Transgenic Research (2005) 14:429–440
DOI 10.1007/s11248-005-5076-0

Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants

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Received 2 December 2004; accepted 27 March 2005

Key words: drought tolerance, HD-Zip, homeodomain-leucine zipper, inducible promoter, plant homeodomain, sunflower, transcription factor, water stress

Abstract

Homeodomain-leucine zipper proteins constitute a family of transcription factors found only in plants. *Hahb-4* is a member of *Helianthus annuus* (sunflower) subfamily I. It is regulated at the transcriptional level by water availability and abscisic acid. In order to establish if this gene plays a functional role in drought responses, transgenic *Arabidopsis thaliana* plants that overexpress *Hahb-4* under the control of the 35S Cauliflower Mosaic Virus promoter were obtained. Transformed plants show a specific phenotype: they develop shorter stems and internodes, rounder leaves and more compact inflorescences than their non-transformed counterparts. Shorter stems and internodes are due to a lower rate in cell elongation rather than to a stop in cell division. Transgenic plants were more tolerant to water stress conditions, showing improved development, a healthier appearance and higher survival rates than wild-type plants. Indeed, either under normal or drought conditions, they produce approximately the same seed weight per plant as wild-type plants under normal growth conditions. Plants transformed with a construct that bears the *Hahb-4* promoter fused to *gusA* show reporter gene expression in defined cell-types and developmental stages and are induced by drought and abscisic acid. Since *Hahb-4* is a transcription factor, we propose that it may participate in the regulation of the expression of genes involved in developmental responses of plants to desiccation.

Introduction

One of the major environmental factors limiting plant productivity is lack of water. Although conventional breeding and marker-assisted selection are currently being used to develop varieties more tolerant to water stress, these methods are time and resource consuming, and do not always give the expected results. Genetic engineering is an attractive alternative to improve water stress tolerance in plants.

Plants respond to water stress with the expression of a specific set of genes, which allow them to adapt to altered environmental conditions (Almoguera et al., 1993; Coca et al., 1996; Bray, 1997; Shinozaki & Yamaguchi-Shinozaki, 1997). The hormone abscisic acid (ABA) plays an important role in a subset of these responses (Skriver & Mundy, 1990; Shinozaki & Yamaguchi-Shinozaki, 1997; Leung & Giraudat, 1998).

Efforts to improve abiotic stress tolerance by means of genetic engineering have relied on the overexpression of genes involved in one of the

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various adaptation transduction signal pathways (Kasuga et al., 1999; Kim et al., 2001; Polidoros et al., 2001; Hsieh et al., 2002a; Tilahun et al., 2003; Kasukabe et al., 2004; Pellegrineschi et al., 2004; Umezawa et al., 2004). Within these overexpressed genes, transcription factors have been shown to produce phenotypic changes, many of which are involved in stress tolerance. This type of proteins can drive a complex alteration in plant metabolism and architecture enhancing or decreasing the expression of a big number of their target genes (Kasuga et al., 1999; Hsieh et al., 2002b; Kasuga et al., 2004; Yi et al., 2004).

Within transcription factors, HD-Zip proteins, unique to plants, have been proposed to be involved in regulating developmental processes associated with the response of plants to environmental conditions (Carabelli et al., 1993; Schena et al., 1993; Chan et al., 1998). These proteins contain a homeodomain associated with a leucine zipper, a coiled-coil structure involved in dimerisation (Ruberti et al., 1991; Mattsson et al., 1992; Schena & Davis, 1992; Gonzalez et al., 1997) and bind DNA efficiently only as dimers (Sessa et al., 1993; Palena et al., 1999).

Previous studies indicated that the sunflower gene encoding the HD-Zip protein Hahb-4 is up-regulated by drought and ABA in roots, stems, and leaves (Gago et al., 2002), suggesting that it may function in the signalling cascade that controls a subset of the ABA-mediated responses of sunflower to water stress.

In order to investigate the function of *Hahb-4* and its possible role in conferring drought tolerance, we decided to overexpress this factor in *Arabidopsis thaliana* plants. Assuming that most basic processes are conserved in dicot plants, we have made use of this heterologous system to evaluate the potential use of *Hahb-4* as a biotechnological tool.

We have observed that Arabidopsis transgenic plants that overexpress *Hahb-4* have a characteristic phenotype that affects stem and internode length, leaf shape and form, inflorescence development and growth rate. Under water stress conditions, transgenic plants show an enhanced tolerance during vegetative developmental stages both in soil and on culture medium. This fact opens the possibility of using *Hahb-4* as a biotechnological tool to improve water stress tolerance in agricultural crops.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). Plants were grown in 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 $\mu E\ m^{-2}\ s^{-1}$. Plants used for the different treatments were grown in 8 cm diameter × 7 cm height pots during the time indicated in the figures. Since transgenic plants expressing *Hahb-4* show phenotypic characteristics that resemble those of the Landsberg erecta ecotype, genotypic analysis using SSLP with several pairs of oligonucleotides, as suggested in the Arabidopsis Biological Resource Center (ABRC) web page, was performed to assess their Columbia background.

Water stress treatments

Water stress treatment in soil was carried out as follows: to a 35 cm plastic square tray, a defined amount of water was added (1, 1.5 or 2 l). Sixteen 8 × 7 cm pots, each with 100 g soil, were placed in the tray. One or three seeds, depending on the experiment, were sowed in each pot and the trays were transferred to culture conditions as described above. Additional water was added only when severe damage of non-transformed plants was observed. Plants were analysed after rewatering.

