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Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb-4 transcription factor

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Summary

Hahb-4 is a member of the *Helianthus annuus* (sunflower) subfamily I of HD-Zip proteins that is transcriptionally regulated by water availability and abscisic acid. Transgenic *Arabidopsis thaliana* plants overexpressing this transcription factor (TF) exhibit a characteristic phenotype that includes a strong tolerance to water stress. Here we show that this TF is a new component of ethylene signalling pathways, and that it induces a marked delay in senescence. Plants overexpressing *Hahb*-4 are less sensitive to external ethylene, enter the senescence pathway later and do not show the typical triple response. Furthermore, transgenic plants expressing this gene under the control of its own inducible promoter showed an inverse correlation between ethylene sensitivity and *Hahb*-4 levels. Potential targets of *Hahb*-4 were identified by comparing the transcriptome of *Hahb*-4-transformed and wild-type plants using microarrays and quantitative RT-PCR. Expression of this TF has a major repressive effect on genes related to ethylene synthesis, such as *ACO* and *SAM*, and on genes related to ethylene signalling, such as *ERF2* and *ERF5*. Expression studies in sunflower indicate that *Hahb*-4 transcript levels are elevated in mature/senescent leaves. Expression of *Hahb*-4 is induced by ethylene, concomitantly with several genes homologous to the targets identified in the transcriptome analysis (*HA-ACOa and HA-ACOb*). Transient transformation of sunflower leaves demonstrated the action of *Hahb*-4 in the regulation of ethylene-related genes. We propose that *Hahb*-4 is involved in a novel conserved mechanism related to ethylene-mediated senescence that functions to improve desiccation tolerance.

Keywords: HD-Zip, drought tolerance, sunflower transcription factor, senescence avoidance, ethylene signalling, ethylene biosynthesis.

Introduction

HD-Zip proteins constitute a family of transcription factors characterized by the presence of a homeodomain associated with a leucine zipper. The association of this DNA-binding domain (HD) with an adjacent dimerization motif (LZ) is a combination found only in plants, although the domains are found individually in a large number of eukaryotic transcription factors (Schena and Davis, 1992). This large family has been divided into four subfamilies (I–IV) according to sequence similarity in and outside the conserved domains and the intron/exon patterns of the corresponding genes (Schena and Davis, 1994; Sessa *et al.*, 1994). Members of subfamily I interact with the pseudo-palindromic sequence CAAT (A/T)ATTG; subfamily II proteins recognize the motif CAAT (C/G)ATTG (Palena *et al.*, 1999; Sessa *et al.*, 1993). In all cases, the formation of homo- or hetero-proteins is a

prerequisite for DNA binding (Gonzalez *et al.*, 1997; Sessa *et al.*, 1993).

Genes of subfamilies I and II are good candidates for triggering developmental processes in response to changes in environmental conditions, a characteristic feature of plants. Several studies have reported that expression of members of the HD-Zip family of transcription factors is regulated by various external factors such as illumination, ABA, salt stress or water stress (Carabelli *et al.*, 1993; Carabelli *et al.*, 1996; Chan *et al.*, 1998; Gago *et al.*, 2002; Henriksson *et al.*, 2005; Lee and Chun, 1998; Schena and Davis, 1992; Schena *et al.*, 1993; Söderman *et al.*, 1994; Söderman *et al.*, 1996; Söderman *et al.*, 1999). Studies in which HD-Zip I and II genes were overexpressed in transgenic plants further support the proposed role of this protein family as developmental regulators that are responsive to environmental conditions (Carabelli *et al.*, 1996; Dezar *et al.*, 2005a; Olsson *et al.*, 2004; Rueda *et al.*, 2005; Schena *et al.*, 1993).

Sunflower Hahb-4 is a member of subfamily I of HD-Zip proteins that binds, as a dimer, to the target sequence CAAT(A/T)ATTG *in vitro* (Palena *et al.*, 1999); its expression is regulated by water stress and ABA at the transcriptional level (Dezar *et al.*, 2005b; Gago *et al.*, 2002). We have recently shown that transgenic Arabidopsis plants overexpressing this gene under the control of the 35S cauliflower mosaic virus promoter show a phenotype in normal growth conditions that is characterized by shorter stems and internodes, rounder leaves and more compact inflorescences compared to control plants; this phenotype ultimately leads to growth retardation. Nevertheless, these transgenic plants show a strong tolerance to water stress at all developmental stages, both on soil and in culture medium (Dezar *et al.*, 2005a).

In this study, we developed transgenic plants expressing the *Hahb*-4 cDNA under the control of its own stress-inducible promoter; these transgenic palnts also exhibited water-stress tolerance, but without any major phenotype. Furthermore, the transgenic plants exhibited a marked delay in senescence and were less sensitive to ethylene. In order to investigate the molecular mechanism of action of this transcription factor, we performed a transcriptome analysis using a CATMA array containing 24 576 gene-specific tags (GSTs) from Arabidopsis (Hilson *et al.*, 2004). Among >3500 genes whose expression levels changed depending on the presence of the transgene and/or the stress condition, a significantly repressed group of genes were found to be involved in ethylene synthesis or signalling pathways. Experiments both in sunflower and Arabidopsis demonstrated that this TF acts on specific ethylene-related targets and is regulated by this hormone. This revealed a conserved cross-talk mechanism involving ethylene and drought-stress signalling through the *Hahb*-4 TF.

Results

Transgenic plants expressing Hahb-4 regulated by its own stress-inducible promoter are tolerant to water stress and do not display developmental defects

Constitutive expression of *Hahb*-4 by the 35S promoter of the cauliflower mosaic virus induced significant morphological changes and strong drought tolerance in Arabidopsis plants (Dezar *et al.*, 2005a). Physiological parameters measured in transgenic and wild-type genotypes under normal or stress conditions indicated that the transpiration level is slightly higher in transgenic plants than in wild-type ones, eliminating a decrease in water loss in these plants as a possible explanation for the acquired tolerance (data not shown). To avoid the morphological changes induced by this transgene, we prepared transgenic plants expressing the sunflower gene under the control of its own inducible promoter. The coding region of *Hahb*-4 was fused to two previously described allelic promoter regions (LPF and SPF; Dezar *et al.*, 2005b). Several homozygous lines were recovered, and three transgenic independent lines for each construct, *LPF:Hahb4-A*, *-B* and *-C* and *SPF:Hahb4-A*, *-B* and *-C*, were selected for more detailed analysis. Figure 1(a) shows a Northern blot hybridized with a *Hahb*-4 probe, in which total RNAs from transgenic plants under normal growth conditions or subjected to drought were analysed. As expected, strong signals were observed in independent transgenic plants that were subjected to water stress for 6 h, whereas no signal was detected under normal growth conditions (Figure 1a). The expression levels under stress were as high as those achieved with the constitutive (35S CaMV) promoter (Dezar *et al.*, 2005b). Further analysis with ten additional transgenic lines showed similar results.

Compared with wild-type plants or 35S:GUS controls, *LPF:Hahb4* and *SPF:Hahb4* transgenic plants exhibited the same morphology when cultured under standard conditions (Figure 1b). No measurable differences were detected in leaves, petioles, inflorescence shapes or forms, or stem elongation rate, in contrast to plants overexpressing 35S:*Hahb4* (Figure 1b). Flower bud formation occurred at the same time (18 days after germination) in both genotypes (wild-type or transgenic), and the number of rosette leaves was the same. In several independent experiments, no delay in anthesis, or changes in final plant height or the total weight of dried seeds produced, were detected between the genotypes.

