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Research

Arabidopsis thaliana HomeoBox 1 (AtHB1), a Homedomain-Leucine Zipper I (HD-Zip I) transcription factor, is regulated by PHYTOCHROME-INTERACTING FACTOR 1 to promote hypocotyl elongation

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Summary

- *Arabidopsis thaliana* HomeoBox 1 (AtHB1) is a homeodomain-leucine zipper transcription factor described as a transcriptional activator with unknown function. Its role in *A. thaliana* development was investigated.
- *AtHB1* expression was analyzed in transgenic plants bearing its promoter region fused to reporter genes. Knock-down mutant and overexpressor plant phenotypes were analyzed in different photoperiod regimes.
- *AtHB1* was mainly expressed in hypocotyls and roots and up-regulated in seedlings grown under a short-day photoperiod. *AtHB1* knock-down mutants and overexpressors showed shorter and longer hypocotyls, respectively, than wild type (WT). *AtHB1* transcript levels were lower in *PHYTOCHROME-INTERACTING FACTOR 1* (*PIF1*) mutants than in controls, suggesting that *AtHB1* is regulated by *PIF1* in hypocotyls. β-glucuronidase (GUS) activity in *Nicotiana benthamiana* leaves cotransformed with *PromAtHB1::GUS* and *35S::PIF1* indicated that PIF1 induces *AtHB1* expression. Hypocotyl lenght was measured in seedlings of *athb1*, *pif1*, or double *athb1/pif1* mutants and *PIF1* or *AtHB1* overexpressors in WT, *athb1* or *pif1* backgrounds, both in short- or long-day. These analyses allowed us to determine that AtHB1 is a factor acting downstream of PIF1. Finally, a transcriptome analysis of *athb1* mutant hypocotyls revealed that AtHB1 regulates genes involved in cell wall composition and elongation.
- The results suggest that AtHB1 acts downstream of PIF1 to promote hypocotyl elongation, especially in response to short-day photoperiods.

Introduction

Plants, as sessile organisms, are continuously affected by environmental factors. Light is one of the most important of these factors, both providing the source of energy and acting as an environmental modulating signal of plant growth and development. Hypocotyls are plastic organs in which elongation is inhibited by light and influenced by external and internal cues, such as temperature changes and hormones (Vandenbussche *et al.*, 2005). This inhibition of hypocotyl elongation by light is mainly achieved through the activation of plant photoreceptors, primarily phytochromes (phys) and cryptochromes (crys), which absorb red/far-red and blue light, respectively (Vandenbussche *et al.*, 2005). The five *Arabidopsis thaliana PHY* genes (named A–E) encode 125-kDa proteins that form homodimers each carrying a phytochromobillin chromophore molecule. Phytochromes are synthesized in their inactive Pr (Pr is the red light-absorbing form of phytochromes) form and are converted reversibly to the active Pfr (Pfr is the far-red light-absorbing form of phytochromes) form after red light absorption. These proteins sense illumination changes and are able to regulate photomorphogenesis (Franklin & Quail, 2010).

One of the ways in which phytochromes affect plant growth and development is by modulating the activity and abundance of transcription factors (TFs) belonging to basic helix-loop-helix (bHLH) subfamily 15, named PHYTOCHROME-INTERACTING FACTORS (PIFs; Castillon *et al.*, 2007; Bae & Choi, 2008). PIF3 was the first of these proteins identified by a yeast two-hybrid screen using phyB as bait. After this identification of PIF3, PIF4 was isolated by both genetic and reverse-genetic approaches (Ni *et al.*, 1998; Huq & Quail, 2002). Next, using sequence homology, four other members of the family, PIF1, PIF5, PIF6 and PIF7, were identified (Huq *et al.*, 2004; Khanna *et al.*, 2004). In

darkness, PIFs accumulate in the nucleus and promote cell elongation in the hypocotyl, whereas in the light, they interact with the Pfr form of phys, which triggers their rapid phosphorylation. This phosphorylation can label PIFs for ubiquitination and degradation by the proteasome or, alternatively, inhibit their DNA-binding activity, arresting hypocotyl growth (Bauer *et al.*, 2004; Park *et al.*, 2004; Shen *et al.*, 2005, 2007, 2008; Oh *et al.*, 2006; Nozue *et al.*, 2007; Al-Sady *et al.*, 2008; Lorrain *et al.*, 2008; Li *et al.*, 2012).

