

RESEARCH PAPER

# The sunflower HD-Zip transcription factor HAHB4 is up-regulated in darkness, reducing the transcription of photosynthesis-related genes

Pablo A. Manavella<sup>1</sup>, Carlos A. Dezar<sup>1</sup>, Federico D. Ariel<sup>1</sup>, María F. Drincovich<sup>2</sup> and Raquel L. Chan<sup>1,\*</sup>

<sup>1</sup> Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CONICET, CC 242 Ciudad Universitaria, 3000, Santa Fe, Argentina

<sup>2</sup> Centro de Estudios Fotosintéticos y Bioquímicos (CEFOTBI), Universidad Nacional de Rosario, CONICET, Suipacha 531, 2000, Rosario, Argentina

Received 18 March 2008; Revised 19 May 2008; Accepted 22 May 2008

## Abstract

**HAHB4 belongs to the sunflower subfamily I of HD-Zip proteins and is involved in drought-tolerance response and ethylene-mediated senescence. Cross-talk between these two processes through this transcription factor was recently described. In this study it is shown that the expression of HAHB4 is induced in darkness and quickly disappears when plants are exposed to light. This regulation of HAHB4 was confirmed at the transcriptional level through the use of transgenic *Arabidopsis* plants bearing constructs in which different segments of the HAHB4 promoter were fused with the reporter gene *GUS*. Together with electrophoretic mobility shift assays performed with sunflower nuclear proteins, these experiments allowed a *cis*-acting element involved in this response to be located. Transient overexpression of the HAHB4 cDNA in sunflower leaf discs and HAHB4 knockdown by iRNA were performed, demonstrating the participation of this transcription factor in the transcriptional down-regulation of a large group of photosynthesis-related genes. In accordance with the reduction in the transcripts encoding chlorophyll *a/b*-binding proteins, the content of these pigments is diminished in *Arabidopsis* HAHB4-expressing transgenic plants. Thus, it appears that HAHB4 may participate with other factors in the intricate regulation mechanism of the photosynthetic machinery in darkness.**

Key words: Dark-inducible promoter, dark regulation, HAHB4, HD-Zip, promoter analysis, sunflower, transcription factor.

## Introduction

Light plays a critical role in the regulation of plant growth and development, modulating the transcription levels of light-responsive genes. Photoreceptors sense light, activating complex transduction pathways that mediate the transcriptional response (Kuhlemeier *et al.*, 1987; Nagy *et al.*, 1988; Nagatani, 2000). Photosynthesis-related genes form one of the most important groups of genes that are regulated by illumination conditions. Plants depend on light to obtain energy. Photosynthetic machinery is turned off at night as well as during senescence and under particular conditions such as drought. When day begins, phytochrome and blue-light photoreceptors sense the stimulus and efficiently induce transcription of photosynthesis-related genes (Tobin and Silverthorne, 1985; Thompson and White, 1991). Little is known about the mechanisms that control the daily fluctuation of gene expression in response to light (Nozue *et al.*, 2007). It is important to note that there are differences between the molecular mechanisms observed at night in plants grown under normal photoperiods compared with those that occur in etiolated seedlings. Molecular and genetic studies in *Arabidopsis* helped to identify numerous signalling intermediates that are specific for individual or multiple types of photoreceptors (for reviews, see Neff *et al.*, 2000; Quail, 2002). Notably, many of these signalling intermediates encode transcription factors (TFs) or transcriptional regulators, including proteins that belong to the b-Zip, FRS, Myb, bHLH, GRAS, and DOF families (Oyama *et al.*, 1997; Ni *et al.*, 1998; Hudson *et al.*, 1999; Bolle *et al.*, 2000; Fairchild *et al.*, 2000; Ballesteros *et al.*, 2001;

\* To whom correspondence should be addressed. E-mail: rchan@fbc.unl.edu.ar

Holm *et al.*, 2002; Huq and Quail, 2002; Wang and Deng, 2002; Park *et al.*, 2003; Huq *et al.*, 2004; Ward *et al.*, 2005). However, the majority of the light-responsive TFs identified to date have not been functionally characterized.

More than 1500 putative TFs have been identified in plants and have been classified into several families. Members of the HD-Zip family (especially those belonging to subfamilies I and II) are involved in regulating developmental processes related to the response of plants to environmental conditions (Carabelli *et al.*, 1993; Schena *et al.*, 1993; Söderman *et al.*, 1994, 1996, 1999; Lee and Chun, 1998; Deng *et al.*, 2002; Henriksson *et al.*, 2005; Ariel *et al.*, 2007). In fact, several members of the HD-Zip family have been shown to be regulated by different light conditions and to be involved in the modulation of light responses (Morelli and Ruberti, 2002; Wang *et al.*, 2003; Rueda *et al.*, 2005; Sessa *et al.*, 2005). Henriksson *et al.* (2005) have shown that the expression of some members of sub-family I, especially *ATHB7* and *ATHB52*, is strongly induced by dark.

Sunflower is an important agronomic crop in warm regions of the world. In many countries, it is the main crop. It is used for oil production as well as for other products related to animal and human food. The sunflower genome is approximately  $3 \times 10^9$  bp, most of which remains unknown. Neither ordinary transforming techniques nor mutant libraries or other genomic tools are available for this plant despite its great economic importance. Therefore, *Arabidopsis* is a heterologous system that could be considered a suitable first approach toward elucidating molecular mechanisms, as well as the functionality of sunflower genes.

HAHB4 is a sunflower member of the HD-Zip I sub-family, which bears the closest resemblance to *Arabidopsis* genes *ATHB7* and *ATHB12*. This TF is positively regulated by drought, ethylene, and abscisic acid (ABA) (Gago *et al.*, 2002; Dezar *et al.*, 2005a; Manavella *et al.*, 2006). Recently cross-talk between ethylene signalling and water deficit response that is mediated by this gene has been described (Manavella *et al.*, 2006). A previously reported microarray analysis of *Arabidopsis* plants that constitutively express *HAHB4* first suggested that it would participate in the regulation of photosynthesis-related genes. The closest *Arabidopsis* HD-Zip gene, *ATHB7*, is also regulated by darkness (Henriksson *et al.*, 2005). For these reasons, it was decided to study the function of *HAHB4* in response to illumination conditions; this became the main aim of the present work.

This paper presents experimental evidence that supports a role for this sunflower TF in the down-regulation of some photosynthesis-related genes. It is reported that the expression of *HAHB4* in sunflower is up-regulated in darkness. The overexpression of *HAHB4* cDNA in sunflower, together with knockdown by iRNA, clearly shows that this TF helps to prevent the accumulation of

transcripts of a large group of genes related to photosynthesis. This TF caused no detectable effects on the rate of photosynthesis, but chlorophyll content was reduced in *Arabidopsis* transgenic plants constitutively expressing *HAHB4*. It seems likely that the transcriptional regulation of several photosynthesis-related genes by *HAHB4* and other TFs in darkness helps to achieve a fine level of control of the transcription of photosynthesis-related genes in response to illumination conditions and other external factors like drought.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* Heyhn ecotype Columbia (Col-0) was 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 by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of  $\sim 150 \mu\text{E m}^{-2} \text{s}^{-1}$ , in 8 cm diameter  $\times$  7 cm height pots during the time indicated in the figures.