The drought tolerance of these plants was evaluated until damage became evident in both genotypes (35S:GUS/WT or *LPF/SPF:Hahb4*. Both young (3-week-old) and reproductive-state (5-week-old) plants showed increased tolerance. The survival rates of non-transformed or empty-vector controls transformed with pBI 121 and transgenic Hahb-4 plants under conditions of severe water deficit were examined by

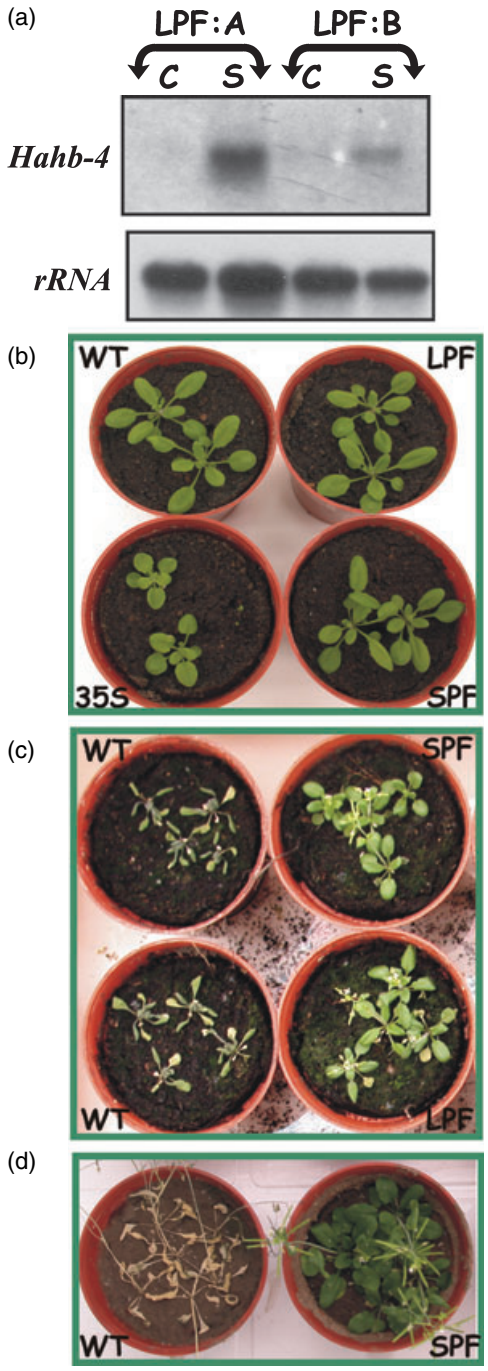

Figure 1. Expression of *Hahb*-4 in Arabidopsis transgenic plants bearing the construction *LPF/SPF:Hahb4*.
(a) Northern blot analysis of transgenic Arabidopsis plants. Total RNA (10 μg) was extracted from independent transgenic plants (lines LPF:A and LPF:B are shown) expressing *Hahb*-4 under the control of its own promoter under normal growth conditions (C) or subjected to water-deficit stress (S). Probes specific for *Hahb*-4 or rRNA were used as described in Experimental procedures.
(b) Representative phenotypes of the same transgenic plants. Comparison between 21-day-old transformed and control plants of the four genotypes: WT, 35S (35S:*Hahb4*), LPF (*LPF:Hahb4*), SPF (*SPF:Hahb4*).
(c) Photographs were taken 2 days after re-watering of 21-day-old plants subjected to severe water-deficit stress as described in Experimental procedures. WT, wild-type genotype; LPF, *LPF:Hahb4* and SPF, *SPF:Hahb4* transgenic genotypes.
(d) 45-day-old plants subjected to severe hydric stress. Left: WT, non-transformed genotype; right: SPF (*SPF:Hahb4*) genotype. In several assays, 35S:GUS-transformed plants were used instead of WT plants and gave identical results (not shown).

Table 1 Survival rates (drought tolerance) of 4-week-old transgenic plants

Type of plant	No. survivors	%
LPF:Hahb4 A	27 ± 3.51	84
WT	9 ± 2.08	28
LPF:Hahb4 B	28 ± 1.73	88
WT	7 ± 1.53	22
SPF:Hahb4 A	26 ± 2.65	81
WT	5 ± 2.52	16
SPF:Hahb4 B	28 ± 2.08	88
WT	10 ± 1.15	31

Average numbers of 4-week-old *Hahb*-4-expressing transgenic plants surviving after exposure to water stress (from a total of 32 plants in each case). Each set of transgenic plants shared a tray with non-transformed (WT, wild-type) plants and is compared with them. These are representative examples of experiments using two transgenic lines for each construction (*SPF:Hahb4* and *LPF:Hahb4*) and each represents the average of triplicate biological experiments. The same approach was taken with ten independent lines and using 35S:*GUS* transgenic plants as controls, yielding similar results.

extending drought stress for 5 days (until severe damage was visible in young or mature plants). At this time, plants were watered and, 2 days later, plant survival was assessed in each population (Figure 1c,d). Transgenic plants were clearly more tolerant at all developmental stages tested (Table 1). In all cases, the percentage of plants surviving under severe stress conditions was higher for transgenic plants than for non-transformed ones. With the inducible promoter, survival rate differences compared with the wild-type genotype were about 50–60%, while with the constitutive promoter, this difference increased to 70–90% on average (Table 1; Dezar *et al.*, 2005a). Therefore, the inducible genotypes are more tolerant of water stress than wild-type plants, and do not show any significant morphological alterations under normal growth conditions. Drought tolerance assays carried out with 10 independent transgenic lines yielded similar results (data not shown).

A delay in senescence is observed in both types of transgenic plants

Senescence occurs at the last developmental stage of a given organ, and ethylene promotes this process as well as programmed cell death. We observed that transgenic plants expressing *Hahb*-4, either constitutively or in an inducible form, exhibit a clear delay in their entry into senescence

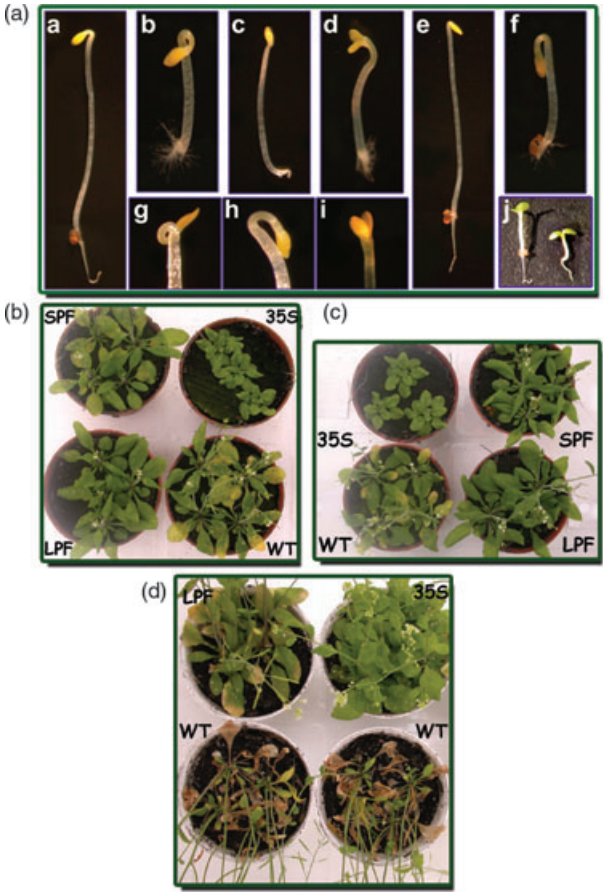

Figure 2. Transgenic plants enter into the senescence program later and are insensitive to ethylene.
(A) Three-day-old seedlings grown in the dark: (a) WT; (c) 35S:*Hahb4*; (e) *LPF:Hahb4*, untreated; (b) WT; (d) 35S:*Hahb4*; (f) *LPF:Hahb4* grown on 5 μM ACC; (g), (h) and (i) apical hook of WT, *LPF:Hahb4* and 35S:*Hahb4*, respectively, treated with 5 μM ACC; (j) 3-day-old seedlings grown under a normal photoperiod (left, WT; right, 35S:*Hahb4*).
(B,C) Plants of the same genotypes treated with 100 μM or 300 μM ethylene.
(D)45-day-old plants: WT, wild-type genotype; 35S, 35S:*Hahb4*; LPF, *LPF:Hahb4*; SPF, *SPF:Hahb4*.