Maximal growth of hypocotyls occurs in continuous darkness, and it has been reported that the quadruple mutant *pif1/pif3/pif4/pif5* (known as *pifq*) displays a constitutive photomorphogenic phenotype (Leivar *et al.*, 2008; Shin *et al.*, 2009). However, under alternating light/dark cycles, the extent of hypocotyl growth depends on the length of the dark period and the actions of PIF1, PIF3, PIF4 and PIF5, especially in short-day conditions (Nozue *et al.*, 2007; Niwa *et al.*, 2009; Soy *et al.*, 2012, 2014). Moreover, PIF1 transcripts are constitutively expressed in short-day conditions, and phy-imposed oscillations in PIF1 protein abundance determine the accumulation and action of this TF during the night (Soy *et al.*, 2014).

TFs are modular proteins that play key roles in the regulation of gene expression and orchestrate complex physiological events, such as organ development and growth. They are especially abundant in plants, representing *c.* 6% of the *A. thaliana* and rice (*Oryza sativa*) genomes (Riechmann *et al.*, 2000; Xiong *et al.*, 2005; Mitsuda & Ohme-Takagi, 2009). These proteins are classified in families and subfamilies according to their DNA-binding domains and other structural and functional features. Their modular characteristics allow these proteins to interact with others to finely tune gene expression. Some TF families are unique to plants, such as the homeodomain-leucine zipper (HD-Zip) family (Schena & Davis, 1992). Among other features, its members are characterized by the presence of a homeodomain associated with a leucine zipper, which acts as a dimerization motif and is necessary for DNA binding.

The HD-Zip family is composed of four subfamilies named I–IV (Ariel *et al.*, 2007; Ribichich *et al.*, 2014). In *A. thaliana*, subfamily I has 17 members encoding proteins of *c.* 35 kDa, which are mainly involved in developmental processes associated with abiotic stress (Ariel *et al.*, 2007; Ribichich *et al.*, 2014). In a recent phylogenetic reconstruction using 178 HD-Zip I proteins from different species that considered not only the HD-Zip motif but also the carboxy-terminal regions (CTRs), *A. thaliana* HD-Zip I proteins were classified into six groups, named I–VI (Arce *et al.*, 2011). These CTRs were demonstrated to be responsible for the interaction with other TFs and for transactivation (Capella *et al.*, 2014).

Arabidopsis thaliana HomeoBox 1 (AtHB1) belongs to group III of HD-Zip subfamily I and acts as a transcriptional activator (Arce *et al.*, 2011; Capella *et al.*, 2014). Point mutations in AtHB1 CTR followed by activation assays in yeast and plants indicated that this TF can interact with *Arabidopsis thaliana* TATA BINDING PROTEIN 2, a component of the basal transcription machinery (Capella *et al.*, 2014). The expression of this gene is down-regulated by NaCl and low temperatures and up-regulated in darkness (Henriksson *et al.*, 2005). Aoyama *et al.* (1995) showed that the overexpression of *AtHB1* in *Nicotiana benthamiana* plants affected the development of palisade parenchyma and conferred a constitutive photomorphogenic phenotype when the plants were grown in darkness. Therefore, AtHB1 has been proposed to be involved in leaf development and light sensing (Aoyama *et al.*, 1995). Although these studies contributed to knowledge of the function of AtHB1, no further reports have been published on this gene, and its role in plant development remains largely unknown.

In this study, we present experimental data showing that AtHB1 is primarily expressed in hypocotyls and regulates the growth of these organs, especially in short-day conditions. The results obtained using expression and phenotypic analyses of *AtHB1* and *PIF* knock-down and overexpressor plants allowed us to postulate that PIF1 positively regulates *AtHB1* expression to promote hypocotyl elongation in seedlings. Finally, a transcriptome analysis of *athb1* mutant plants revealed that *AtHB1* regulates hypocotyl growth by modifying cell elongation-related gene expression.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana L. Heynh. ecotype Columbia (Col-0) plants were grown in soil in a growth chamber at 22–24°C under a long-day photoperiod at an intensity of *c.* 120 $\mu mol\ m^{-2}\ s^{-1}$ in 8 × 7 cm pots. When particular photoperiod conditions were used, they are indicated in the corresponding figure legend.