Plants used in some experiments (as indicated in the figure legends) were grown in Petri dishes containing Murashige and Skoog (MS) medium, with 0.8% agar. Dishes were kept at 4 °C for 2 d and then transferred to growth-chamber conditions and maintained for variable periods of time.

The characterization of plants bearing *HAHB4* cDNA controlled by the 35S cauliflower mosaic virus promoter or by the promoter of *HAHB4* (LPF) has been described previously (Dezar *et al.*, 2005a; Manavella *et al.*, 2006).

*Helianthus annuus* L. (sunflower cv. Contiflor 15, from Zeneca) seeds were surface sterilized and grown on filter paper inside Petri dishes for 4 d in the dark or under different illumination conditions as described in the figure legends. Seedlings were then transferred to plastic supports containing soil and grown for different periods of time depending on the experiment.

### Darkness treatments

Different periods of incubation in complete darkness were applied to 15-d-old plants grown under 16 h light periods at a light intensity of  $150 \mu\text{E m}^{-2} \text{s}^{-1}$  in MS medium. After incubation in darkness, plants were harvested immediately (also in darkness) in liquid nitrogen until RNA or proteins were isolated as described below.

When sunflower plants were analysed, 4-week-old plants grown under culture conditions were used and the tissues of interest were harvested after each period of incubation.

### Constructs

*LPF-GUS* and *SPF-GUS* constructs (bearing *HAHB4* promoter regions that direct the expression of the reporter gene *GUS*; Dezar *et al.*, 2005b) in pBI 101.3 were obtained as previously described.

Constructs containing upstream deletions or chimeras of the promoter were made by PCR amplification with the oligonucleotides described in Table S2 in Supplementary data available at *JXB* online, and digested with *Bam*HI/*Sa*II in order to be cloned in the same restriction sites of pBI101.3.

Constructs for iRNA assays were made as follows: a PCR-amplified 300 bp fragment bearing a segment of the *HAHB4* non-conserved coding region plus a segment of the 3' non-coding region was cloned into the *Xho*I/*Kpn*I sites (sense) and the *Hind*III/*Xba*I sites (antisense) of the pHANNIBAL vector. This clone was

digested with *NorI* in order to subclone into the pART27 plasmid, then used to transiently transform sunflower leaves as described below. The oligonucleotides used for these constructs are detailed in Table S2 in Supplementary data available at *JXB* online.

#### 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 PCR was carried out on genomic DNA with specific oligonucleotides for each construct in order to verify the selected lines. To assess *GUS* expression, northern blot analyses and real-time RT-PCR were performed on T<sub>2</sub> transformants as described below. At least three positive independent lines for each construct (arising from two different transformation experiments) were used to select homozygous T<sub>3</sub> and T<sub>4</sub> plants in order to analyse the expression levels of *GUS*. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way and used as negative and positive controls, respectively.

#### Transient transformation of sunflower leaves

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

#### RNA isolation and analysis by northern blot

Total RNA for northern blots and real-time PCR was prepared with Trizol<sup>®</sup> reagent (Invitrogen<sup>™</sup>). Specific amounts of RNA were electrophoresed through 1.5% (w/v) agarose/6% formaldehyde gels. The integrity of the RNA and equality of RNA loading were verified by ethidium bromide staining. RNA was transferred to Hybond-N nylon membranes (Amersham Corp.) and hybridized overnight at 65 °C with <sup>32</sup>P-labelled 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), then with 0.1×SSC plus 0.1% (w/v) SDS at 37 °C for 15 min; filters were then dried and exposed to Kodak BioMax MS films. To check the amount of total RNA loaded and transferred in each lane, filters were then re-probed with a 25S rRNA from *Vicia faba*. For *GUS* detection, a full-length cDNA probe was obtained by digest of pBI101.3 with *Bam*HI/*Sac*I. Hybridization was performed at 68 °C to avoid nonspecific reactions.

#### Real-time RT-PCR measurements

RNA (1 µg, prepared as described above) was used for the RT reactions with M-MLV reverse transcriptase (Promega). Quantitative PCRs were carried out using a MJ-Cromos 4 apparatus in 25 µl final volume containing 1 µl SyBr green (×1), 10 pmol of each primer, 3 mM MgCl<sub>2</sub>, 5 µl of the diluted RT reaction, and 0.15 µl Platinum Taq (Invitrogen Inc.). Fluorescence was measured at 80–84 °C over 40 cycles. Sunflower RNA was also prepared with

the Trizol technique. All samples were tested in triplicate and three biological replicates were used for each experiment.

Specific oligonucleotides for each gene were designed using publicly available sequences (Arabidopsis.org web page for *Arabidopsis* genes and tigrblast.tigr.org/tgi for sunflower genes). The oligonucleotide sequences are listed in Table S1 in Supplementary data available at *JXB* online.

#### Measurement of contents of chlorophyll and other pigments

To determine the pigment content of transgenic and wild-type (WT) plants, 200 mg of 21-d-old leaves of each genotype were collected and frozen with liquid nitrogen. Chlorophyll and carotenoid concentrations were determined according to the method described by Whatley *et al.* (1963).

#### Confocal microscopy and image analysis

Protoplast or entire leaf confocal images were obtained using a Carl Zeiss LSM5 Pascal laser scanning confocal microscope equipped with an argon/helium/neon laser (Zeiss, Jena, Germany), 40× C-Apochromat (1.2 numerical aperture) oil immersion objective, and a 560-nm-long pass filter to capture chlorophyll fluorescence. The confocal pinhole of the microscope was set to obtain an optical slice of 5 µm. Images were acquired and processed with the Zeiss LSM image software. Chloroplast number and estimation of chloroplast size were performed using the ImageJ 1.35h software (<http://rsb.info.nih.gov/ij/>).

#### Microarray experiments

Transcriptome analysis has been performed with the CATMA array containing 24 576 gene-specific tags from *Arabidopsis thaliana* (Crowe *et al.*, 2003; Hilson *et al.*, 2004) and previously analysed by Manavella *et al.* (2006).

#### Preparation of nuclei

Sunflower nuclear extracts were prepared from control or dark-treated 7-d-old seedlings according to the technique described by Maliga *et al.* (1995). Protein patterns were analysed by SDS-PAGE and total protein concentrations were measured as described (Sedmak and Grossberg, 1977).

#### DNA binding assays

For electrophoretic mobility shift assays (EMSAs), aliquots of purified nuclear proteins (8–10 µg) were incubated with double-stranded DNA (0.3–0.6 ng, 10 000 cpm, labelled with [ $\alpha$ -<sup>32</sup>P]dATP by filling in the 3' ends using Taq DNA polymerase) generated by hybridization of complementary oligonucleotides dark3', dark3'c, dark5', dark5'c, mut3', and mut3'c (Table S2 in Supplementary data available at *JXB* online). Binding reactions (40 µl) containing 20 mM HEPES-NaOH (pH 7.6), 40 mM NaCl, 0.2 mM EDTA, 1.0 mM DTT, 0.5% Triton X-100, 20% glycerol, and 1.5 µg poly(dI-dC), were incubated for 15 min at 4 °C, supplemented with 2.5% (w/v) Ficoll, and immediately loaded onto a running gel (5% acrylamide, 0.08% bis-acrylamide in 0.5×TBE plus 2.5% glycerol; 1×TBE is 90 mM TRIS-borate, pH 8.3, 2 mM EDTA). The gel was run in 0.5×TBE at 30 mA for 1.5 h at 4 °C and dried prior to autoradiography.