(Figure 2D). In both transgenic genotypes, siliques start their maturation while leaves are still green and photosynthetically active. Leaves of non-transformed plants became yellow and ultimately brown at the same age, in contrast to transgenic plants. These observations suggest that the function of this transcription factor may be related to ethylene-mediated senescence. To test this hypothesis, we assayed ethylene sensitivity in wild-type and transgenic plants using external ethylene or ACC at two concentrations (Figure 2A). Three-day-old seedlings treated with 300 μM ethylene or grown on 5 μM ACC show an inhibition of the triple response in both types of transgenic plants (constitutive or inducible genotypes) in contrast to wild-type plants. Neither typical apical hook formation nor marked inhibition in root and hypocotyl elongation were observed in transgenic plants. Furthermore, a different effect in each genotype was observed 48 h after the treatment of mature plants (Figure 2b,c). Wild-type plants immediately started senescence, whereas the constitutive genotype plants appeared to be almost insensitive. The inducible genotype plants showed an intermediate behaviour, more closely resembling the constitutively *Hahb*-4-expressing plants than the non-transformed plants (Figure 2b,c). Together, these results reveal that sensitivity to ethylene has an inverse relationship with *Hahb*-4 transcript levels.

Further evidence for a relationship between *Hahb*-4 and senescence processes mediated by ethylene was obtained by analysis of *Hahb*-4 expression in senescent leaves of sunflower (Figure 3). Figure 3(a) shows images of the leaves used for RNA extraction, with the level of *Hahb*-4 expression shown in Figure 3 (b). Transcript levels increase concomitantly with leaf age, indicating a possible function of the gene in aging and maturing processes, an observation without precedent for this or other members of the HD-Zip family.

Hahb-4 promoter has been previously characterized as inducible by both water stress and ABA in transgenic Arabidopsis plants (Dezar *et al.*, 2005b). In order to test whether this promoter is also regulated by ethylene, we analysed *Hahb*-4 transcript levels in transgenic Arabidopsis plants bearing *LPF:Hahb4* constructs and in sunflower plants treated or untreated with this hormone. Figure 4(a) shows a

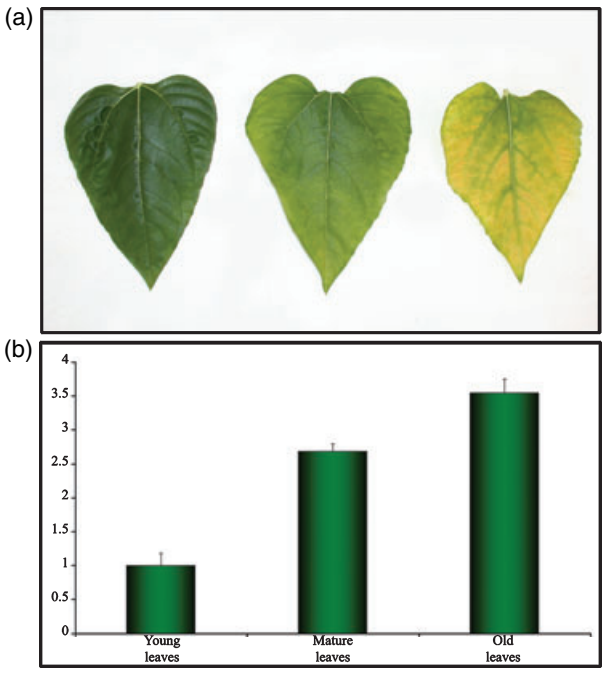

Figure 3. *Hahb*-4 is involved in maturation/senescence processes.
(a) Sunflower leaves of a single plant at different developmental stages.
(b) *Hahb*-4 transcript levels in these leaves were measured by quantitative RT-PCR and related to transcript levels in young leaves. This experiment was repeated three times with similar results (standard deviations were calculated with three repetitions).

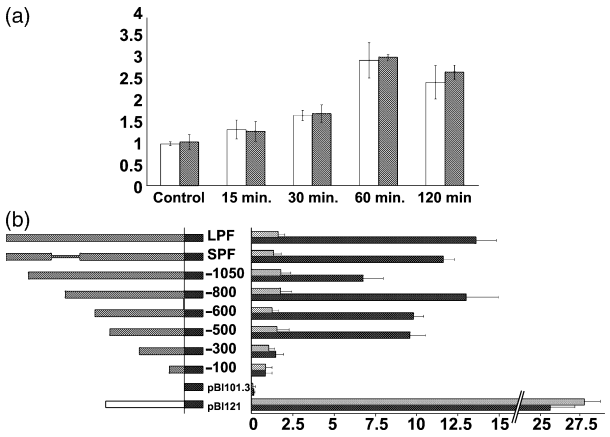

Figure 4. Ethylene regulation of *Hahb*-4 occurs at the transcriptional level.
(a) *Hahb*-4 is regulated at the transcriptional level in both species. Transcript levels measured by real-time RT-PCR of *Hahb*-4 in sunflower (grey bars) or directed by *LPF* (white bars) in Arabidopsis at various indicated times, after treatment with 500 μM ethylene. Their levels in untreated plants were defined as 1 (relative units).
(b) Induction of the GUS reporter gene expression in plants transformed with different segments of the *Hahb*-4 promoter. Left, a schematic representation of the constructs used to transform Arabidopsis plants; right, grey bars and black bars represent expression levels without or with ACC treatments respectively.

good correlation between the transcript behaviour in these two species, indicating the existence of a transcriptional regulation of *Hahb*-4 by ethylene. A maximal induction of *Hahb*-4 transcript level was observed in leaves 60 min after treatment with the hormone.

In order to identify a *cis*-acting element responsible for this induction, constructs with different portions of the promoter fused to the *uidA* reporter gene were generated and transformed into Arabidopsis plants (Figure 4b). Transgenic plants were treated with 20 μM ACC for 60 min and *uidA* transcript levels were then measured by quantitative RT-PCR. The results indicate that an ethylene-responsive element is present in the sequence located between 300 and 500 bp upstream of the transcription initiation site in both allelic forms (Figure 4b).

Together, these molecular and physiological observations led us to conclude that (1) *Hahb*-4 seems to temporally repress the senescence program regulated by ethylene, and (2) this regulation occurs at the transcriptional level and is mediated by *cis*-acting promoter elements that are responsive to this hormone.

Expression of Hahb-4 induces significant changes in the Arabidopsis transcriptome

In order to investigate the mechanisms involved in these responses at the molecular level, a transcriptome analysis was carried out using RNA obtained from transgenic (35S:*Hahb4*) or wild-type plants grown in normal conditions or subjected to water stress. From a total of 25 316 Arabidopsis genes, 3641 showed altered transcription levels between at least two samples (Table S1). Particularly when stress and control conditions are compared, a large group of previously characterized water-stress responsive genes were identified, as expected. In contrast, only a limited group of genes (815) changed their behaviour due the presence of the transgene. We have validated these results by quantitative RT-PCR with oligonucleotides designed for a selected group of genes on two biological replicates. Figure 5 illustrates the results obtained for a group of 22 genes (3rd column), and the good correlation between transcript levels measured by RT-PCR and the data obtained in the microarray analysis (first column). Of the 22 validated genes, only three (*EIN-3*, *ERF-2* and *ADC-2*) showed slight differences between array data and real-time RT-PCR measurements. These values are included in Table 2 and in Table S2. The transcript values for *EIN-3* are very similar between one of the biological replicates of the array and the quantitative RT-PCR, but differ from the average of the biological replicates. At the whole-transcriptome level, genes regulated by this transcription factor belong to several metabolic/signalling pathways, for example the biosynthesis of osmoprotectants.