For *Hahb-4* promoter analysis, 14 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 the same periods of time and then harvested for RNA isolation.

Genetic constructs

The cDNA corresponding to *the Hahb-4* gene cloned in vector pUC119 as previously described was amplified with oligonucleotides T1 (5′-GCG*GGATCC*ACC**ATG**TCTCTTCAACAA-GTA) and T2 (5′-GCC*GAGCTC***TTA**GAACT

CCAACCACTTTTG), restricted with *Bam*HI and *Sac*I and cloned in plasmid pBI121 digested with the same enzymes. In this way, only the coding region of *Hahb-4* (nucleotides representing start and stop colons are shown in bold in T1 and T2, respectively) was inserted between the 35S promoter and the *nos* termination sequence with an extra ACC triplet just before the ATG to improve translation efficiency (Fütterer & Hohn, 1996). *E. coli* DH5α cells were transformed with this construct and, once positive clones were obtained and sequenced, *Agrobacterium tumefaciens* cells were transformed (Höfgen & Willmitzer, 1988) with the same plasmid.

A 1015-bp region upstream of the transcription initiation site of *Hahb-4* was amplified by PCR with oligonucleotides PROM1 (5′-GCG*GTCGAC*-ACCTGGCACATCGTATCTT-3′) and PROM2 (5′-CGC*GGATCC*GAGGGTTTGATAAGTGA-T-3′) using genomic sunflower DNA as template. This DNA fragment was restricted with *Sal*I and *Bam*HI and cloned in plasmid pB1101.3 previously digested with the same enzymes. In this way, the promoter region of *Hahb-4* controls the expression of the reporter *gusA* gene when plants are transformed with this construct.

Transformation and identification of transformed plants

Transformed *Agrobacterium tumefaciens strain* GV2260 was used to obtain transgenic Arabidopsis plants by the floral dip procedure (Clough & Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR carried out on genomic DNA with oligonucleotides T1 and T2 described below. To assess *Hahb-4* expression, northern blot analysis was performed on T2 transformants. Three positive independent lines (arising from two different transformation experiments) were developed further and homozygous T3 and T4 plants were used to analyse the expression levels of *Hahb-4* and the phenotype of transgenic plants. Plants transformed with pBI101.3 or pBI121, used as negative or positive controls, respectively, or with a construction bearing the *Hahb-4* promoter region fused to the reporter gene *gusA*, were obtained in a similar way. In all cases three independent homozygous transformed lines (T3 and/or T4) were analysed.

RNA isolation and analysis

Total RNA was isolated as described by Carpenter and Simon (1998). 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 uniformity 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-labeled probes in buffer containing 6 × SSC, 0.1% (w/v) polyvinylpyrrolidone, 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 in each lane, filters were then re-probed with a 25S rDNA from *Vicia faba* under similar conditions as those described above, except that hybridisation was performed at 62°C and the wash with 0.1× SSC was omitted. For *Hahb-4*, the probe used was an *Spe*I/*Eco*RI cDNA fragment (from +424 to +674), corresponding to the 3′-noncoding region plus the last 177 nucleotides of the coding region, which does not include the HD-Zip domain. For *Athb-7* and *-12*, full-length cDNA clones (RAFL05-20-M16 and RAFL11-01-J18) obtained from the RIKFN BRC Experimental Plant Division, Tsukuba, Japan, were used. For *GST8* (clone 116M6T7), *RAB18* (clone 1251M 19T7), *UBQ10* (clone 193N23T7) and *RD21A* (clone 187D5T7), EST clones obtained from the Arabidopsis Biological Resource Center (ABRC) were used. An EST clone from *RD22* (SQ069b10) was obtained from the Kazusa DNA Research Institute (Japan). For *DREB2A* and *RD29A*, gene specific probes were obtained by PCR. For *gusA* detection, a full-length probe was obtained by restriction of pBI101.3 with *Bam*HI/*Sac*I. In this case, hybridisation was performed at 68°C to avoid unspecific reactions.

DNA isolation and Southern blot analysis

Arabidopsis genomic DNA was isolated according to the method described by Doyle and Doyle (1987). The DNA (10 μg) was digested overnight with *Hin*dIII electrophoresed through 0.7% (w/v) agarose gels and transferred to Hybond-N nylon

membranes (Amersham Corp.). Southern blot analysis was carried out essentially as described in Ausubel et al. (1983) using as probe the same fragment described for northern analysis. Filters were washed with 2× SSC plus 0.1% (w/v) SDS at 65°C (4 times, 15 min each) and 0.1× SSC plus 0.1% (w/v) SDS at 37°C during 15 min, dried and exposed to Kodak BioMax MS films.

Histochemical GUS staining

In situ assays of GUS activity were performed as described by Jefferson et al. (1987). 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 the plant tissues by immersing them in 70% ethanol.