The mutant seeds *athb1-1* (SALK_123216C), *athb1-2* (SALK_207381C; Alonso *et al.*, 2003), *pif3-7* (CS66042), *pif4-2* (CS66043), *pif5-3* (CS66044, also known as *pil6-1*) and *pifq* (CS66049) in the Col-0 ecotype background were obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH, USA; http://www.arabidopsis.org). Homozygous lines were selected after two complete growth cycles.

Seeds of *A. thaliana pif1* and *pil5* mutants (SALK_071677, known as *pif1-2* or *pil5-1*; SALK_131872C, known as *pil5-2*) were kindly provided by Dr Pablo Cerdan from the Leloir Institute, Buenos Aires, Argentina.

Plants used for hypocotyl length evaluation were grown in Petri dishes containing 0.5% Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) and 0.9% agar. The dishes were kept at 4°C for 3 d and then transferred to a growth chamber during the periods indicated in the respective figure legends.

PCR genotyping of SALK insertional lines

Rosette leaves from 30-d-old mutant (F_2) or wild-type (WT) plants were detached, and genomic DNA was extracted. PCR reactions were conducted using genomic DNA of each genotype, and the corresponding oligonucleotides were designed for each mutant (Supporting Information Table S1).

Hypocotyl length measurement

For each treatment and point, 30 seedlings were placed horizontally on an acetate sheet and scanned at a resolution of 1200 dots inch^{-1}. Hypocotyl length was determined using ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA) by measuring the distance from the most basal root hair to the 'V' shape made by the cotyledons. Statistical analyses of the mean values were performed using R statistical language (R Development Core Team, 2008). Each experiment was repeated at least three times.

Genetic constructs

PromAtHB1::GUS was constructed as follows: the promoter region of *AtHB1* and the 5′ untranslated region (UTR) (1416 bp) were amplified using *A. thaliana* genomic DNA as a template and the oligonucleotides promAtHB1-F and promAtHB1-R (Table S1). The PCR product was cloned into the *Sal*I and *Xba*I sites of *pENTR3C* and finally into the *pKGWFS7* vector to generate the construct by GATEWAY recombination.

The *pCAMBIA-His* vector was constructed by cloning the segment of the *pBI122* vector (Capella *et al.*, 2014), containing the *35S* cauliflower mosaic virus (*CaMV*), the His-tag, the multiple cloning site and the *NOPALINE SYNTHASE* into the *Hin*dIII and *Eco*RI sites of *pCAMBIA1380*.

To generate *35S::AtHB1*, the *GST::AtHB1* clone was digested with *Bam*HI to clone it into the unique *Bam*HI site of *pBI122* (Capella *et al.*, 2014). The insert orientation was determined by sequencing. The *35S::AtHB1* construct used to transform the *pif1-2* background was obtained by digesting the *BD-AtHB1* clone (Arce *et al.*, 2011) with *Eco*RI and *Bam*HI and cloning it into the *pGADT7* vector. This construct was then digested with *Bgl*II and *Xho*I and cloned into the *Bam*HI and *Xho*I sites of *pBluescript* SK(−). Finally, this last clone was digested with *Xba*I and *Xho*I, and the product was cloned into the *Xba*I and *Sal*I sites of *pCAMBIA-His*.

The *35S::PIF1* construct was obtained after amplifying *PIF1* cDNA using total seed RNA as a template with specific oligonucleotides (AtPIF1 cDNA F and AtPIF1 cDNA R; Table S1) and inserting the amplification products into the *Xba*I/*Sal*I sites of the *pCAMBIA-His* vector.

PromIRT1::GUS and *PromPLP4::GUS* were obtained as follows: the promoter regions of *IRON-REGULATED TRANSPORTER 1* (1639 bp; At4g19690) and *PATATIN-LIKE PROTEIN 4* (1650 bp; At4g37050) were amplified using *A. thaliana* genomic DNA as a template and the oligonucleotides promIRT1-F and promIRT1-R, and promPLP4-F and promPLP4-R, respectively (Table S1). The *IRT1* PCR product was cloned into the *Sal*I and *Xba*I sites of *pBI101.3*, whereas the *PLP4* PCR product was cloned into the *Hin*dIII and *Bam*HI sites of the same vector.