#### Gas exchange analysis

CO<sub>2</sub> exchange measurements were performed on detached leaves using an open gas-exchange system with infrared gas analyser (IRGA, Qubit Systems Inc., Kingston, ON, Canada). A minimum of three expanded leaves were sealed in the chamber illuminated with

PPF of  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The temperature was maintained between  $25^\circ\text{C}$  and  $27^\circ\text{C}$ . Room air was used in air flows with an average  $\text{CO}_2$  concentration of  $380\text{--}400 \mu\text{l l}^{-1}$ . Measurements of gas exchange were taken at least five times for each independent transgenic or WT plant using four different individuals of each. The measurements were repeated at least three times. The leaf surface area was calculated by scanning and  $\text{CO}_2$  assimilation rate, expressed as  $\mu\text{mol s}^{-1} \text{m}^{-2}$ .

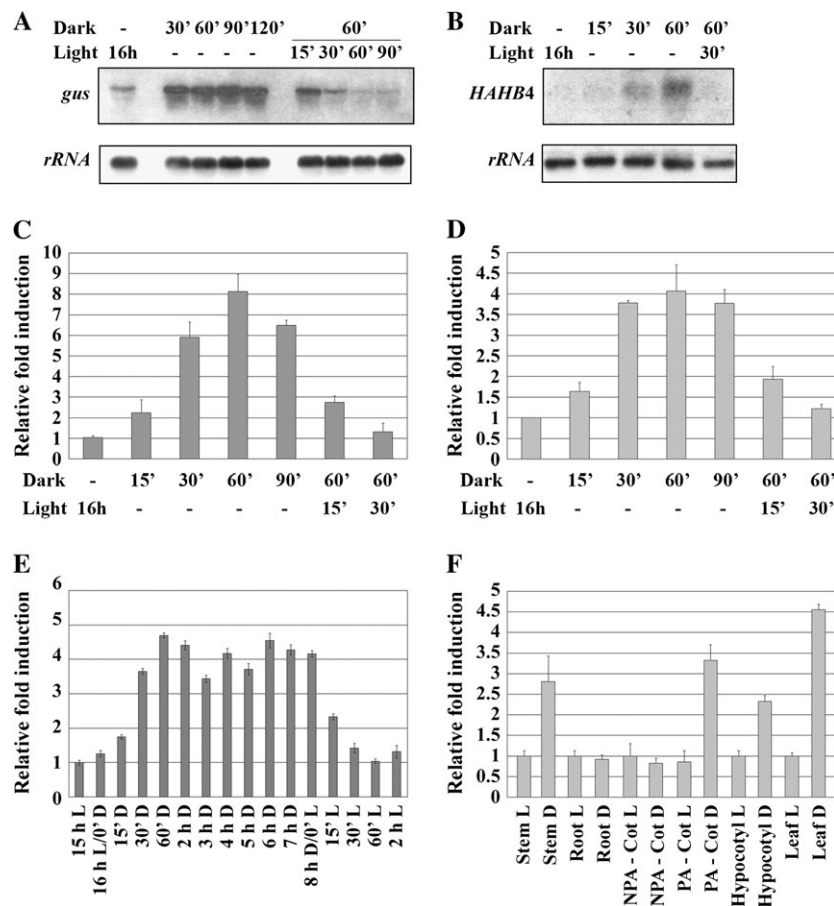
## Results

### *HAHB4* is up-regulated in darkness

*HAHB4* transcript levels in the sunflower were practically undetectable under normal conditions. However, GUS activity as directed by the *HAHB4* promoter (analysed by histochemistry) in *Arabidopsis* transgenic plants was constantly high. Considering that histochemical processing required incubation in total darkness, and knowing

that *Arabidopsis ATHB7* is up-regulated by darkness, it seemed likely that *HAHB4* gene expression could be also regulated by this condition. In order to test this hypothesis, *Arabidopsis* transgenic plants bearing the *LPF:GUS* construct (the *HAHB4* promoter directing GUS activity) were incubated in the dark for several periods of time. Total RNA samples were extracted and *GUS* transcript levels were analysed by northern blot hybridization and qPCR. As shown in Fig. 1A (northern blot analysis) and Fig. 1C (qPCR measurements), the *HAHB4* promoter responds rapidly to darkness, reaching the highest accumulation of the reporter gene transcripts at 30 min, and remaining almost constant until at least 2 h.

In order to determine whether the same type of regulation occurs in sunflower, *HAHB4* transcript levels were measured by northern blot (Fig. 1B) and by qPCR (Fig. 1D) after subjecting sunflower plants to different



**Fig. 1.** *HAHB4* expression is transcriptionally regulated by dark/light conditions. (A) Northern blot hybridization (total RNAs, 10  $\mu\text{g}/\text{line}$ ) of transgenic *Arabidopsis* bearing the *LPF:GUS* construct maintained in the darkness for the indicated periods or re-illuminated after 60 min of darkness. (B) Northern blot of sunflower total RNA (10  $\mu\text{g}$ ) hybridized with the *HAHB4* probe. In both cases, filters were rehybridized with a ribosomal probe as a control for loading and transfer (lower panel). (C–F) Transcript levels measured by real-time RT-PCR: (C) RNAs obtained from three independent lines of transgenic plants (*LPF:GUS*) subjected to different periods of absolute darkness, as indicated, and transferred to normal illumination conditions (the oligonucleotides used correspond to the *GUS* reporter gene); (D) similar kinetics as in (C), but with sunflower RNA measuring *HAHB4* transcript levels; (E) sunflower plants subjected to longer periods of absolute darkness and/or re-illuminated for the indicated periods of time; (F) dark treatment isolating different tissues and organs of the sunflower plant. NPA-Cot, Non-photosynthetically active cotyledon; PA-Cot, photosynthetically active cotyledon. In all cases: D, darkness; L, light. All differences were considered significant when  $P < 0.05$ .

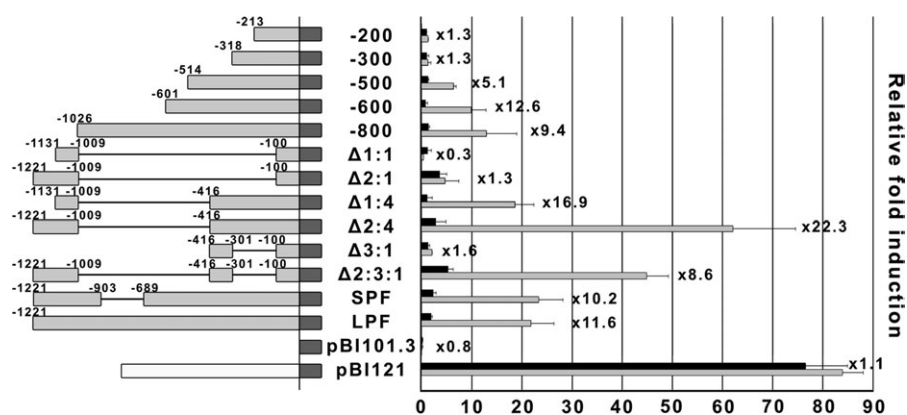
illumination conditions. These experiments, in both systems, clearly determined that induction of *HAHB4* gene expression is maximal after 60 min of incubation. Accordingly, when plants incubated in darkness were transferred to an illuminated culture chamber, the expression levels of *HAHB4* in sunflower and *GUS* in *Arabidopsis* decreased. Thirty minutes of light exposure were enough to reduce either *GUS* or *HAHB4* transcript levels to their initial values. It was then of interest to determine whether the expression of this gene was influenced by the day/night cycle, for which *HAHB4* transcript levels were measured in sunflower plants during a whole night. The results supported what had been previously observed. High levels of *HAHB4* transcripts were almost constant throughout the night (8 h), and decreased immediately when the day started (Fig. 1E).