Microscopic analysis

Tissue preparation for staining was carried out essentially as described by Dixon and Klessig (1995) with slight modifications. Plant material was fixed for 3 h in phosphate buffer/12% glutaraldehyde at room temperature, washed twice with phosphate buffer (Na_2HPO_3, pH 7.1) for 20 min and kept at 4°C in fresh phosphate buffer. The tissue was then dehydrated through an ethanol series and embedded in Histoplast (Biopack). Sections (8–10 μm thick) were mounted on slides coated with 50 μg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris–HCl, pH 8.0 and dried overnight at 37°C. After removing the paraffin with xylene, sections were rehydrated by an ethanol series and stained with Fast Green. After a brief wash with H_2O/ethanol/xylol, sections were dried at room temperature for 3 days. Digital images were generated using a Coolpix 995 digital camera (Nikon) mounted on an optical microscope, and processed with Adobe Photoshop 5.0.

Results

Hahb-4 overexpressing Arabidopsis *lines*

To investigate the *in vivo* functions of *Hahb-4*, we used an overexpression approach. The coding region of *Hahb-4* was fused to the 35S promoter of Cauliflower Mosaic Virus, and the construct was used to transform Arabidopsis plants. Several homozygous lines were recovered and, after preliminary analysis, three transgenic independent lines, named *35S:Hahb4-A*, *-B* and *-C*, respectively, were selected for more detailed analysis. Figure 1a shows a northern blot hybridised with an *Hahb-4* probe, where total RNA from wild type or transgenic plants was analysed. The probe did not hybridise with RNA extracted from wild-type plants, indicating no cross-reaction with members of the Arabidopsis HD-Zip family. Strong signals

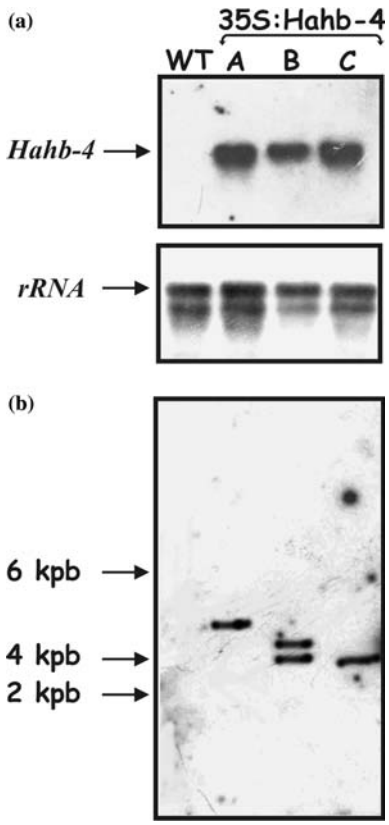

Figure 1. Expression of *Hahb-4* in Arabidopsis transgenic plants. (a) Northern blot analysis of transgenic Arabidopsis plants. Total RNA (10 μg) was extracted from wild-type (WT) and three independent transgenic plants (A, B and C) overexpressing *Hahb-4*. Probes specific for *Hahb-4* or rRNA were used. (b) Southern blot analysis of transgenic Arabidopsis plants. Total genomic DNA (10 μg) from wild type (first lane) or transgenic plants A, B and C (lanes 2, 3, and 4, respectively) was isolated, digested, electrophoresed and analysed as described in 'Materials and methods'. Lambda phage DNA digested with *Hin*dIII was used as molecular weight marker.

of similar intensity were observed with RNA from the different transgenic plants suggesting that they express the *Hahb-4* mRNA at high levels. Southern blot analysis (Figure 1a) indicated the presence of one copy of the transgene in lines A and C, while two copies seem to be present in line B. This difference in copy number does not seem to influence significantly expression levels, since the three lines (as well as others that were analysed, not shown) show similar *Hahb-4* transcript levels.

Phenotype of Hahb-4 overexpression lines

Compared with wild-type plants, *35S:Hahb4* transgenic plants exhibited a characteristic phenotype when cultured under standard irrigation conditions. When grown in soil, the rosette of transgenic plants was smaller, leaf petioles were shorter, and leaves were rounder in shape (Figure 2a and b). Transgenic plants also showed reduced stem elongation rate compared to wild-type plants (Figure 2c). This gives rise to a more compact inflorescence as shown in Figure 2d. Flower bud formation occurs with a difference of approximately 2 days between wild type and transgenic plants: 18 days after germination for wild-type plants and 20 days for transgenic counterparts. In both cases, the number of rosette leaves was the same. A delay of 5 days in anthesis was also observed in transformed plants with respect to non-transformed plants. The difference between wild type and transgenic plants became less evident upon progression of the reproductive stage of development, with transgenic plants having about 87% of the wild-type height when seed maturation begun (approximately 50 days after germination). The difference in stem and internode length between transgenic and non-transformed plants is probably due to a slower longitudinal enlargement of cells. In Figure 3, longitudinal sections of internodes from the two genotypes are shown. It can be appreciated that cells from transgenic plants are considerably smaller than their wild type counterparts.

Interestingly, seed production was not affected in transgenic plants with respect to non-transformed plants. In five independent experiments, the total weight of dried seeds produced by plants of both genotypes was measured. The observed difference in weight was smaller than the standard deviation calculated in each experiment performed with 20 individuals of each genotype. Significant differences were observed between experiments performed with either one or three plants per pot, but both genotypes behaved similarly. This indicates that no loss in seed productivity is caused by the transgene, as it has been observed with other transcription factors when they were overexpressed under the control of the 35S promoter (Kesuga et al., 1999; Hsieh et al., 2002). This agrees with the fact that the phenotypic difference is reduced when plants reach the reproductive stage.