The constructs were used to transform *Agrobacterium tumefaciens* strain LBA4404 and then either to obtain transgenic *A. thaliana* plants by the floral dip procedure (Clough & Bent, 1998) or for *Nicotiana benthamiana* transient transformation as described below.

Transient transformation of *Nicotiana benthamiana* leaves

Leaves were cotransformed by infiltration with a syringe, as previously described (de Felippes & Weigel, 2010), with cultured *A. tumefaciens* LBA4404 transformed with the constructs indicated in the corresponding figures and mixed with *A. tumefaciens* cells transformed with the silencing inhibitor p19. Two days after infiltration, samples were harvested starting 2 h before the end of the day and used for total protein extraction and β-glucuronidase (GUS) activity quantification.

GUS assays

In situ assays of GUS activity were performed as described by Jefferson *et al.* (1987). Whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-b-glucuronic acid solution in 100 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100, and after applying vacuum for 5 min, the plants were incubated at 37°C overnight. Chlorophyll was cleared from green plant tissues by immersing them in 100% ethanol.

Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) essentially as described by Jefferson *et al.* (1987). Total protein extracts were prepared by grinding the tissues in extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA and 10 mM β-mercaptoethanol) containing 0.1% (w/v) SDS and 1% Triton X-100, followed by centrifugation at 13 000 ***g*** for 10 min. GUS activity in supernatants was measured in extraction buffer containing 1 mM MUG and 10% methanol. Reactions were stopped with 0.2 M Na_2CO_3, and the amount of 4-methylumbelliferone was calculated by normalizing relative fluorescence units with those of a standard of known concentration. Protein concentrations were determined as described by Bradfort (1976).

RNA isolation and expression analyses performed using real-time reverse transcription (RT)-PCR

RNA for real-time RT-PCR was extracted from seedlings and harvested 2 h before the end of the night with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using oligo(dT)$_{18}$ and Moloney Murine Leukemia Virus reverse transcriptase II (Promega, Fitchburg, WI, USA). Quantitative RT-PCR (RT-qPCR) was performed with the Mx3005P Multiplex qPCR system (Stratagene, La Jolla, CA, USA) in a 20 μl final volume containing 2 μl of SYBR green (4×), 8 pmol of each primer, 2 mM $MgCl_2$, 10 μl of a 1 : 15 dilution of the RT reaction and 0.12 μl of Platinum Taq (Invitrogen). Fluorescence was measured at 78–80°C during 45 cycles. The gene-specific primers used in the experiment are shown in Table S1. Quantification of the mRNA levels was performed by normalization with *Actin* transcript levels (*ACTIN2* and *ACTIN8*) according to the $\Delta\Delta C_t$ method. All the reactions were performed with at least three replicates.

For expression kinetics assays, 3-d-old seedlings grown in a short-day regime were harvested at different times of the night

and the following day (when seedlings were 4 d old). The night started at 17:00 h and ended at 09:00 h. Samples were harvested at 03:00, 05:00, 07:00, 09:00, 12:00, 15:00, 17:00 and 21:00 h.

RNA-sequencing (RNA-Seq) analysis

Total RNAs from 4-d-old seedlings grown in short-day conditions and harvested 1 h before the end of the night were isolated using the RNeasy Plant Mini Kit (Qiagen, Venlo, the Netherlands). Ten micrograms of RNA per sample was submitted to the Genome Technology Access Center at the Washington University School of Medicine, and mRNA was extracted using a Dynal mRNA Direct Kit (Life Technologies, Carlsbad, CA, USA). Messenger RNAs were fragmented and reverse-transcribed; double-stranded cDNA was obtained using random primers, and then adapters were added. Sequencing was performed on HiSeq 2500 equipment (Illumina, San Diego, CA, USA). The fastQ files were then aligned to the latest *A. thaliana* Col-0 genome assembly (TAIR10; released in June 2009) with TopHat version 2.0.8 (Center for Bioinformatics and Computational Biology, College Park, MD, USA) using Bowtie2 version 2.1.0 (Center for Bioinformatics and Computational Biology). Normalized read counts were then subjected to a Kruskal–Wallis one-way analysis of variance test.