Hahb-4 binds the pseudo-palindromic sequence CAAT (A/T)ATTG *in vitro* (Johannesson *et al.*, 2001; Palena *et al.*, 1999). We investigated which of the genes whose transcript levels were altered due to the presence of the transgene have this target sequence in their promoter region by analysing their 5′ upstream sequences deposited in Arabidopsis databases. This enabled us to identify possible direct target genes of this transcription factor. On average, 4.5% of the genes that change their expression level in plants overexpressing *Hahb*-4 represent possible direct targets of this transcription factor. Among all Arabidopsis promoters (TAIR6 genome release), only 2.8% possess this sequence, indicating that genes with this structural characteristic are enriched in the group of genes regulated by the transgene.

Genes involved in ethylene biosynthesis or perception pathways are repressed in transgenic plants

A significant group of genes encoding both ethylene biosynthesis and signal transduction pathway components are repressed in *Hahb*-4-expressing transgenic plants (Table 2). We then tested by quantitative RT-PCR other key genes that changed their transcription level in at least one of the two independent biological replicates used for the microarray analysis. Figure 6 schematizes these pathways, their regulation points, and the variation in transcription level of the genes in transgenic plants compared with their non-transformed counterparts (deduced from the microarray and quantitative RT-PCR analysis). The expression of several ethylene-related regulatory genes is strongly

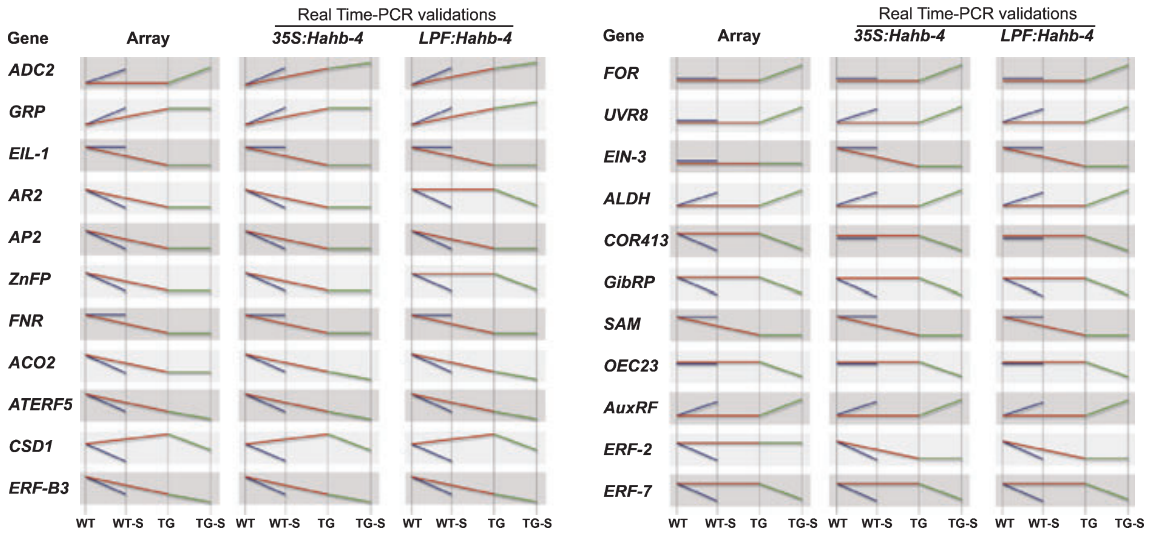

Figure 5. Schematic comparison between transgenic plants constitutively expressing *Hahb*-4 (35S:*Hahb*4) or under a stress-inducible promoter (*LPF:Hahb4*) with regard to the behaviour of selected induced or repressed genes detected in the microarray analysis. Differences in transcript levels between WT and WT-S (wild-type plants grown under normal or stress conditions, respectively) are indicated in blue; those between TG and TG-S (*Hahb*-4-expressing plants grown under normal or stress conditions, respectively) are indicated in green; those between WT and TG grown under normal conditions are indicated in red. The analysed transcripts correspond to ADC-2 (arginine decarboxylase), GRP (glycine-rich protein), EIL-1 (ethylene-insensitive 3-like 1), AR-2 (NADPH cyt-reductase), AP2 (AP2 domain-containing ethylene response factor), ZnFP (zinc finger protein), FNR (ferredoxin NADP oxidoreductase), ACO (ACC oxidase), ERF-5 (ethylene response transcription factor 5), CSD-1 (Cu/Zn superoxide dismutase), ERF-B3 (ethylene response factor subfamily B-3), FOR (FAD-dependent oxidoreductase family), UVR-8 (UVB resistance protein), EIN3 (ethylene-insensitive 3), ALDH (betaine aldehyde dehydrogenase 10A8), COR413 (cold-acclimatization family protein), GibRP (gibberelin-regulated family protein), SAM (*S*-adenosylmethionine synthetase), OEC23 (photosystem II oxygen-evolving complex), AuxRF (auxin response factor), ERF-2 (ethylene response transcription factor 2), ERF-7 (ethylene response transcription factor 7). Numerical values corresponding to the schematic representations are shown in Supplementary Tables b and c.

transcriptionally repressed, including, for example, *EIN3* and *EIL1*, which are involved in the ethylene signalling pathway, *ERF2* and *ERF5*, which are ethylene- responsive transcription factors, and *ACO* and *SAM*, which participate in ethylene biosynthesis.

As we were unable to detect *Hahb*-4 by Northern blot hybridization when its expression was controlled by its own promoter under normal conditions (Figure 1a), we expected that at least a subset of the analysed potential target genes would not change their transcription levels in the inducible genotype. However, real-time RT-PCR of the previously validated genes (Figure 5, third column) showed that all of the genes analysed behave similarly in both transgenic genotypes. Even though *Hahb*-4 expression is not detected when driven by the inducible promoter, we could detect low but significant *Hahb*-4 transcript levels in these plants by using quantitative RT-PCR (data not shown). .Therefore, Hahb-4 may be present at a high enough concentration to induce or repress the expression of target genes, such as those of the ethylene signalling and biosynthesis pathways.