#### Regulation of *HAHB4* expression by darkness is restricted to photosynthetic organs

Aiming to determine whether the regulation of *HAHB4* by darkness is ubiquitous throughout the plant, 25-d-old sunflower plants were incubated in the dark and RNA was obtained from several organs, including roots, cotyledons, hypocotyls, stems, and leaves. Northern blot assays indicated that *HAHB4* transcript levels increased in stems and leaves, and could be clearly detected after 30 min in the dark (not shown). Real-time RT-PCR revealed that the induction was almost 5-fold in leaves and 2.5-fold in hypocotyls and stems. No induction was observed in roots or in non-photosynthetically active cotyledons, but *HAHB4* transcript levels were clearly detectable in photosynthetically active cotyledons (Fig. 1F).

#### The proximal promoter of *HAHB4* is responsible for regulation by darkness

The sequences responsible for the activity of *HAHB4* promoter in darkness were localized by making use of previously obtained transgenic plants bearing alternative deletions of the promoter (Dezar *et al.*, 2005b) and plants transformed with additional mutated constructs specially obtained for this purpose. The transcript levels of the reporter gene were measured by real-time RT-PCR. Figure 2 shows that both allelic forms of this promoter (*LPF* and *SPF*; Dezar *et al.*, 2005b) increase activity in the dark compared with the activity in transgenic plants collected during the day (11.6- and 10.2-fold for *LPF* and *SPF*, respectively). Shorter constructs are also inducible by darkness when they bear at least the first 514 bp upstream of the transcription initiation site. Neither the -300 nor the -200 constructs were regulated by illumination conditions. The behaviour of the  $\Delta 2:1$  and  $\Delta 6:1$  constructs showed that the region upstream from positions -600 and/or -1009 have no elements that are responsive to dark. With these results, it is possible to localize the induction region to the area between bases -514 and -301. In order to obtain a more detailed characterization of this segment, several chimeras were constructed, as shown in Fig. 2. Plants were transformed with these constructs and *GUS* transcript levels were measured in the T<sub>2</sub> transgenic plants by real-time RT-PCR. Taking into account that the 5' region of the promoter has no known dark-responsive elements (see above), a comparison between the  $\Delta 2:4$  construct and the -300 construct indicated that the essential elements responsive to darkness are located between -416 bp and -301 bp. As shown in Fig. 2, the constructs bearing the first 416 bp from the transcription initiation site plus a further upstream 5' segment of 200 bp ( $\Delta 2:4$ )



**Fig. 2.** A *cis*-acting element responsible for dark response is located between -316 and -401 of the *HAHB4* promoter. Left panel: a schematic representation of the mutant and chimeric constructs of the *HAHB4* promoter (*LPF*). Numbers on grey boxes indicate the positions contained in each construct. Characters on the left of this panel indicate the name of each construct. Right panel: *GUS* transcript levels measured by real-time RT-PCR of three independent lines of transgenic plants bearing the constructs represented in the left panel, related to the value obtained with -200 construct in light, taken as a unit. Grey bars correspond to RNA obtained after dark treatment, while black bars correspond to samples obtained from plants cultured in light. Fold induction by dark treatment as related to each light control is indicated on the right of the bars. Standard deviation and statistical analyses were calculated from three independent experiments.

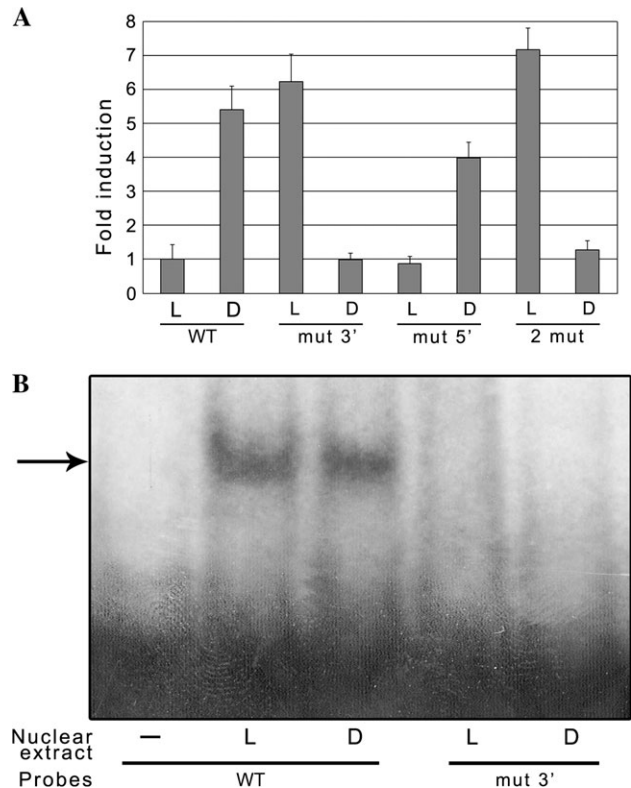
show the highest induction by incubation in the dark, reaching values of almost 20-fold, supporting the observation that dark-responsive elements are located between  $-416$  bp and  $-301$  bp. The dark response is strengthened by certain enhancer sequences between  $-1221$  and  $-1009$ . This idea is supported by the finding that, for every construct, basal transcript levels were higher when the 5' extreme of the promoter was included.

The localization of the dark-responsive element between  $-416$  bp and  $-301$  bp was supported by a new chimeric construct,  $\Delta 2:3:1$ , which contains only a minimal promoter, followed by the  $-416$  bp to  $-301$  bp region and the detected enhancer (Fig. 2). Northern blots performed on all the samples confirmed these results (not shown).

*Mutant constructs of the promoter indicate that a cis-acting element located at position  $-320/-315$  is responsible for induction by darkness*

The analysis of putative *cis*-acting elements located between  $-416$  bp and  $-301$  bp in the promoter region revealed the existence of two GT boxes, TTAACC (GGTTAA in the complementary strand). These 6 bp elements were described as being responsible for light-dependent regulation of gene expression (Green *et al.*, 1988; Dehesh *et al.*, 1990). In order to determine whether one or both elements are necessary for dark induction, mutations of the  $-600$  construct were made by deleting the 6 bp segments. *Arabidopsis* plants were transformed with these constructs and *GUS* transcript levels were analysed using real-time RT-PCR. Figure 3A shows the *GUS* expression in these mutant-constructs as compared with the non-mutated construct. Deletion of the 3' GT element completely abolished promoter induction by darkness. Moreover, transcript levels of this mutant were higher in light as compared with the non-mutated control. By contrast, the 5' mutant was still able to be normally induced by darkness. The double mutant maintained the behaviour of the 3' mutant. These results made it clear that the 3' segment is essential for regulation, both in light and in darkness. Moreover, these results indicated not only that *HAHB4* is up-regulated by darkness, but also that it is repressed by light. This opposite regulation of the TF by light conditions seems to be mediated by the same *cis*-acting element in its promoter. Mobility shift assays were performed and showed that the GT element between  $-320$  and  $-315$  is recognized both in light and in darkness.