RNA-Seq data obtained in this study can be found in the Gene Expression Omnibus database under the accession number GSE66666.

Results

AtHB1 is primarily expressed in roots and hypocotyls

The first step in characterizing *AtHB1* function was to determine its expression pattern throughout the plant life cycle. For this purpose, *A. thaliana* plants were transformed with a genetic construct (*PromAtHB1::GUS*) bearing the *AtHB1* promoter (1052 bp) and 5′ UTR (365 bp) and directing the expression of *Green Fluorescence Protein* (*GFP*) and *GUS* reporter genes. Homozygous transgenic plants were obtained and analyzed by histochemistry, and *GUS* expression could be detected only in hypocotyls, root vasculatures and tips of 4-d-old seedlings (Fig. 1a) and anthers of mature plants (data not shown).

A previous study showed that *AtHB1* transcript levels are induced in darkness (Henriksson *et al.*, 2005), so we tested whether its expression is modulated by the photoperiod. Transgenic *PromAtHB1::GUS* seedlings were grown under either long-day or short-day conditions for 4 d. Sample collection was performed 2 h before the end of the day, and then total proteins were extracted and subjected to fluorometric assays. An analysis of GUS activity showed that *AtHB1* expression was up-regulated in short-day conditions compared with a long-day regime (Fig. 1b). Consistently, when *GUS* and *AtHB1* transcript levels were quantified by RT-qPCR in RNAs obtained from the same seedlings, both genes (*AtHB1* and *GUS*) showed higher levels when seedlings were grown in short-day conditions than under a long-day photoperiod. Finally, *PromAtHB1::GUS* transgenic plants grown in short-day conditions were analyzed by histochemistry. GUS activity was detected in the same tissues as in plants grown under the long-day regime (Fig. 1a), but the staining was more intense.

AtHB1 plays a role in hypocotyl elongation

Considering the *AtHB1* expression pattern and the influence exerted by the photoperiod, we investigated whether this TF regulates hypocotyl elongation. Two T-DNA insertional *athb1* mutants (named *athb1-1* and *athb1-2*, respectively) were obtained as homozygotes and analyzed. T-DNA presence and homozygosis were corroborated by PCR using genomic DNA. In both mutants, the T-DNA was inserted in the 5′ UTR, which resulted in a reduction of the transcripts by *c.* 20-fold compared with the WT control (Fig. 2a). Hypocotyl lengths of 4-d-old WT and *athb1* mutant seedlings grown either under long-day or under short-day conditions were measured. Under a short-day regime, *athb1-1* and *athb1-2* mutants developed shorter hypocotyls than WT seedlings, whereas under long-day conditions, no significant differences were detected between the genotypes (Fig. 2b). To further investigate *AtHB1* function, transgenic plants expressing this gene under the control of the *35S CaMV* promoter (*35S::AtHB1-A* and *35S::AtHB1-B*) were obtained and characterized in the same growth conditions. These plants exhibited longer hypocotyls than WT controls in both long- and short-day conditions (Fig. 2c). However, no other obvious phenotypes were observed in *AtHB1* mutant or overexpressor plants grown under normal conditions.

A previous study reported that ectopic expression of *AtHB1* in tobacco plants resulted in a constitutive photomorphogenic phenotype when seedlings were grown in complete darkness (Aoyama *et al.*, 1995). To evaluate whether this phenotype is conserved in *A. thaliana* overexpressors and whether AtHB1 is involved in skotomorphogenesis, the expression pattern of *AtHB1* and the phenotype of etiolated seedlings of mutants and overexpressors were analyzed. GUS histochemical staining indicated that *AtHB1* was expressed in hypocotyls and roots when *PromAtHB1::GUS* seedlings were grown in absolute darkness for 4 d (Fig. S1a), similar to what was observed with de-etiolated plants (Fig. 1a). In contrast to the results obtained in tobacco plants (Aoyama *et al.*, 1995), *athb1* and *35S::AtHB1* plants did not show significant differences compared with WT controls when grown in complete darkness (Fig. S1b,c).