Sunflower genes homologous to those identified in Arabidopsis are co-regulated by Hahb-4

To analyse the conservation of the regulatory networks involving *Hahb*-4 in sunflower, we analysed the expression pattern of specific target genes homologous to those identified in our microarray analysis. Even though genomic information is rather sparse in this agricultural species, we were able to identify sunflower homologs. Specific oligonucleotides were designed to measure transcript levels of these genes in sunflower plants under water-stressed, ethylene-treated or control conditions (Figure 7a). When sunflower plants were treated with ethylene, the *Hahb*-4 expression level increased to levels comparable to those reached in plants subjected to water stress (Figure 7a, first panel). *AT-ACO* (a gene repressed in *Hahb*-4-expressing transgenic plants) has two sunflower homologs (Figure 7a; *HA-ACOa* and *HA-ACOb*) that are repressed both in drought-stressed or ethylene-treated sunflower plants. In contrast, a gene encoding a Cu/Zn superoxide dismutase (Iturbe-Ormaetxe *et al.*, 1998) that is normally repressed by water stress in wild-type Arabidopsis plants is induced in the transgenic lines. Similarly, a sunflower homolog is induced in plants subjected to water stress or ethylene treatments. Other sunflower genes (Figure 7) also show correlative expression patterns with *Hahb*-4, and seem to be targets of *Hahb*-4-dependent drought and senescence signalling. A different but consistent picture is observed with ALDH, a gene implicated in betaine biosynthesis: in Arabidopsis, this gene is induced only in water-stressed transgenic plants; accordingly, in sunflower, it is also induced in water-stressed plants, but not in ethylene-treated plants, indicating that

Table 2 *Hahb*-4-regulated genes involved in ethylene biosynthesis and signalling pathways

		WT, dry versus wet		WT versus HAHB-4, wet		HAHB-4, dry versus wet	
Gene ID	Gene name/description	Ratio	*P*-value	Ratio	*P*-value	Ratio	*P*-value
Data obtained in the microarray analysis							
At1g62380	1-aminocyclopropane-1-carboxylate oxidase	−1.179	1.E-09	−0.786	2.E-01	−0.713	1.000
At1g06620	2-oxoglutarate-dependent dioxygenase	−0.548	1.000	1.374	2.E-10	−1.864	–
At2g36880	*S*-adenosylmethionine synthetase	0.192	1.000	−1.021	2.E-04	0.333	1.000
At2g27050	Ethylene-insensitive 3-like 1 (EIL1)	−0.388	1.000	−0.885	1.E-02	0.766	0.756
At5g47220	Ethylene-responsive element-binding factor 2 (ERF2)	−1.441	–	−0.542	1.000	−0.667	1.000
At5g47230	Ethylene-responsive element-binding factor 5 (ERF5)	−2.666	–	−1.029	1.E-04	−1.983	–
At5g61600	Ethylene-responsive element-binding protein (ERF B3)	−2.804	–	−1.701	–	−1.494	1.E-11
At3g20310	Ethylene-responsive element-binding protein (ERF 7)	−0.850	3.E-09	0.419	1.000	−1.154	8.E-06
At5g51190	AP2 domain-containing transcription factor	−1.977	–	−1.314	2.E-09	−0.287	1.000
At3g62550	Universal stress protein (USP) family protein	−0.827	3.E-03	0.858	0.028	−1.174	4.E-06
AT3G20770	Ethylene-insensitive 3 (EIN3)	0.50	1.000	−0.13	1.00	0.77	0.763

ID	Description	WC	WS	TC	TS
Data obtained by quantitative RT-PCR					
AT1G62380	1-aminocyclopropane-1-carboxylate oxidase (ACO)	1 ± 0.083	0.50 ± 0.021	0.62 ± 0.019	0.46 ± 0.041
AT5G47230	Ethylene-responsive element-binding factor 5 (ERF5)	1 ± 0.043	0.26 ± 0.009	0.50 ± 0.008	0.13 ± 0.009
AT3G20770	Ethylene-insensitive 3 (EIN3)	1 ± 0.045	0.96 ± 0.106	0.17 ± 0.087	0.20 ± 0.031
AT2G36880	*S*-adenosylmethionine synthetase (SAM)	1 ± 0.114	1.12 ± 0.139	0.32 ± 0.082	0.30 ± 0.121
AT5G47220	Ethylene-responsive element-binding factor 2 (ERF2)	1 ± 0.123	0.32 ± 0.120	0.41 ± 0.076	0.48 ± 0.083
AT3G20310	Ethylene-responsive element-binding family protein (ERF 7)	1 ± 0.067	0.24 ± 0.074	1.17 ± 0.126	0.58 ± 0.071
AT5G61600	Ethylene-responsive element-binding family protein (ERF B3)	1 ± 0.098	0.45 ± 0.035	0.36 ± 0.012	0.13 ± 0.068
AT2G27050	Ethylene-insensitive 3-like 1 (EIL1)	1 ± 0.138	0.89 ± 0.099	0.21 ± 0.042	0.25 ± 0.076
AT5G51190	AP2 domain-containing transcription factor	1 ± 0.099	0.38 ± 0.041	0.42 ± 0.067	0.50 ± 0.101

Expression analysis of genes involved in ethylene biosynthesis and signalling. WT, wild-type plants; HAHB4, transgenic plants overexpressing the Hahb-4 sunflower gene. *P*-values were calculated according to the Bonferroni test (see *Experimental procedures*). Where no *P*-value is given, this means that it is less than E^{-10}. WC, wild-type plants grown under normal conditions; WS, wild-type plants subjected to drought stress; TC, transgenic plants grown in normal conditions; TS, transgenic plants subjected to drought stress. All values are expressed relative to transcript values from wild-type plants grown under control conditions (WC) taken as control samples (relative value 1). The standard deviation was calculated for each sample from technical triplicates. Samples were considered different when *P*-values were <0.01.

it may be involved only in *Hahb*-4-dependent drought-induced responses.

To assess the role of *Hahb*-4 in the regulation of these genes, a novel transient expression assay in sunflower leaves was developed using the 35S:*Hahb4* construct, in which 25-day-old sunflower plants were transiently transformed as described in Experimental procedures, and the transcript levels of target genes were determined in triplicate experiments. Controls experiments were performed using 35S:GUS constructs or non-transformed plants. The overexpression of *Hahb*-4 in transiently transformed leaves was verified by quantitative RT-PCR. These results, shown in Figure 7(b), clearly indicate that *Hahb*-4 overexpression regulates the target genes, validating the usefulness of the transcriptome analysis performed in Arabidopsis.

These results clearly indicate the existence of conserved mechanisms of response to water stress and ethylene treatment in Arabidopsis and sunflower, and, furthermore, strongly suggest that *Hahb*-4, and possible Arabidopsis orthologs, act in similar signal transduction pathways in both plants. In addition, *Hahb*-4 seems to have a conserved role in the cross-talk between ethylene and drought-stress signalling pathways.

Discussion

Plants possess several alternative and cooperative molecular mechanisms to adapt to adverse environmental conditions. One of the most stressful adverse environmental conditions is caused by dehydration, which activates pathways that may or may not involve ABA (Leung and Giraudat, 1998; Skriver and Mundy, 1990). Transcription factors are proteins that regulate the expression of entire pathways – HD-Zip transcription factors in particular have been proposed to be good candidates to regulate developmental processes associated with changes in environmental conditions (Dezar *et al.*, 2005a; Henriksson *et al.*, 2005; Olsson *et al.*, 2004). In this paper, using genomic approaches, we elucidate a novel cross-talk mechanism involving ethylene and drought signalling pathways that is mediated by a specific HD-Zip in sunflower.

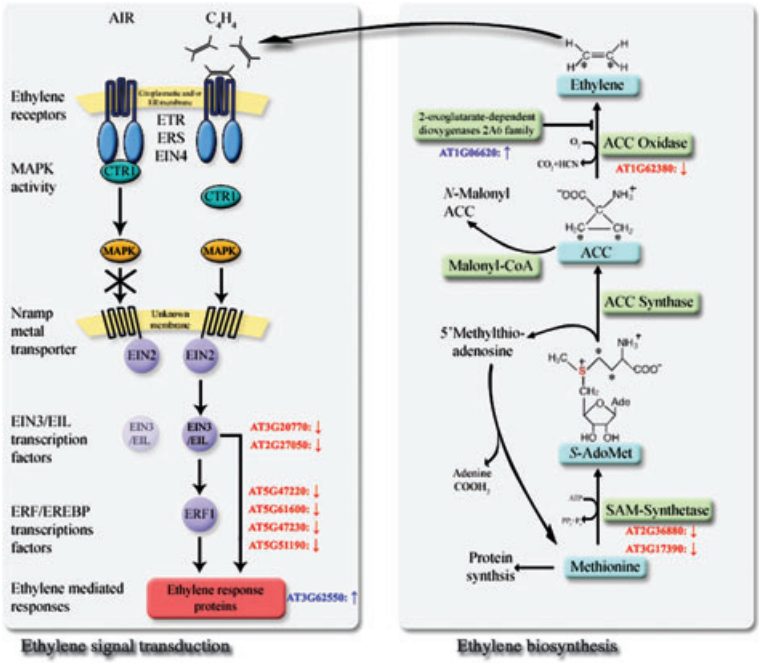

Figure 6. Schematic representation of ethylene synthesis and signalling pathways.
Genes repressed or induced in 35S:*Hahb4* transgenic plants are indicated in red or blue with their respective notation. Numerical values corresponding to the genes involved in ethylene pathways that are represented here are detailed in Table 2.