For EMSA, double-stranded synthetic oligonucleotides containing the putative light-responsive *cis*-acting element at position  $-320/-315$  (in which the box GGTTAA was replaced by ACCGTT) were radiolabelled and mixed with dark- or light-purified sunflower nuclear proteins. Figure 3B shows EMSA done with WT or mut-3' probes; the 3' functional element was recognized by at least one protein complex. Moreover, the shifted band appears when either



**Fig. 3.** (A) Real-time RT-PCR measurement of RNA obtained from transgenic plants bearing the wild type (WT) or mutated DNA segments directing *GUS* activity. 2 mut represents a double mutant where both the 3' and 5' boxes were mutated. Values were referred to the one obtained in light with the WT promoter segment taken arbitrarily as one. (B) EMSA performed with 8  $\mu$ g of sunflower nuclear protein and WT or mutant radiolabelled oligonucleotides representing the 3' region, respectively, of the  $-416/-300$  segment. In all cases: L, plants incubated in light; D, in absolute darkness.

dark- or light-purified sunflower nuclei are added, indicating that this site is recognized equally in both conditions. These shifted bands remain almost invariant in the presence of an excess of poly-dIdC or nonspecific DNA used as competitors, indicating a high binding specificity (data not shown). Therefore, even though it was not possible to identify the protein/s interacting with the *cis*-acting element, these results supported the functionality of this GT-element, showing that it is recognized by a protein complex.

*The transcriptional levels of some photosynthesis-related genes decreased in Arabidopsis plants constitutively expressing HAHB4*

In order to determine what role *HAHB4* plays once it is induced by darkness, it was of great importance to identify the genes controlled by this TF. The data obtained from the previously reported microarray experiments (Manavella *et al.*, 2006) shed light on this area. The microarray was performed with 3-week-old *Arabidopsis* transgenic plants that constitutively express *HAHB4*. This

transcriptome analysis indicated that many genes involved in photosynthetic pathways are down-regulated as a result of the constitutive expression of *HAHB4*. Table 1 shows the expression values of photosynthesis-related genes extracted from the microarray data (Manavella *et al.*,

2006). Within the set of repressed genes there were some that encode components of the electron transport chain (including the ATP-synthase complex) and others that encode components of the Calvin cycle. As shown in Table S1 in Supplementary data available at *JXB* online,

**Table 1.** Photosynthesis-related genes regulated by *HAHB4*

Microarray analysis. Genes down-regulated by *HAHB4* detected in the transgenic plants microarray analysis as components of the photosynthetic machinery. ID, *Arabidopsis* notation; rat, is expressed as the  $\log_2$  of the ratio between transcript values of transgenic (bearing *35S:HAHB4*) versus WT plants.

ID	Description	WT/Hahb-4	
		$\log_2$ rat	<i>P-Val</i>
At5g01530	Chlorophyll <i>a/b</i> -binding protein CP29 (LHCB4)	-1.736	-
At2g34420	Chlorophyll <i>a/b</i> -binding protein / LHCII type I (LHB1B2)	-1.243	4.4E-08
At3g08940	Chlorophyll <i>a/b</i> -binding protein (LHCB4.2)	-1.109	7.7E-06
At2g34430	Chlorophyll <i>a/b</i> -binding protein/LHCII type I (LHB1B1)	-1.432	-
At3g54890	Chlorophyll <i>a/b</i> -binding protein/LHCI type I (CAB)	-2.221	-
At1g61520	Chlorophyll <i>a/b</i> -binding protein/LHCI type III (LHCA3.1)	-2.294	-
At3g61470	Chlorophyll <i>a/b</i> -binding protein (LHCA2)	-1.803	-
At1g15820	Chlorophyll <i>a/b</i> -binding protein (LHCB6)	-0.938	0.003
At2g06520	Photosystem II light reaction centre subunit	-1.865	-
At1g67740	Photosystem II core complex proteins psbY (PSBY)	-2.723	-
At4g28660	Photosystem II reaction centre W (PsbW)	-1.605	1.0E+00
AtCg00720	Cytochrome <i>b<sub>6</sub></i>	-1.527	4.2E-10
At1g76100	Plastocyanin	-1.708	-
At4g09650	ATP synthase delta chain (OSCP)	-1.112	6.9E-06
At4g04640	ATP synthase gamma chain I(ATPC1)	-1.781	-
At4g02770	Photosystem I reaction centre subunit II 20 kDa/PSI-D (PSAD1)	-1.302	3.8E-09
At2g20260	Photosystem I reaction centre subunit IV/PSI-E (PSAE2)	-0.985	5.7E-04
At1g55670	Photosystem I reaction centre subunit V/PSI-G (PSAG)	-1.440	5.5E-12
At4g12800	Photosystem I reaction centre subunit XI/PSI subunit V (PSI-L)	-1.121	5.0E-06
At1g52230	Photosystem I reaction centre subunit VI/PSI-H (PSAH2)	-1.156	1.3E-06
AtCg00340	Photosystem I P700 apoprotein A2	-1.274	3.8E-06
At1g31330	Photosystem I reaction centre subunit III	-4.189	-
At1g20020	Ferredoxin-NADP(+) reductase/adrenodoxin reductase	-1.406	3.8E-11
At1g32060	Phosphoribulokinase (PRK)	-1.461	5.5E-12
At1g67090	Ribulose biphosphate carboxylase small chain 1A/RuBisCO small subunit 1A (RBCS-1A) (ATS1A)	-1.272	1.3E-08
At5g38410	Ribulose biphosphate carboxylase small chain 3B/RuBisCO small subunit 3B (RBCS-3B) (ATS3B)	-1.417	2.2E-11
At2g39730	Ribulose biphosphate carboxylase/oxygenase activase/RuBisCO activase	-1.832	-
At1g42970	Glyceraldehyde-3-phosphate dehydrogenase B (GAPB)	-1.518	-
At2g24270	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	-1.189	3.8E-07
At4g26530	Fructose-biphosphate aldolase	-2.153	-
At4g38970	Fructose-biphosphate aldolase	-1.060	4.4E-05
At2g45290	Transketolase	-1.750	-
At5g61410	Ribulose-phosphate 3-epimerase	-1.622	-
At5g36790	Phosphoglycolate phosphatase	-1.022	1.7E-04
At5g26030	Ferrochelatase I	-0.868	2.1E-02
At4g30210	NADPH-cytochrome p450 reductase/NADPH-ferrihemoprotein reductase	-0.850	3.5E-02
At4g25080	Magnesium protoporphyrin IX methyltransferase-chlorophyll biosynthesis	-0.760	4.0E-02
At1g08520	Magnesium chelatase-chlorophyll biosynthesis	-0.989	4.9E-04
At3g56460	Putative chlorophyll biosynthesis	-1.791	6.2E-04
At1g74470	Geranylgeranyl reductase-chlorophyll biosynthesis	-1.25	5.8E-04
At1g23740	Putative chlorophyll biosynthesis	-1.41	2.7E-11
At5g43940	Formaldehyde dehydrogenase (glutathione)-putative chlorophyll biosynthesis	-1.29	5.1E-09

Real-time validations. Validation of the results was performed by real-time RT-PCR using specific oligonucleotides.