Transgenic Arabidopsis *plants are more tolerant to water deficit than wild-type plants*

The strong induction of *Hahb-4* under water stress led us to evaluate the capacity for drought resistance of transgenic plants during vegetative development. For this purpose, transgenic and wild-type Arabidopsis plants were grown in pots intercalated in the same tray. After cold treatment for dormancy break, the pots were watered by adding either 1, 1.5 or 2 l of water to the trays and then transferred to a culture chamber. Since plants were not further watered until severe damage was observed in controls, these treatments produced water stress at different developmental stages. The first symptom of water deficit was a reduction in growth rate. In fact, wild-type plants under these conditions showed a phenotype that resembled transgenic plants under normal conditions (i.e. reduction in leaf size, delayed inflorescence, stem elongation and flowering) suggesting that the effect of Hahb-4 expression is to enhance a natural defence mechanism present in a wild-type plant. Upon prolonged water deficit, damage became evident in wild-type plants. After 20/25 days, it was observed that leaves of wild-type plants initially irrigated with 1 l water became wilted and curled, whereas transgenic plants were not affected (Figure 4a and c). Similar observations, but at later stages of development, were done when the initial water supply was 1.5 l (Figure 4b and d) or 2 l (Figure 4e). To examine the survival rates of non-transformed and transgenic plants under conditions of severe water deficit, the drought treatment was extended 5 days until severe damage was visible. At this stage, plants were watered and, 2 days after that, survivors were observed and counted in the two populations. Compared with

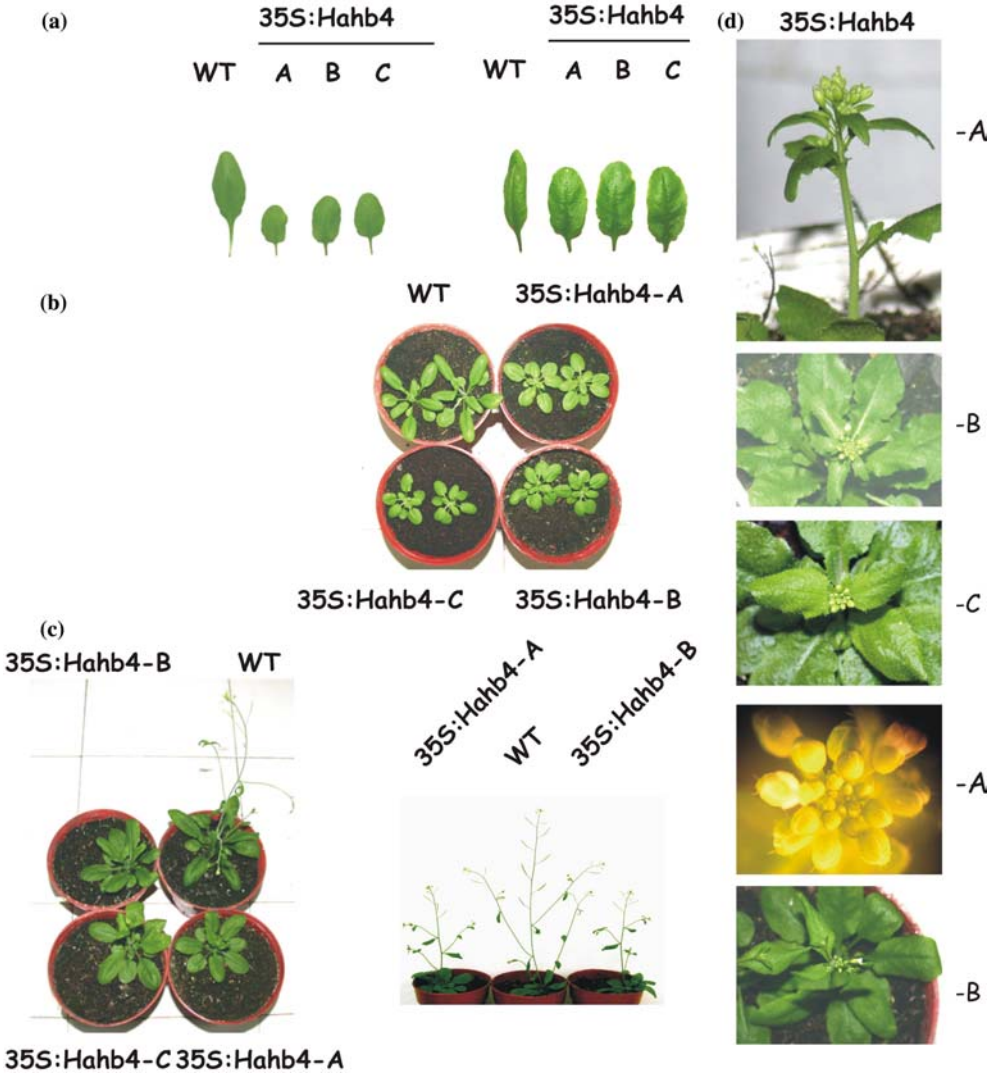

Figure 2. Phenotype of *35S:Hahb4* transgenic plants. (a) Leaves from *Hahb-4* overexpressing transgenic plants and non-transformed plants at 21 days of development (left) and 45 days of development (right). (b) Rosette size and leaf shape of transgenic and non-transformed plants during the vegetative stage. (c) Rosette size, leaf shape and inflorescence stern of transgenic and non-transformed plants during the reproductive stage. (d) Compact inflorescence observed in transgenic plants.