Hahb-4 confers drought tolerance to Arabidopsis plants even when its expression is controlled by its own promoter. In this case, the transgenic plants showed no distinguishable phenotype from controls, thereby allowing us to discard the possibility that water-stress tolerance is associated with specific morphological changes induced by the constitutively expressed *Hahb*-4 transgene (Dezar *et al.*, 2005a). In *Arabidopsis thaliana*, two *Hahb*-4-like genes (*Athb*-7 and -12) are also regulated by water stress and ABA (Lee and Chun, 1998; Söderman *et al.*, 1996). It has been reported, however, that these genes do not exhibit the same behaviour as *Hahb*-4 when overexpressed; furthermore, neither bind the same target sequence *in vitro*, leading us to expect a difference in the pathways that these genes regulate (Olsson *et al.*, 2004).

Sunflower, like many crops, is not easily manipulated. Hence, functional genomic approaches in model plants such as Arabidopsis using sunflower genes may provide clues to the mechanisms in which these genes operate. In this work, we have assumed that most of the important metabolic and functional processes are conserved in the plant kingdom, and that we could obtain evidence for the function of *Hahb*-4 in *Arabidopsis thaliana*. The use of heterologous systems in these cases can be particularly useful (Chang *et al.*, 2003; Hsieh *et al.*, 2002a,b; Polidoros *et al.*, 2001). In addition, we were able to use the information obtained from Arabidopsis to demonstrate the existence of conserved mechanisms in sunflower. The molecular mechanisms involved in the water-stress response involving *Hahb*-4 were first analysed by microarray analysis of transgenic Arabidopsis plants; the conservation of the identified mechanism was then tested in sunflower. Microarray analysis based on the knowledge gained in the model Arabidopsis allowed us to identify genes that are induced or repressed under given conditions or in mutants due to a point mutation or to the presence of a transgene. Microarray analysis of plants overexpressing *Hahb*-4 indicates, as expected, that *Athb*-7 and -12 are induced in water-stressed wild-type or transgenic plants, but neither gene exhibited altered transcription levels due to the presence of the transgene (Table c). A different behaviour was observed for *ATHB-6*, previously described as a repressor of the ABA response (Himmelbach *et al.*, 2002). This gene is repressed in transgenic plants, and repressing a repressor may thus indirectly allow the plant to positively respond to this stress-related hormone. No other members of the Arabidopsis HD-Zip family change their expression level due to the presence of *Hahb*-4.

Genes involved in osmoprotection are induced in *Hahb*-4 transgenic plants. Among them is arginine decarboxylase, an enzyme involved in polyamine synthesis, known to be regulated by osmotic stress (Capell *et al.*, 2004). The gene encoding this enzyme contains a promoter *cis*-element that is recognized by Hahb-4 *in vitro*. In addition, expression of a gene encoding betaine-aldehyde dehydrogenase, a protein involved in the biosynthesis of betaine, a well-known osmoprotectant (Sakamoto and Murata, 2000), is also induced. More experimental work must be done to corroborate the role of these genes in water-stress tolerance in our transgenic plants.

It is clear from this and our previous work (Dezar *et al.*, 2005a) that drought tolerance conferred by *Hahb*-4 does not fit into the known signal transduction pathways that are regulated by *DREB*-related genes (Chini *et al.*, 2004;

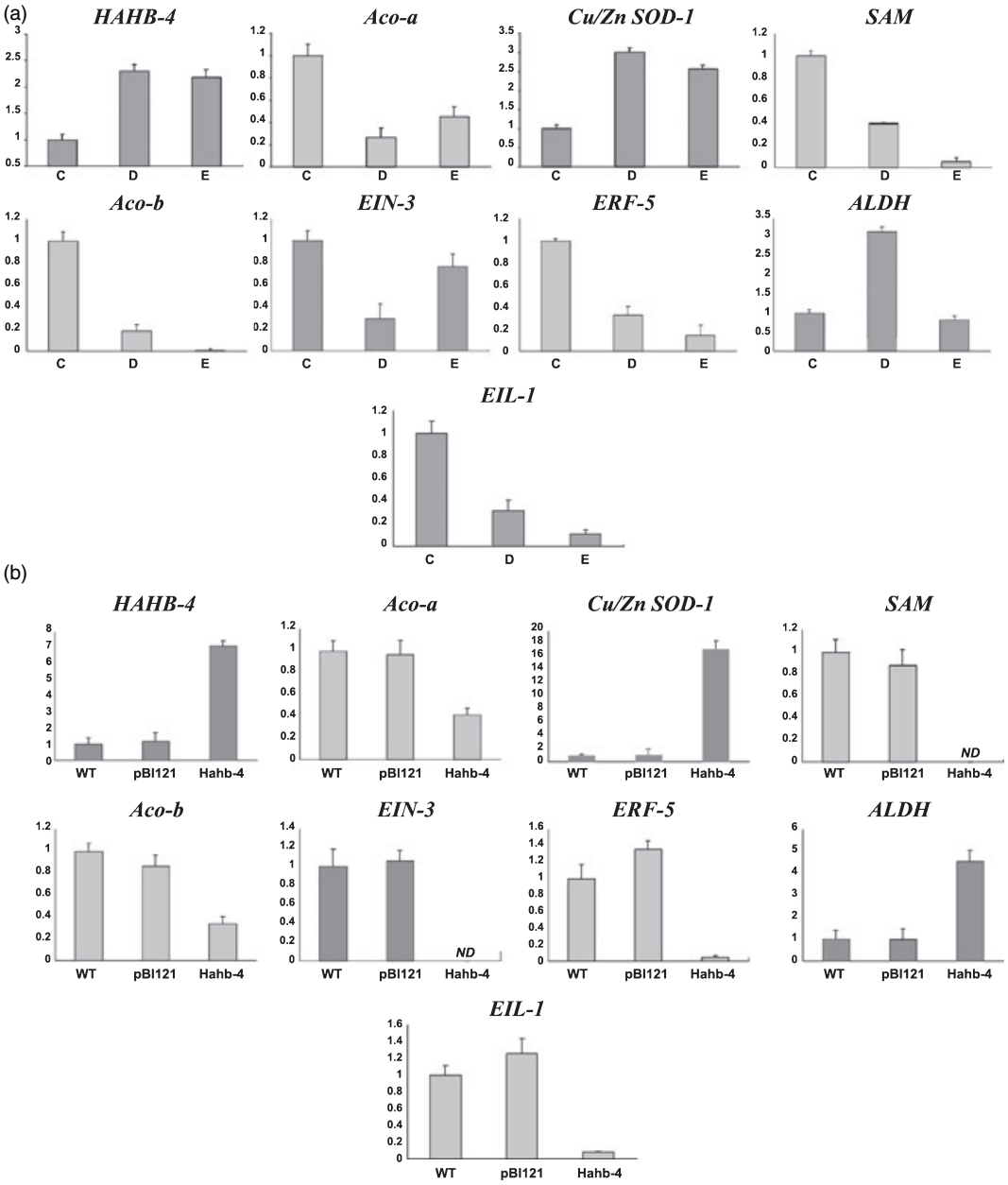

Figure 7. Sunflower homologs of the Arabidopsis genes, identified through the microarray analysis as being regulated by Hahb-4, are involved in drought response and senescence programs.