ID	Description	WT	Hahb-4	<i>P-Val</i>
At2g06520	Photosystem II light reaction centre subunit (PSB-X)	1±0.28	0.35±0.05	<0.01
At1g61520	Chlorophyll <i>a/b</i> -binding protein/LHCI type III (LHCA3.1)	1±0.03	0.45±0.19	<0.05
At4g12800	Photosystem I reaction centre subunit XI/PSI subunit V (PSI-L)	1±0.29	0.44±0.10	<0.05
At1g32060	Phosphoribulokinase (PRK)	1±0.03	0.47±0.11	<0.01
At2g39730	Ribulose biphosphate carboxylase/oxygenase activase (RuBisCO act)	1±0.20	0.37±0.12	<0.01
At5g36790	Phosphoglycolate phosphatase (PhG-P)	1±0.24	0.38±0.09	<0.01
At1g20020	Ferredoxin-NADP(+) reductase (FNR)	1±0.13	0.30±0.06	<0.01

transcript levels of several genes with altered expression levels were measured by qPCR in several independent transgenic lines in order to validate the results. Remarkably, a significant group of genes encoding photosystem I and components of the light-harvesting complexes are down-regulated in the transgenic plants. Based on the information obtained from the transcriptional approach in *Arabidopsis*, subsequent studies were focused on the same group of genes in sunflower.

*Sunflower genes homologous to those identified in Arabidopsis are co-regulated with HAHB4 during darkness*

Although a large portion of the sunflower genome remains unknown, the information available in databases made it possible to identify genes probably orthologous to those identified in *Arabidopsis*. Specific oligonucleotides were designed to measure transcript levels of a selected group of these genes in sunflower, either in control conditions or when *HAHB4* expression is induced. Figure 5A shows the results for the analysed genes when *HAHB4* expression levels increase due to treatment with ethylene or drought. As shown, *PSBx*, *LHCA*, *PSI-1*, *PRK*, *RubisCO* act, *PhGP*, and *FNR* are clearly repressed. Since the regulation of these genes is generally related to ethylene and drought stimuli, it was crucial to confirm that this down-regulation is due to the action of *HAHB4*. Therefore, sunflower leaves were transiently transformed by agro-infiltration with a construct that constitutively expresses *HAHB4* (*35S:HAhb4*) or with iRNA constructs that target the *HAHB4* sequence. Controls were carried out either with WT leaves infiltrated with buffer or agro-infiltrated with empty vector. RNA samples were taken from plants incubated 1 h in the dark and compared with those exposed to light. The results, similar to those obtained with external treatments (Fig. 4A), are shown in Fig. 4B. In every case, when *HAHB4* is up-regulated, the analysed sunflower genes were transcriptionally down-regulated. Moreover, plants unable to express *HAHB4* (H4-i) were less efficient in down-regulating the expression of the same genes in the dark (Fig. 4B, last columns). When *HAHB4* was silenced by iRNA, the transcript levels of the target genes were similar to those in plants grown in the light, even after being incubated in the dark for 1 h. Control assays to check iRNA efficiency were carried out and are included in the supplementary data as Fig. S1 in Supplementary data available at *JXB* online.

*In Arabidopsis plants that overexpressed HAHB4, pigment content decreased, but the effects on CO<sub>2</sub> fixation were not significant*

In light of the molecular observations described above, it was decided to perform some physiological measurements in *Arabidopsis* transgenic plants overexpressing *HAHB4*

in order to establish a direct relationship with the transcriptome analysis. As controls, the same physiological parameters were measured in *Arabidopsis* transgenic plants expressing *HAHB4* under its own inducible promoter and in WT plants.

Since most of the chlorophyll-binding proteins are apparently down-regulated by *HAHB4*, the content of chlorophylls *a* and *b* and carotenoids was quantified. This analysis indicated a general decrease in the pigment contents in the transgenic plants, especially for chlorophyll *a* (Fig. 5B). Confocal microscopy revealed that the number and size of chloroplasts were similar in leaves and protoplasts from all three genotypes (Fig. 5A). Together, these results indicated that the decrease in the pigment content in constitutively expressing transgenic plants is not a consequence of differences in chloroplast size or number (Fig. 5A, lower panel).

In order to validate these results in the sunflower, leaves were transiently transformed either to overexpress or repress *HAHB4* with *35S:HAHB4* or iRNA constructs, respectively. Results shown in Fig. 5C indicate a significant reduction in the average chlorophyll content in plants overexpressing the TF. Accordingly, leaves transformed with an iRNA construct did not show a significant change in pigment content. Chlorophyll content is expressed in relation to the values obtained from plants transformed with empty vector.