the survival rate of non-transformed plants, transgenic plants were clearly more resistant at the three developmental stages. Drought resistance was more pronounced when the stress was applied at earlier developmental stages (2/3-week-old plants) than in the reproductive stage (4/5-week-old plants). Table 1 illustrates experiments done with the three independent lines subjected to water stress as described in 'Materials and methods'. In all cases the percentage of plants surviving under severe stress conditions was considerably higher for transgenic plants than for non-transformed plants. Small variations were observed between experiments, probably due to differences in the soil quality and its initial water content. This is reflected in higher survival rates observed for wild-type plants in a given experiment which were always correlated with a similar increase in survival rate for transformed plants present in the same fray, independently of the line tested. Consequently, significant differences in survival rates were observed between transformed and

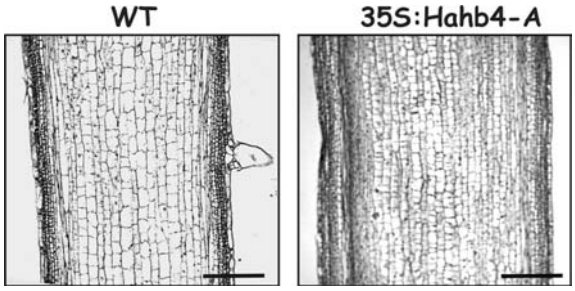

Figure 3. Microscopic views of internode cells of *35S:Hahb4* overexpressing plants and control plants. Tissue sections were prepared as described in 'Materials and methods' and stained with fast green. Scale bar: 100 μm.

non-transformed plants in all experiments, with mean values of 80 ± 15% for transformed plants versus 19 ± 11% for wild-type plants.

We have also analysed seed production in surviving transgenic plants subjected to drought. Seeds from 100 transformed plants in five independent drought experiments were collected individually and seed weight after dehydration was determined. Total seed weight was unaffected in transformed plants that were watered after a long period of water stress with respect to plants that did not suffer drought.

Increased drought resistance was also observed in plants grown in Petri dishes. To subject plants to water deficit, transgenic or wild-type plants were grown in Petri dishes whose cover was larger than normal in diameter and placed in the culture chamber under low humidity conditions. Evaporation of water under these conditions was slow but constant and the MS-agar medium progressively dried. Twenty five days after germination, wild-type plants showed retarded growth with respect to transgenic plants subjected to the same treatment (Figure 4f and g). In addition, leaves of wild-type plants showed visible damage while transgenic plants remained healthy. These results indicate that overexpression of *Hahb-4* can significantly improve water deficit tolerance in Arabidopsis plants.

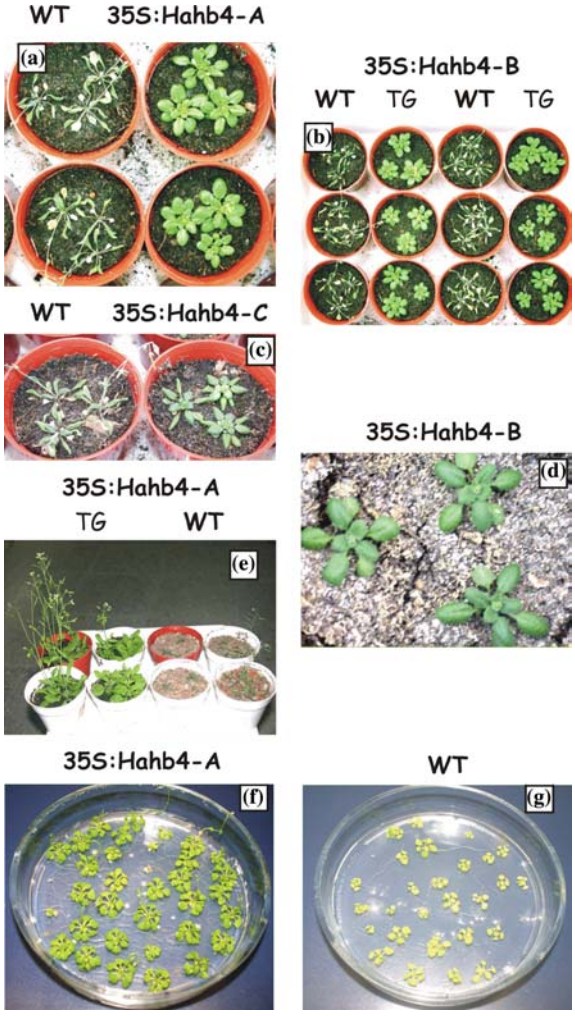

Figure 4. Drought tolerance of *35S:Hahb4* transgenic plants. (a, b and c) Transgenic lines A, B and C (TG) and wild-type (WT) plants were grown on soil in the same container, plants were re-watered when damage was observed. The photographs were taken 2 days after rewatering. (d) A closer view of 3-week-old transgenic plants subjected to a strong water deficit stress (the same plants as in 'b' without rewatering). (e) A group of 45-day-old plants previously subjected to water deficit stress and re-watered (transgenic plants are on the left and non-transformed plants on the right). (f and g) Transgenic (f) or wild-type (g) plants were grown in Petri dishes with slightly larger covers on MS medium as described in 'Materials and methods'. Photographs were taken after 30 days.