(a) Transcript levels of *Hahb*-4, ACC oxidase (ACOa), Cu/Zn superoxide dismutase 1 (CSD1), ACC oxidase (ACOb), ethylene-insensitive 3 (EIN3), ethylene responsive element binding factor 5 (ERF5), *S*-adenosylmethionine synthetase (SAM), betaine aldehyde dehydrogenase (ALDH), ethylene-insensitive 3-like 1 (EIL1) for Sunflower plants grown in control conditions (C), subjected to water stress (D) or treated with ethylene (E).

(b) Transcript levels of the same genes as in (a), in transiently transformed sunflower leaves: non-transformed plants (WT), plants transformed with 35S:GUS (pBI 121) or with 35S:*Hahb4* (*Hahb*-4). Note that Hahb-4 is induced concomitantly with the target genes.

Kasukabe *et al.*, 2004; Umezawa *et al.*, 2004). These genes and their known targets do not change their transcription levels due to the presence of *Hahb*-4, suggesting the existence of a novel unrelated pathway. A reduction of ethylene synthesis and the inhibition of its signal transduction pathways generate a marked delay in senescence processes, which may constitute significant elements contributing to drought tolerance in the transgenic plants. These plants may be healthier at all developmental stages due to their inability to enter into a senescence program. In fact, Hahb-4 transgenic plants were almost insensitive to ethylene treatment at various developmental stages. Concomitant results show that *Hahb*-4 is positively regulated by ethylene and during the normal leaf senescence process. Interestingly, neither

Hahb-4, nor any other HD-Zip protein, was previously though to be involved in ethylene-mediated senescence.

Our microarray analysis clearly demonstrates that the *Hahb*-4 transgene has a major effect on the ethylene pathway. Biosynthesis of ethylene was inhibited at two important points: the first one repressing SAM transcription, and the second repressing ACO, one of the enzymes responsible for transforming ACC into ethylene. Furthermore, *2-ODD* (*2-oxoglutarate-dependent dioxygenase*, 2A6 family), a gene homologous to the tomato *E8* gene, is induced in transgenic plants. *E8* is a gene regulated by ethylene during tomato fruit ripening, and has been shown to have a negative effect on ethylene biosynthesis (Peñarrubia *et al.*, 1992). In addition, the ethylene signalling pathway is also negatively regulated in transgenic plants through repression of the EIN3 and EIL1 transcription factors. Chao *et al.* (1997) reported that a loss-of-function EIN3 mutant was partially insensitive to ethylene; its insensitivity could be partly compensated for by the action of the EIL1 transcription factor, leading to a partly insensitive phenotype. Repression of both transcription factors in our plants provides a plausible explanation for their strong insensitivity to ethylene treatment and senescence. Related to these observations, Mayda *et al.* (1999) reported that antisense suppression of H52 (an HD-Zip protein member) in transgenic tomato plants produces a conditional lethal phenotype. The transgenic lines that survive exhibit spontaneous mis-regulation of cell death control in leaves and the overaccumulation of ethylene among other pathogen-related effects (Mayda *et al.*, 1999). These authors suggested a role for this gene in cellular protection through limiting programmed cell death. It was also reported that plants lacking ACC synthase presented delayed leaf senescence under normal growth conditions and inhibited drought-induced senescence (Young *et al.*, 2004). Our study indirectly supports a connection between HD-Zip transcription factors and ethylene responses.

The utility of model heterologous systems to identify mechanisms requires the analysis of related genes in the original plant for validation. By examining the expression of related genes in the sunflower, we have demonstrated correlation between a pathway dealing with *Hahb*-4 and ethylene action in this crop. *Hahb*-4 may play a similar role as a non-identified possible ortholog in Arabidopsis plants, suggesting that a link between water-stress responses and ethylene-regulated pathways involving this transcription factor is conserved between species. Even though sunflower is less amenable to the transgenic approach, we were able to correlate transcript levels of homologous genes with *Hahb*-4 expression in sunflower plants treated with ethylene or during drought stress or by transient transformation of sunflower leaves.

In summary, although the water-stress tolerance observed in *Hahb*-4 transgenic plants is due to a combination of factors, the most significant seems to be the inhibition of ethylene-induced senescence. This senescence delay may function to maintain active photosynthesis for longer periods, allowing plants to synthesize osmoprotectants, among other metabolites. Hence, *Hahb*-4 represents a new component in the cross-talk between drought-effect and ethylene signalling pathways.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) seeds were purchased from Lehle Seeds (Tucson, AZ, USA). Plants were grown directly on soil in a growth chamber at 22–24°C under long-day photoperiods (16 h of illumination with a mixture of cool-white and GroLux fluorescent lamps, Sylvania, Madrid, Spain) at an intensity of approximately 150 $\mu E\ m^{-2}\ sec^{-1}$ in 8 cm diameter, 7 cm high pots for the time periods indicated in the figures.

Preparation and characterization of plants bearing the *Hahb*-4 cDNA controlled by the 35 S cauliflower mosaic virus were previously described (Dezar *et al.*, 2005a).

Helianthus annuus L. (sunflower cv. Contiflor 15) seeds (Zeneca, Balcaree, Argentina) were grown on soil in a culture room at 28°C for variable times depending on the purpose of the experiment (as detailed in the figure legends).

Water-stress treatments

Early water-stress treatment in soil was carried out as follows: 16 pots (8 × 7 cm), each with 120 g soil and four seeds, water-saturated, were placed in a 35-cm plastic square tray and cultured as described above except that further water was not added until severe damage was observed. Plants were harvested for RNA isolation when stress was evident by visual inspection. RNA was then used for expression analysis of the specific *rd29A* gene, a well-characterized water-stress-inducible gene (Kasuga *et al.*, 2004) as described below. Water-stress treatment was also performed on mature 4-week-old plants grown under the same culture conditions. At this age, no water was added again until stress became evident (approximately 17 additional days). In both cases, photographs were taken 2 days after re-watering.

Ethylene treatments

For experiments with young seedlings, seeds were surface-sterilized and plated with MS medium in Petri dishes. After 2 days of incubation at 4°C, dishes were placed in a growth chamber at 22–24°C. Dark-grown seedlings were sprayed with 100 or 300 μM Ethephon (Sigma, St. Louis, Missouri, USA) every 24 h for 3 days, or grown for 3 days on 5 μM ACC. They were then observed and photographed. Thirty-day-old plants grown on soil under a normal photoperiod as described above were treated with ethylene, applied by spraying with 100 or 300 μM Ethephon (Sigma).

Constructs

LPF-GUS and SPF-GUS (constructs bearing *Hahb*-4 promoter regions directing the expression of the reporter gene *uidA*; Dezar *et al.*, 2005b) in pBI 101.3 were digested with *Bam*HI and *Sac*I to delete

uidA cDNA, and the *Hahb*-4 cDNA fragment was restricted with the same enzymes (Dezar *et al.*, 2005a) and introduced into this vector by standard procedures. The construct was used to transform *Agrobacterium tumefaciens* cells (Höfgen and Willmitzer, 1988).

LPF-GUS, SPF-GUS and successive 5′ deletions of these constructs have been described previously by Dezar *et al.* (2005b).