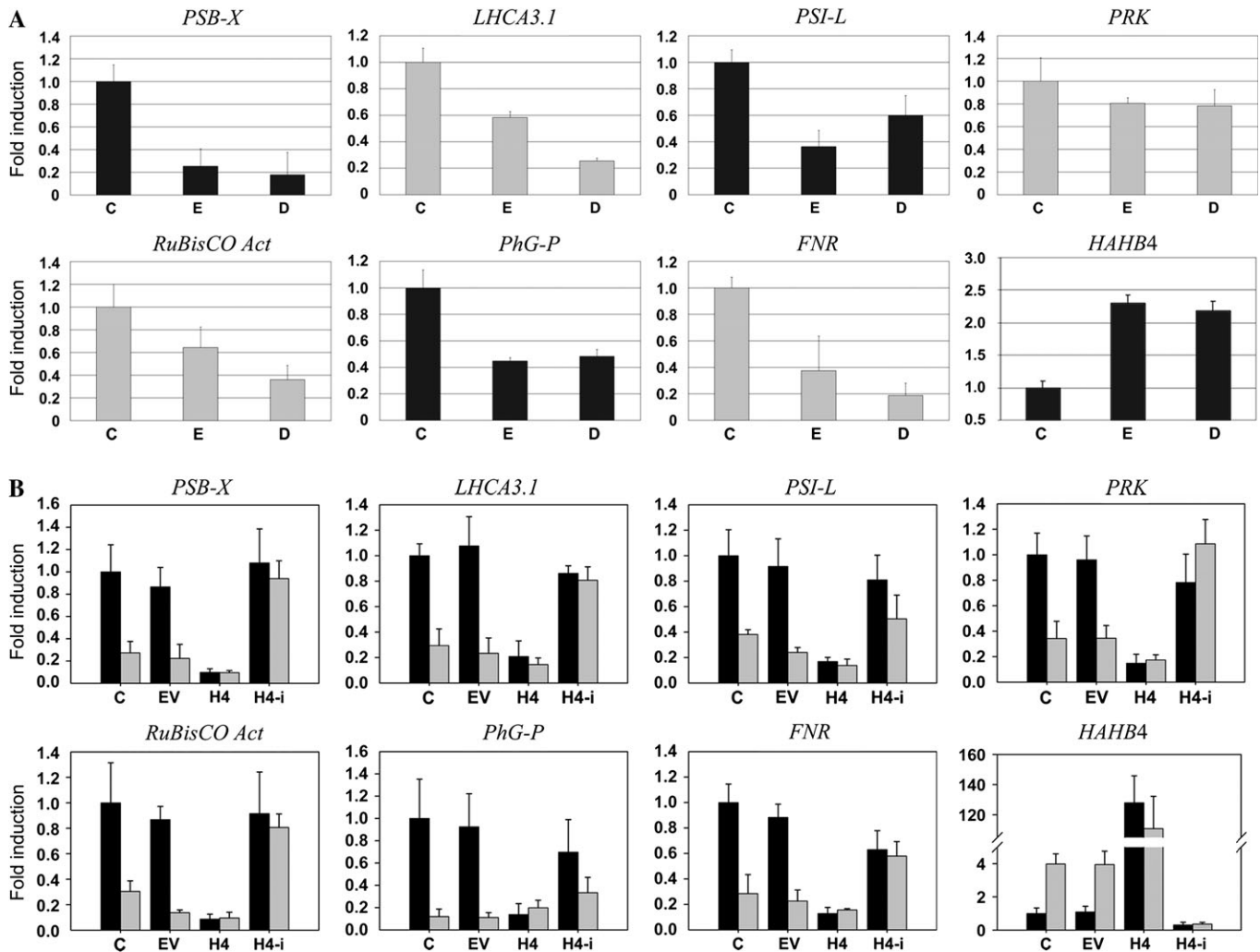
The CO<sub>2</sub> fixation rate was determined for plants expressing *35S:HAHB4* (H4) or transformed with empty vector (EV). The results from this experiment indicated that both genotypes exchange gases at similar rates (EV, 25.89 ± 1.58 μmol m<sup>-2</sup> s<sup>-1</sup>; H4, 29.95 ± 2.34 μmol m<sup>-2</sup> s<sup>-1</sup>). A difference in the CO<sub>2</sub> fixation rate was only detectable if the whole plant was considered as an individual. Given that transgenic individuals are smaller than untransformed controls, they assimilate less CO<sub>2</sub> (EV, 25.61 ± 1.32 nmol s<sup>-1</sup>; H4, 14.48 ± 2.31 nmol s<sup>-1</sup> per plant, respectively; *P* > 0.01).

## Discussion

Plants are constantly maintaining a fine balance between CO<sub>2</sub> assimilation and water loss, controlled by the intricate pathway of signals that involve hormones, kinases, and TFs. TFs have evolved to play a major role in minimizing the loss of water during CO<sub>2</sub> fixation, thereby preserving the physiological stability of the plant (Chaves and Olivera, 2004).

In the sunflower, *HAHB4* transcript levels increase in the dark, peaking after 60 min and remaining constant through the night. This up-regulation is detectable only in photosynthetic tissues. Transcript levels diminish soon after the start of the day, suggesting that this gene would have an acute effect on light-mediated processes. This



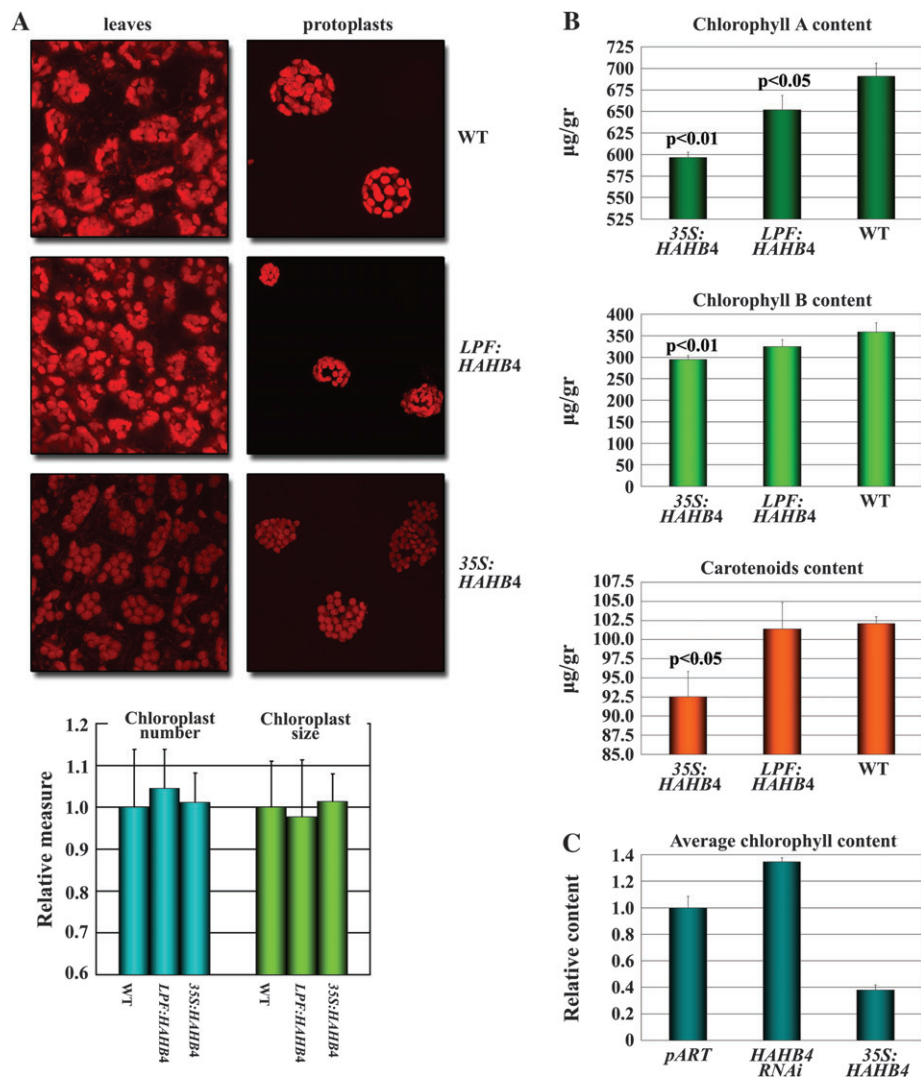


**Fig. 4.** Sunflower homologues of the *Arabidopsis* genes identified through the array analysis as being regulated by *HAHB4* are involved in dark response. (A) Transcript levels of photosystem II light reaction centre subunit (*PSB-X*); chlorophyll *a/b*-binding protein/LHCI type III (*LHCA 3.1*); photosystem I reaction centre, subunit XI/PSI subunit V (*PSI-L*); phosphoribulo-kinase (*PRK*); ribulose biphosphate carboxylase/oxygenase activase (*RuBisCO Act*); phosphoglycolate-phosphatase (*PhG-P*); ferredoxin-NADP reductase (*FNR*), and *Helianthus annuus* homeobox 4 (*HAHB4*), measured in 2-week-old sunflower seedlings grown in control conditions (C), treated with 30  $\mu$ M ethylene (E), or incubated in darkness (D) for 60 min. (B) Transcript levels of the same genes as in (A) in transiently transformed sunflower leaves (as described in Materials and Methods). Black or grey columns represent transcript levels under control conditions or after incubating leaves for 1 h in absolute darkness. For all genes tested, C represents non-transformed leaves; EV, leaves transformed with the empty vector; H4, leaves transformed with *35S:HAhb4*; and H4-i, leaves transformed with the *HAHB4*/RNAi cassette (an average of five independent transient expression events).

regulation occurs at the transcriptional level, as it can be seen in the *GUS* transcript levels of *Arabidopsis* plants transformed with a construct bearing the *HAHB4* promoter; this indicates the recognition of a conserved *cis*-acting element. Together, these results suggest that *HAHB4* may exert a role in dark responses.