Expression of drought-related genes is unaffected in Hahb-4 overexpressing plants

Since *Hahb-4* belongs to the sunflower HD-Zip family, we tested the expression levels of its closest Arabidopsis homologues, *Athb-7* and *-12*, in transgenic and wild-type plants. As deduced from northern blot experiments, neither *Athb-7* nor *Athb-12* transcript levels were significantly affected (Figure 5). This result enhances the conclusion that *Hahb-4* overexpression is directly responsible for the phenotype observed and for water stress tolerance.

Table 1. Survival rates of transgenic plants

Transgenic line	Survival	Total	%
Drought tolerance in 4-week-old plants			
35S:Hahb4 A	15	18	83
WT	1	22	4.5
35S:Hahb4 B	24	24	100
WT	5	24	21
35S:Hahb4 C	8	22	36
WT	0	24	0

Number of 4-week-old plants overexpressing *Hahb-4* surviving exposure to water stress. Each set of transgenic plants is compared with non-transformed plants that shared the tray with them.

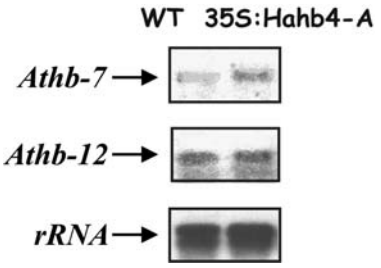

Figure 5. Arabidopsis *Athb-7* and *Athb-12* expression is not affected by overexpression of *Hahb-4*. Total RNA (10 μg) was extracted from 2-week-old wild-type or transgenic plants, electrophoresed, blotted onto nylon membranes, and probed with ^{32}P-labelled *Athb-7* or *Athb-12* cDNA. The same filter was hybridised with an rRNA probe as a control for RNA loading and transfer (lower panel).

In order to investigate the mechanism of action *of Hahb-4*, we also analysed expression levels of several genes that are induced by water stress, some having in their promoter region the target DNA sequence bound by *Hahb-4 in vitro*. We have prepared probes for *RAB18* (At5g66400), *RD22* (At5g25610), *DREB2A* (At5g05410), *RD29A* (At5g52310), *RD21A* (At1g47128), *GST8* (At1g78380) and *UBQ10* (At4g05320), the last three having the target sequence of *Hahb-4* in their promoters, to investigate this point. Notably, neither of the genes under study showed significant changes in RNA steady-state levels in *Hahb-4* overexpressing plants compared to control plants (data not shown). This indicates that the phenotype conferred by *Hahb-4* originates from changes in gene expression that do not include the usual drought-responsive genes regulated by other transcription factors.

Expression patterns driven by the Hahb-4 promoter

The phenotype described here indicates that *Hahb-4* is involved in water deficit stress responses, conferring tolerance especially during vegetative stages of plant development. It has been previously observed that this gene is expressed in roots, seedlings and leaves of sunflower and regulated by drought and ABA (Gago et al., 2002). These studies have been performed with the RNAse protection technique and northern blot hybridisation. To assess the physiological relevance of the results presented here, we investigated the spatial and temporal expression patterns conferred by the *Hahb-4* promoter region. For this purpose, we have cloned a 1015 bp fragment, containing sequences upstream of the +1 transcription initiation site of *Hahb-4*, in front of the *gusA* reporter gene. We have obtained several independent transgenic Arabidopsis homozygous lines with this construct, and analysed three by histochemical β-glucuronidase (GUS) staining. GUS activity was already clearly detectable 20 h after germination and in the emerging radicle some hours later (not shown). Expression was also observed in vegetative tissues including hypocotyls, stems and leaves until approximately 40 days after germination (Figure 6a). Cotyledons, as well as young and mature leaves were strongly stained in all the lines analysed but expression decreased after 20–25 days of germination (Figure 6a and b). Roots exhibited strong staining at the tips, in the vascular cylinder and in the nascent secondary roots (Figure 6c). GUS activity was not detected in reproductive organs (not shown). We were also interested in testing if the *Hahb-4* promoter is inducible by drought and ABA. For this purpose, we analysed *gusA* expression in transgenic plants subjected to drought stress or treated with 100 μM ABA. Expression levels, analysed by northern blot hybridisation using a *gusA* probe, were compared with those of untreated *Hahb-4* promoter-*gusA* plants or of plants transformed with the T-DNA region of plasmid pBI121, which contains the *gusA* gene under the control of the strong 35SCaMV promoter (Figure 6d). *Hahb-4* promoter activity is strongly induced by ABA and drought stress, reaching in both cases transcriptional levels similar to those of the 35S Cauliflower Mosaic Virus promoter. We conclude that the *Hahb-4* promoter is recognised in Arabidopsis plants and the