Promoter analysis

Plants transformed with constructs bearing different segments of the *Hahb*-4 promoter region were grown under normal conditions and treated (or not) with 20 μM ACC as described below. One hour after the treatment, plants were harvested and RNA was then prepared and analysed by quantitative RT-PCR using *uidA*-specific oligonucleotides.

Stable transformation and identification of transformed plants

Transformed *Agrobacterium tumefaciens* strain LBA4404 was used to obtain transgenic Arabidopsis plants by the floral dip procedure (Clough and Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR which was carried out on genomic DNA using oligonucleotides F (5′-CCATGTCTCTTCAACAAGTA) and R (5′-TTAGAACTCCAACCACTTTTG) specific for the *Hahb*-4 cDNA. To assess *Hahb*-4 expression, Northern blot analysis and real-time RT-PCR were performed on T_2 transformants, as described below. Three positive independent lines for each construction (arising from two different transformation experiments) were used to select homozygous T_3 and T_4 plants in order to analyse phenotypes and the expression levels of *Hahb*-4. Plants transformed with pBI 101.3 were used as negative controls.

Transient transformation of sunflower leaves

Sunflower leaves (in R1 developmental state; Schneiter and Miller, 1981) were infiltrated with 5 ml of *Agrobacterium tumefaciens* strain LBA4404 and then transformed with either pBI 121 or 35S:*Hahb4*. After infiltration, plants were left in the growth chamber for an additional 48 h; 1 cm diameter disks (50 mg each) were excised from the infiltrated leaves and RNA was then extracted with Trizol (see below). For each gene transcript measurement, two disks originating from different plants were analysed and the experiment repeated at least twice. In order to test the infiltration in these experiments, GUS reporter gene expression was measured by histochemical assays as previously described (Dezar *et al.*, 2005b).

RNA isolation and analysis by Northern blot

Total RNA was isolated for Northern blots as described by Carpenter and Simon (1998). RNA was 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. Buckinghamshire, UK) and hybridized 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 (four times, 15 min each), then 0.1× SSC plus 0.1% w/v SDS at 37°C for 15 min, dried and exposed to Kodak BioMax MS film (Rochester, New York, USA). To check the amount of total RNA loaded in each lane, filters were then re-probed with a 25S rRNA from *Vicia faba*. The *Hahb*-4 probe was a *Spe*I/*Eco*RI cDNA fragment (from +424 to +674), corresponding to the 3′-non coding region plus the last 177 nucleotides of the coding region, which does not include the HD-Zip domain (Gago *et al.*, 2002). The RD29A gene-specific probe was obtained by cloning a PCR product that was amplified using oligonucleotides RD1: 5′-CACACAAACAAGGAATTATACC-3′ and RD2 5′-GGAAGAGGAAGTGAAAGGAGG-3′, from Arabidopsis DNA, into the TOPO plasmid (Invitrogen, Carslbad, CA, USA).

Real-time RT-PCR measurements

RNA for real-time RT-PCR was prepared with Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA (1 μg) was used for reverse transcription reactions using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative PCRs were carried out using a MJ-Cromos 4 apparatus (BIO-RAD, Hercules, CA, USA) in a 25 μl final volume containing 1 μl SyBr green (10×), 10 pmol of each primer, 3 mM $MgCl_2$, 5 μl of the reverse transcription reaction and 0.20 μl platinum *Taq* polymerase (Invitrogen). Fluorescence was measured at 80–84°C during 40 cycles. Sunflower RNA was also prepared with the Trizol technique.

Specific oligonucleotides for each gene were designed using publicly available sequences (http://www.Arabidopsis.org). The sequences are specified in Table S3.

Microarray experiments

Transcriptome analysis was performed with the CATMA array containing 24 576 gene-specific tags from *Arabidopsis thaliana* (Crowe *et al.*, 2003; Hilson *et al.*, 2004). The GST amplicons were purified on Multiscreen plates (Millipore, Bedford, MA, USA) and resuspended in TRIS-EDTA dimethyl sulfoxide at 100 ng μl^{-1}. The purified probes were transferred to 1536-well plates in a Genesis workstation (Tecan, Männedorf, Sweden) and spotted onto UltraGAPS slides (Corning, NY, USA) using a Microgrid II (Genomic Solutions, Huntingdon, UK). The current CATMA version printed at the Unité de Recherche en Genomique Végétale (INRA, France) consists of three metablocks, each composed of 64 blocks of 144 spots. A block is a set of spots printed with the same print-tip. In these arrays, a print-tip is used three times to print a block in each metablock. For the transcriptome studies, four pooled samples of young plants were harvested at stage 5 (according to Boyes *et al.*, 2001), representing a biological replicate of each pool. One dye swap was performed for each comparison: Col-0 under normal versus stress conditions, 35S:*Hahb4* under normal versus stress conditions, and Col-0 versus 35S:*Hahb4* under normal conditions. RNA was extracted from these samples using Trizol extraction (Invitrogen) followed by two ethanol precipitations, and then checked for RNA integrity using a bioanalyser from Agilent (Waldbronn, Germany). cRNAs were produced from 2 μg of total RNA from each pool using the 'Message Amp aRNA' kit (Ambion, Austin, TX, USA), and 5 μg of the cRNAs were then reverse-transcribed in the presence of 200 U SuperScript II (Invitrogen), cy3-dUTP and cy5-dUTP (NEN, Boston, MA, USA) according to the method described by Puskas *et al.* (2002) for each slide. Samples were combined, purified and concentrated using YM30 Microcon columns (Millipore). Slides were pre-hybridized for 1 h and then hybridized overnight at 42°C in 25% formamide. Slides were washed in 2× SSC plus 0.1% SDS for 4 min, 1× SSC for 4 min, 0.2× SSC for 4 min, and 0.05× SSC for 1 min, dried by centrifugation (5 min, 60 ***g***). Six hybridizations (three dye swaps) were carried out. The arrays were scanned on a GenePix

4000A scanner (Axon Instruments, Foster City, CA, USA) and images were analysed by GenePix Pro 3.0 (Axon Instruments).

Transcriptome analysis was repeated with two biological replicates for each sample in separate experiments. Data shown in Table c indicate the results of each experiment and the average values for each gene.

Statistical analysis of microarray data

The statistical analysis was performed as described by Lurin *et al.* (2004) based on dye swaps, i.e. two arrays each containing the 24 576 GSTs and 384 controls. The controls were used for assessing the quality of the hybridizations, but were not included in the statistical tests. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between the mutant and the control. It is either $\log_2$ (red/green) or $\log_2$ (green/red), according to the experiment design. An array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features introduced by the experimenter. We then performed a global intensity-dependent normalization using the Loess procedure (see Yang *et al.*, 2002) to correct the dye bias. Finally, on each block, the log ratio median was subtracted from each value of the log ratio of the block to correct a print-tip effect on each metablock. To determine differentially expressed genes, we performed a paired *t*-test on the log ratios. The number of observations per spot varies between 2 and 6 and was inadequate for calculating a gene-specific variance. For this reason, we assumed that the variance of the log ratios was the same for all genes. The raw *P*-values were adjusted by the Bonferroni method, which controls the family-wise error rate (FWER).

Target gene promoter analysis

Promoter sequences from all the annotated *Arabidopsis thaliana* genes were analysed (loci upstream sequences −1000 bp, ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR6_genome_release). Promoters containing the *Hahb*-4 target sequence, CAAT(N)ATTG, were identified using Microsoft Excel software. These genes were contrasted with the microarray results, and those with differential expression were identified as putative direct target genes of *Hahb*-4.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. Transcriptome analysis of *Hahb*-4 over expressing transgenic plants.

Table S2. Numerical values represented in Figure 5

Table S3. Sequence and notation for the primers used in RT-PCR measurements

This material is available as part of the online article from http://www.blackwell-synergy.com

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