Taken together, the results suggested that *AtHB1* could act as a positive regulator of hypocotyl growth during photomorphogenesis, especially in short-day conditions. However, this TF does not seem to be involved in the skotomorphogenic development of *A. thaliana* plants.

PIF1 acts upstream of AtHB1 and positively regulates its expression

PIFs are bHLH TFs that play a central negative regulatory role in photomorphogenic development and promote hypocotyl

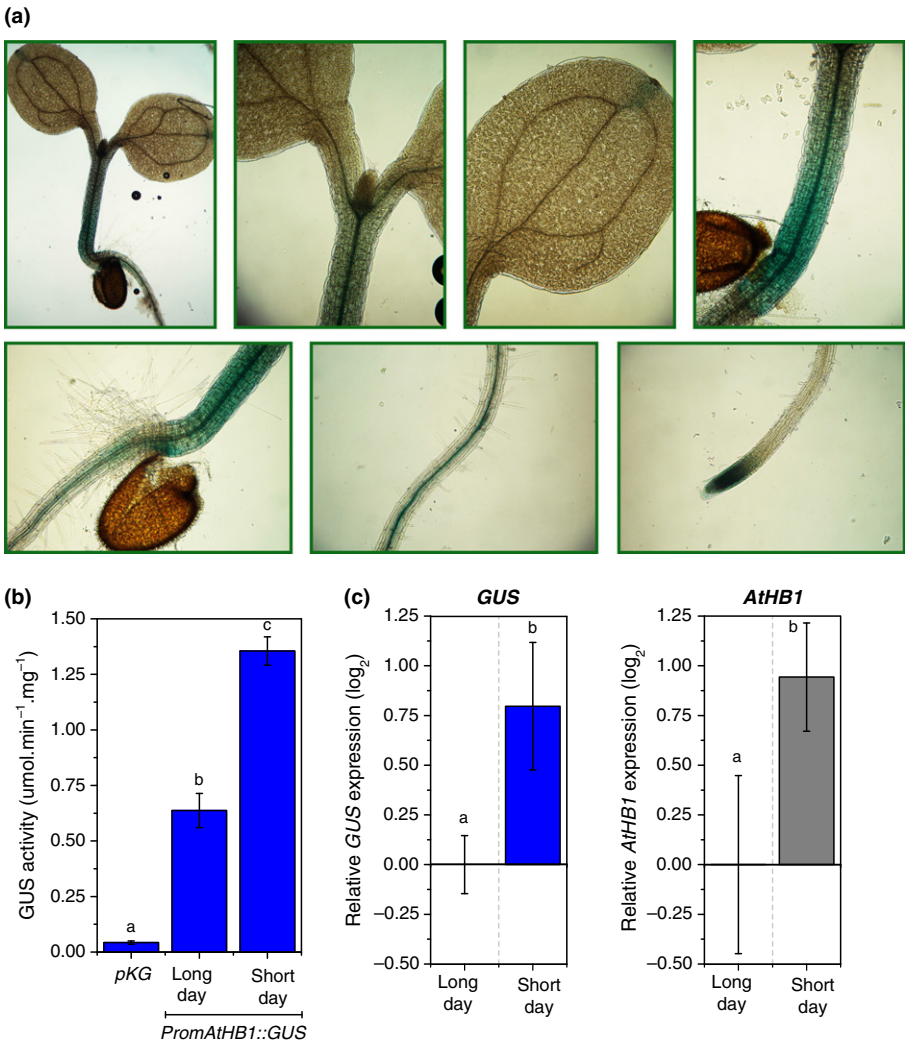

Fig. 1 *Arabidopsis thaliana HomeoBox 1* (*AtHB1*) is expressed in hypocotyls, root vasculatures and tips and induced under short-day conditions in *Arabidopsis thaliana* seedlings. (a) *AtHB1* expression pattern analyzed by histochemical detection of β-glucuronidase (GUS) enzymatic activity in 4-d-old *PromAtHB1::GUS* long-day-grown seedlings. (b) Analysis of GUS activity in protein extracts from 4-d-old seedlings of *PromAtHB1::GUS* or *promoterless::GUS* (pKG, used as a negative control) genotypes grown in long-day or short-day conditions. (c) Transcript levels of *GUS* (left panel) and *AtHB1* (right panel) quantified by quantitative real-time PCR using RNAs isolated from *PromAtHB1::GUS* seedlings grown in short-day or long-day regimes. All the values were normalized with that measured in long-day conditions using the $\Delta\Delta C_t$ method. *Actin* transcripts (*ACTIN2* and *ACTIN8*) were used as a reference. Error bars represent the ± SD of three independent biological replicates. Analysis of variance (ANOVA) was performed, and different letters denote significant differences with a Tukey *post hoc* test at $P < 0.05$.