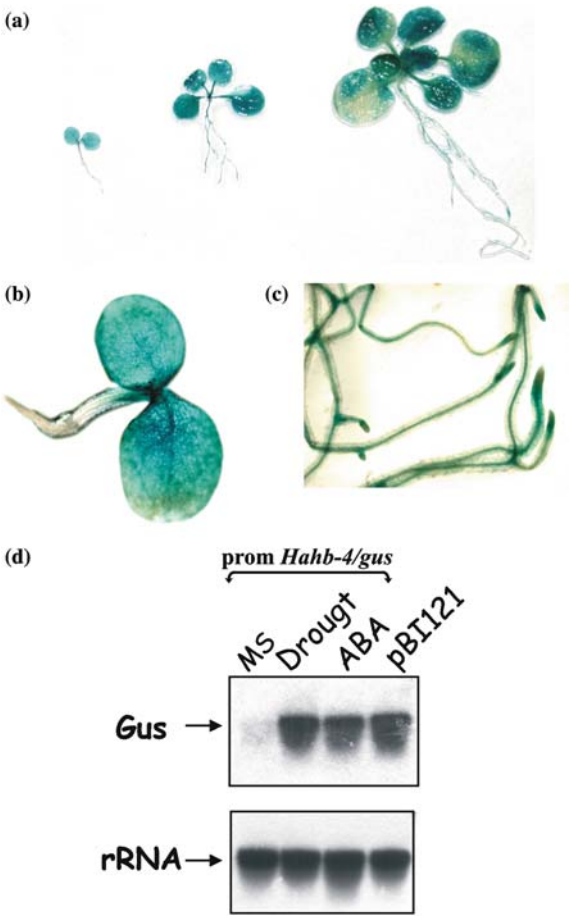

Figure 6. Expression pattern *of Hahb-4*. (a) Arabidopsis plants transformed with the promoter region of *Hahb-4* fused to *gusA*. From left to right: 2-day, 10-day, and 20-day-old plants. (b) Cotyledons of histochemically stained transformed plants. (c) Detail of roots of 20-day-old plants. (d) Induction of *gusA* expression by drought-stress and ABA. Total RNA (10 μg) was extracted from 3-week-old plants transformed with pBI101.3 or with the *Hahb-4* promoter-*gusA* fusion and kept under control conditions or subjected to water stress or 100 μM ABA. RNA was electrophoresed, blotted onto nylon membranes, and probed with a ^{32}P-labelled *gusA* cDNA as detailed in 'Materials and methods'. The same filter was hybridised with an rRNA probe as a control for RNA loading and transfer (lower panel).

expression profile of the reporter gene agrees with that observed in sunflower for the *Hahb-4* mRNA. The expression profile conferred by the promoter also agrees with the effect of *Hahb-4* overexpression, which is more pronounced at earlier developmental stages, during vegetative development. Otherwise, this activity is strongly induced by water stress or ABA treatment, leading us to infer that regulation of this gene occurred at the transcriptional level.

Discussion

HD-Zip proteins, unique to plants, are proposed as good candidates to trigger developmental responses to environmental conditions, a characteristic feature of plants. Several authors have reported that expression of members of the HD-Zip family of transcription factors is regulated by different external factors for example illumination or water stress (Schena & Davis, 1992; Carabelli et al., 1993; Schena et al., 1993; Söderman et al., 1994, 1996; Lee & Chun, 1998).

Recently, *Hahb-4* has been shown to encode an HD-Zip factor that belongs to the sunflower HD-Zip I subfamily. This gene is expressed at early stages of plant development and its expression is induced by water deficit and ABA (Gago et al., 2002). The stress inducibility of *Hahb-4* suggested that it may have a potential role in stress-responsive signalling and, possibly, in conferring drought tolerance to plants. To address this question, we used an overexpression approach choosing Arabidopsis as a model system. The expression levels of *Hahb-4* in the *35S:Hahb4* transgenic lines used in our study are higher with respect to their Arabidopsis counterparts under control conditions and to its own expression level in sunflower. Transgenic plants showed a slightly retarded development during the vegetative stage. Shorter stems and petioles and smaller leaves indicate that *Hahb-4* expression produces a decrease in cell enlargement. Developmental retardation was also observed when non-transformed plants were subjected to water stress deficit, indicating that the phenotype caused by the overexpression of *Hahb-4* is similar to the normal plant response to drought. Another interpretation of these observations, specially the growth retardation, is that they result from the constitutive expression of *Hahb-4* due to the presence of the 35S promoter.

Experiments with non-constitutive promoters (*rd29* and the *Hahb-4* own promoter) are currently under way. Preliminary results indicate that water-stress tolerance is also conferred by these constructions but to a lesser extent, while developmental differences are almost absent in irrigated plants (not shown). Additional analysis of these transgenic plants will also be helpful in determining the relationship between developmental changes and water-stress tolerance. The fact that severe abnormalities were not observed and that

the effect of *Hahb*-4 on development is coincident with the expression patterns directed by its own promoter suggest, however, that the observations made are related to its *in vivo* function rather than to its aberrant expression in incorrect tissues or cells. Additionally, transcriptional levels of *Athb-7* and *-12* are unaffected in transgenic plants, and a similar phenotype to that conferred by *Hahb-4* was not observed comparing the transgenic plants described in this work with plants transformed with either of two other members of the sunflower HD-Zip family (*Hahb-1* and *-10*), nor when plants were transformed with pBI121 as control (data not shown).

The phenotype of *35S:Hahb4* plants includes tolerance to prolonged water deficit, observed in plants of different ages. Again, these observations are coincident with the expression characteristics of *Hahb-4*, which is strongly induced by water stress in sunflower and in Arabidopsis carrying promoter-*gusA* fusions. These facts point to the existence of conserved regulatory mechanisms in the response to drought in both species.

