the root vascular cylinder since deletion of this segment abolishes expression in this cells types, while expression in all other organs and tissues is conserved. Interestingly, the action of the element(s) involved in root vascular cylinder expression is not disrupted by the insertion found in one of the promoter forms, suggesting that it may act as an independent enhancer. The putative TATA-box is present, as usual, at −24 from the transcription initiation site. This means that most relevant promoter elements are rather distal from this site. This is also true for the elements involved in regulation by ABA and dehydration. This fact is not surprising, taking into account that the sunflower genome is about two orders of magnitude larger than the *Arabidopsis* one, i.e.: larger sequence distances between functional boxes is logical.

It is interesting to note that the expression patterns observed in histochemical GUS assays indicate that *Hahb4* is preferentially expressed in proliferating tissues, especially in growing lateral roots and that expression progressively disappears when lateral roots mature. This might indicate that this gene function is related to initial steps of secondary root formation, probably in response to external factors unidentified up to now. It is remarkable that sequences that direct expression in the central cylinder are separated from those that direct expression in proliferating tissues, a fact evident by the loss of activity in the central cylinder in plants transformed with constructs shorter than −818/−1024.

On the other hand, the same promoter region seems to determine expression in organs and inducibility by ABA and water stress. Preliminary data indicate however that the segment located between −514/518 and the 5′ end of this promoter is sufficient to promote ABA-stimulated *gus* expression (data not shown).

The results obtained both in histochemical and Northern blot experiments with the deleted constructs are summarised in Table 1 for better comprehension.

In summary, we have established that the promoter of the *Hahb4* gene directs expression in specific organs and tissues by a combination of elements. Induction by water stress and ABA has also been observed indicating that regulation of this gene occurs at the transcriptional level. Otherwise, these segments are sufficient to generate dehydration stress tolerance in transgenic plants when they direct *Hahb4* expression (data not shown). A detailed analysis of the promoter elements that confer the observed responses will be helpful to elucidate the molecular mechanisms involved in the expression of this and other genes encoding members

Table 1

Construct name	Root central cylinder	Lateral root growing	Leaves	Salt inducibility	Water stress inducibility	ABA inducibility
−1200 LFP/−1000 SFP	+++	+++	++++	++++	++++	++++
−1100 LFP/−900 SFP	+++	+++	++++	+++	+++	+++
−1000 LFP/−800 SFP	−	+++	+++	++	++	++
−600 LFP/−600 SFP	−	+++	++	−	−	−
−500 LFP/−500 SFP	−	+++	+	−	−	−
−400 LFP/−400 SFP	−	+++	+	−	−	−
−300 LFP/−300 SFP	−	−	−	−	−	−
−200 LFP/−200 SFP	−	−	−	−	−	−

Summary of the expression profiles observed in histochemical and Northern blot experiments done with the deletion constructs of *Hahb4* promoter regions. Constructs are schematised in Fig. 3 with their corresponding names. LFP: large fragment promoter; SFP: short fragment promoter. ++++: Strong expression observed; −: none expression observed.

of the HD-Zip family. To the best of our knowledge this is the first report on the inducibility of a sunflower promoter by external conditions. This fact may open the possibility of biotechnological uses of this DNA segments as tools to direct expression of this or others genes in transgenic plants.

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