elongation in response to dark. According to ChIP-chip experiments, PIF1 (also known as PIL5) was able to recognize and bind the *AtHB1* promoter. However, PIF1 did not regulate *AtHB1* expression, at least in imbibed seeds (Oh *et al.*, 2009). Because PIF1 and AtHB1 are involved in the control of hypocotyl growth, we wondered whether PIF1 could regulate *AtHB1* expression in seedlings grown in a long-day or short-day regime. Two *pif1* T-DNA insertional mutants (previously named *pil5-2* and *pif1-2*) were ordered; homozygous lines were obtained and germinated in short- or long-day photoperiods for 4 d. Seedlings were then harvested 2 h before the end of the night, and the expression levels of *AtHB1* were quantified by RT-qPCR. As shown in Fig. 3(a), both *pil5-2* and *pif1-2* mutants showed lower *AtHB1* transcript abundances than controls, in both short- and long-day conditions. Considering the photoperiodic control of hypocotyl elongation and the expression kinetics described for the PIFs (Breton & Kay, 2007; Nozue *et al.*, 2007), which present a maximum at the end of the night, the abundance of *AtHB1* transcripts, as a positive regulator of growth and a putative target of PIF, should also increase at the end of the night. Thus, the expression of *AtHB1* in WT and *pif1-2* mutant seedlings was evaluated throughout the day and night (Fig. 3b). The results revealed that *AtHB1* transcript abundance was lower in *pif1-2* mutants than in WT throughout the night. However, *AtHB1* transcripts peaked 2 h before the end of the night in the WT background, whereas in the *pif1-2* mutants, the maximum presented a delay of *c.* 2 h compared with the WT (Fig. 3b).

As a second strategy to investigate the putative regulation of *AtHB1* by PIF1, transient transformation of *N. benthamiana*