A *cis*-acting element (GT1-like) was identified as essential for *HAHB4* promoter activity and localized in the region between  $-301$  and  $-416$ . However, the presence of additional elements cannot be ruled out even if they were not detected in the experiments performed, due to a hypothetical balance between repressors and enhancers in *HAHB4* promoter. Deletion of this element revealed not

only that induction by darkness was lost, but also that the basal level of expression in light was remarkably higher. In summary, this element seems to mediate induction by dark as well as repression by light. However, and given that the mutation was carried out as a deletion, it cannot be dismissed that other elements changed their position in relation to the transcription initiation site, that could have a certain influence on this response. The inversion of the normal behaviour suggested three alternative explanations: one single protein could bind to this element and exhibit opposite regulatory activities depending on the illumination conditions: two different TFs (an activator and a repressor) bind to this element differentially in dark or



**Fig. 5.** Pigment content is reduced in transgenic plant chloroplasts. (A) Confocal microscopy images of leaves (left) or protoplasts (right) from transgenic (*35S:HAHB4* or *LPF:HAHB4*) or wild-type plants. At the bottom, measures of chloroplasts size and number. (B) Measures of chlorophyll *a*, chlorophyll *b*, and carotenoid content in leaves of WT (non-transformed), *35S:HAHB4* (transgenic plants constitutively expressing *HAHB4*), or *LPF:HAHB4* (transgenic plants expressing *HAHB4* under induction) plants. (C) Total chlorophyll content in transiently transformed sunflower leaves (an average of five independent transient expression events).

in light; or dark/light-specific co-factors interact with a single TF bound to this element both in the light and in the dark. Regarding previous studies that described the functionality of these GT elements and the TFs that interact with them, the latter mechanism seems the most likely. For example, it has been established that GT-1 proteins, which bind to these elements, need to interact with other unknown cofactors to modulate their response (Gilmartin and Chua, 1990). This hypothesis is based on the fact that this protein differentially regulates gene expression in light or in darkness, but its expression and binding capability do not change between those conditions. It has also been shown that depending on the promoter context, GT elements can modulate both light activation/dark repression and light repression/dark activa-

tion in response to specific signalling molecules (Zhou, 1999). The results reported by other groups indicated that even when this element is critical for dark/light regulation, recognition by a single TF is not sufficient to modulate the specific response.

Regarding genes whose expression is regulated by dark/light, the most extensively studied are those that encode the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase and the chlorophyll *a/b*-binding proteins (Tobin and Silverthorne, 1985; Manzara and Grissem, 1988; Dean *et al.*, 1989; Anderson *et al.*, 1997; Humbeck and Krupinska, 2003). In several plant species, an increase in the transcript levels of these genes occurs in etiolated seedlings and in dark-adapted plants, once they are exposed to light. This induction is mediated by the

photoreceptor phytochrome and is regulated at the transcriptional level (Casal and Yanovsky, 2005). Many TFs and their target *cis*-acting elements have been identified and characterized as being involved in the dark/light regulation of photosynthesis-related genes (Gilmartin *et al.*, 1990; Lee and Hahn, 2003; McClung, 2006).

With the aim of unravelling the role of *HAHB4* in dark responses, the transcriptomic analysis of *Arabidopsis* transgenic plants overexpressing this TF was used as a first approach. The use of transiently transformed sunflower leaves allowed those results to be validated in the homologous system, and *HAHB4* expression to be silenced by iRNA, thereby ruling out possible artefacts caused by overexpression of the transgene in the heterologous system. These experiments confirmed that a large group of photosynthesis-related genes is down-regulated by *HAHB4*.

It is worth noting that among the genes transcriptionally down-regulated by *HAHB4* are some that encode components of photosystem I (LHCa), photosystem II (PSBx), genes related to chlorophyll biosynthesis, and others that comprise the Calvin cycle, such as PRK and RuBisCO. A concomitant decrease in the protein level of all these components would result in decreased photosynthetic efficiency in plants that constitutively express *HAHB4*. Accordingly, the pigment content of transgenic plants overexpressing *HAHB4* is rather lower than that of nontransformed plants. However, gas exchange seems to be unchanged; it is likely that the protein levels of the photosynthetic machinery components are not significantly altered, or perhaps the conditions used for this analysis were not those needed to detect a difference.

Apart from a change in the levels of involved transcripts, there must be a difference in the amount of protein, which depends on protein synthesis rate and turnover, in order to observe an impact in a certain biological process. Accordingly, it was recently reported that a set of genes encoding enzymes involved in central carbon and nitrogen metabolism showed marked changes of transcript levels but smaller and slower changes in the enzymatic activity of the corresponding protein products, both in the diurnal cycle and after transfer to continuous darkness (Gibon *et al.*, 2004). In these cases, the change in transcription would not result in an immediate effect on the biological process.

The experimental results reported in this work indicated that *HAHB4* inhibits *per se* the transcription of photosynthesis-related genes, in accordance with reports that other external and intrinsic factors play their respective roles in order to maintain the adaptation to the surrounding environment. Transcription of *HAHB4* is induced by the presence of ABA or ethylene or under water deficit. Constitutive expression of this gene in *Arabidopsis* results in a delay in the adverse effects caused by drought and senescence (Dezar *et al.*, 2005a;

Manavella *et al.*, 2006). Regarding this, when a plant is subjected to water deficit, it suffers photooxidative stress due to an excess of free electrons. In these conditions, *HAHB4* would inhibit the transcription of the main photosynthetic genes involved in light harvesting. Its action would reduce the formation of reactive oxygen species, what partially explains the observed drought tolerance of the transgenic plants.

In summary, given that *HAHB4* is up-regulated in drought or during senescence and that photosynthesis is inhibited under the same conditions, it is likely that *HAHB4* has a role in the down-regulation of photosynthesis observed during senescence and drought.

### Supplementary data

Supplementary material including Fig. S1 and Tables S1 and S2 are available online at the *Journal of Experimental Botany* web site.

### Acknowledgements

We gratefully acknowledge Dr Kimitaka Yakura, Kanazawa University, Japan, for sending us a *Vicia faba* rRNA clone. We also thank Dr Carlos Mas for assistance in microscopic assays, and Drs María Elena Alvarez and Hugo Maccioni for helpful suggestions. This work was supported by ANPCyT (PAV 137/2/2, PICT 2005,38103, PICTO 08-13204), CONICET (PIP 6383), UNL, and CABBIO. PAM and FDA are Fellows of CONICET, CAD, MFD, and RLC, and researchers at the same Institution.

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