Phylogenetic analysis indicates that *Athb-7* and *-12* are the Arabidopsis genes more related to *Hahb-4*. However, they are more related to each other than to *Hahb-4*, suggesting that they have arisen from a recent duplication event. It is noteworthy that both genes are also induced by water stress and ABA (Söderman et al., 1994, 1996; Lee & Chun, 1998). Outside the HD-Zip domain, however, there is no significant homology between the encoded proteins and *Hahb-4*. This fact, and the low percentage of identity at the amino acid level within the homeodomain, makes it difficult to ascertain whether *Hahb-4* is an orthologue of any of the Arabidopsis genes. Another important difference between Hahb-4 and the Arabidopsis proteins is that while the first one is able to bind the sequence CAAT(A/T)ATTG *in vitro* (Palena et al., 1999), *Athb-7* and *-12* do not bind this or related sequences (Johannesson et al., 2001). This may indicate a requirement for post-translational modifications of the Arabidopsis proteins. Recently, it has been reported that both genes, *Athb-7* and *-12*, act as negative regulators of plant growth (Hjellström et al., 2003; Olsson et al., 2004). Transgenic plants that express a reporter gene under the control of the *Athb-7* promoter were obtained (Olsson et al., 2004). Expression patterns seem to be quite different from the ones conferred by the *Hahb-4* promoter, suggesting that the latter is not the orthologue of the Arabidopsis genes. Another gene from the same family, *Athb-6*, is also induced by the same treatments but to a lesser extent (Söderman et al., 1999). Transgenic Arabidopsis plants overexpressing this gene have been obtained by Himmelbach et al. (2002), who reported that they show increased ABA insensitivity. We did not observe differences in ABA-dependent inhibition of germination in the three independent lines of transgenic plants that overexpress *Hahb-4* with respect to non-transformed plants in a range of concentrations from 0.5 to 1.5 μM ABA (data not shown), indicating that *Athb-6* and *Hahb-4* do not share the same mechanism of action.

Changes in gene expression play a central role in the plant adaptive responses to water stress. Many genes whose expression is regulated by stress, generally termed *RD* (responsive to dehydration), *ERD* (early responsive to dehydration) or *LEA* (late embryogenesis abundant protein), as well as transcription factors that interact with them, have been isolated (Shinozaki & Yamaguchi-Shinozaki, 2000; Uno et al., 2000). The mechanism of action and role of these genes seems to be conserved between species. In some cases, transgenic plants overexpressing these genes or transcription factors show enhanced tolerance to water deficit and salt stress, but sometimes also negative phenotypic characteristics, such as severe growth retardation and/or deficiencies in seed production (Xu et al., 1996; Kasuga et al., 1999; Haake et al., 2002; Hsieh et al., 2002; Kang et al., 2002). In this context, it is interesting that transgenic plants that overexpress *Hahb-4* produce the same weight of seeds compared with non-transformed plants under normal conditions, and that seed production is not affected when they are subjected to extreme water deficit stress.

Among the genes involved in water deficit stress responses, transcription factors are thought to be 'master switches'. At present, numerous factors from different families were shown to be related with these responses. Most of these factors act by directly or indirectly inducing the above-mentioned drought-responsive genes. Since the product of *Hahb-4* is a transcription factor, it can be postulated that this protein also regulates a set of genes involved in producing the observed phenotype. The lack of response of typical

drought-responsive genes in *Hahb-4* overexpressing plants, however, leads us to conclude that the mechanism of action of the sunflower gene is different from that of other transcription factors. We speculate that *Hahb-4* may be specially involved in producing developmental changes that allow the plant to survive under water deficit conditions. Two Arabidopsis mutants were described, *ERECTA* (*ER*) and *corymbosa2* (*crm2*), where the mutation results in a compact inflorescence and short pedicels similar to those observed in the *Hahb-4* overexpressing lines (Tiorii et al., 1996; Suzuki et al., 2002). It is possible that the genes responsible for these mutations participate in similar transduction pathways as those affected by *Hahb-4* overexpression. The identification of the genes whose expression is modified when *Hahb-4* is overexpressed will allow to infer the mechanisms used by the plant to increase its drought tolerance. Microarray analysis of plants expressing *Hahb-4* will hopefully allow us to better understand the mechanism followed by this transcription factor to confer drought tolerance to *Arabidopsis* plants.

Great effort to improve plant tolerance to drought, high salinity and other abiotic stresses have been pursued by breeding and genetic engineering. The success was limited owing to the genetic complexity of the stress response. The improved understanding of the role of novel genes in stress adaptation will provide the basis for effective engineering strategies leading to greater stress tolerance. The results reported here allow us to envisage a biotechnological use of *Hahb-4* for the production of water deficit tolerant plants.

Acknowledgements

We thank Dr E. Zabaleta for technical advice with plant transformation and helpful suggestions and Drs Marta and Susana Gattuso for assistance with the microscopic analysis. We also thank Dr Kimitaka Yakura, Kanazawa University, Japan, for sending us a *Vicia faba* rDNA clone and the RIKEN BRC Experimental Plant Division, the Arabidopsis Biological Resource Center and the Kazusa DNA Research Institute for providing us cDNA clones.

This work was supported by grants from CONICET, ANPCyT, Fundación Antorchas and Universidad Nacional del Litoral. RLC and DHG are members of CONICET; CAD and GMG are fellows of the same Institution.

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