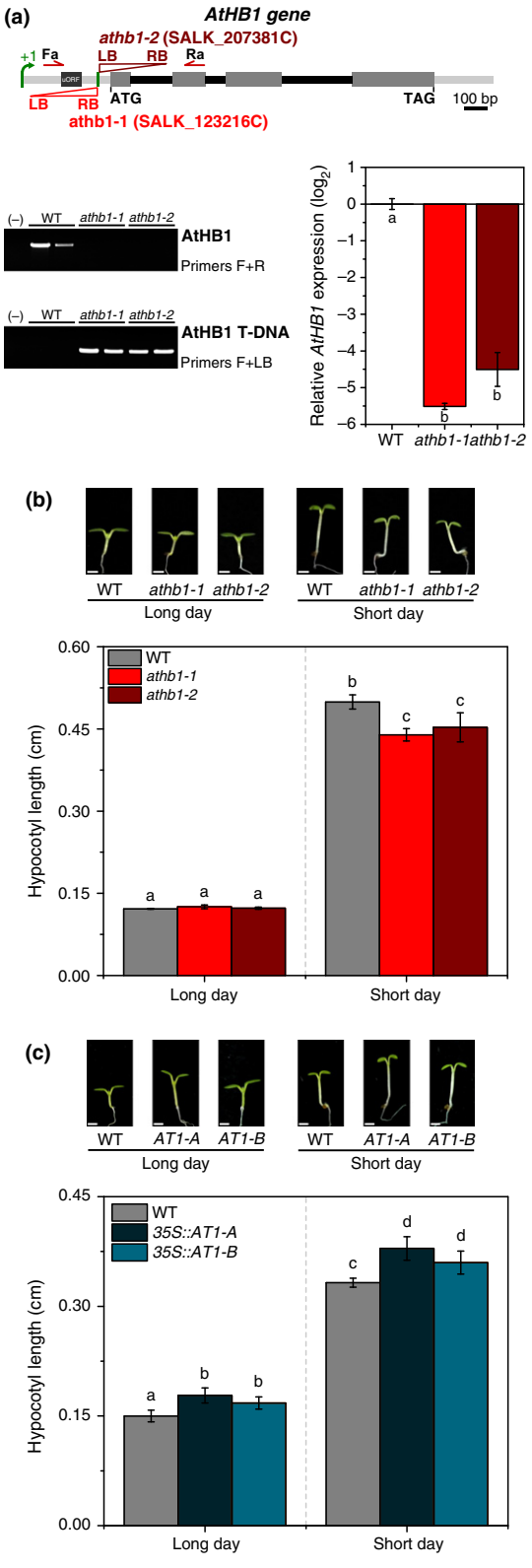

Fig. 2 *Arabidopsis thaliana* HomeoBox 1 (AtHB1) positively regulates *A. thaliana* hypocotyl elongation. (a) Upper panel, schematic representation of the *AtHB1* gene structure. Exons are shown in dark gray, introns in black, and untranslated regions in light gray. The triangles indicate T-DNA insertions in the *athb1-1* and *athb1-2* mutant lines; red arrows indicate the oligonucleotides (Fa, Ra and LB) used in the PCR assays. Lower left panel, genotyping performed using PCR on genomic DNAs from wild type (WT), *athb1-1* and *athb1-2* plants. Right panel, *AtHB1* transcript levels in WT, *athb1-1* and *athb1-2* 4-d-old seedlings grown under long-day conditions. All the values were normalized with that of WT using the $\Delta\Delta C_t$ method. *Actin* transcripts (*ACTIN2* and *ACTIN8*) were used as a reference. (b) Upper panel, illustrative photographs of WT, *athb1-1* and *athb1-2* hypocotyls grown under short-day conditions. Lower panel, quantification of hypocotyl length of WT, *athb1-1* and *athb1-2* seedlings grown under long- or short-day conditions for 4 d. (c) Upper panel, illustrative photographs of WT and two independent transgenic *35S::AtHB1* (*35S::AtHB1-A* and *35S::AtHB1-B*) hypocotyls of seedlings grown under long-day or short-day regimes. Lower panel, quantification of hypocotyl length of WT and *35S::AtHB1-A* and *35S::AtHB1-B* lines grown under long- or short-day conditions for 4 d. Error bars represent the ± SD of three independent biological replicates for each genotype. Analysis of variance (ANOVA) was performed, and different letters denote significant differences with a Tukey *post hoc* test at $P < 0.05$. Bars, 1 mm.

leaves was performed with *A. tumefaciens* carrying the *PromAtHB1::GUS* construct together with constructs in which the *35S CaMV* promoter was fused to *PIF1* or *mGFP* cDNA (*35S::PIF1* and *35S::mGFP*, respectively). The empty vector *pKGWFS7* carrying a promoterless *GUS* was used as a negative control. After infiltration, tobacco plants were grown in long- or short-day conditions, and GUS enzymatic activity was quantified 2 d later. Enzymatic activity from *PromAtHB1::GUS* plus *35S::mGFP* cotransformed leaves was quantified, indicating that the *AtHB1* promoter region directed *GUS* expression in these tobacco tissues. Additionally, these observations helped to corroborate that *AtHB1* expression was up-regulated in a short-day regime compared with the long-day conditions (Fig. 3c). Furthermore, *PromAtHB1::GUS* plus *35S::PIF1* cotransformed leaves showed approximately three-fold higher GUS activity than leaves cotransformed with *PromAtHB1::GUS* plus *35S::mGFP* (Fig. 3c), under both short and long photoperiod conditions. The results indicated that the ectopic expression of *PIF1* induces the *AtHB1* promoter, at least in the tobacco heterologous transient system.

Phenotypic analyses of 4-d-old WT, *athb1-1*, and two *PIF1* mutant (*pil5-2* and *pif1-2*) seedlings showed that *pif1* mutants exhibited shorter hypocotyls than WT when grown in short-day conditions, as observed for *athb1-1* mutants (Fig. 3d). Under a long-day regime, no differences were detected. Taken together, these results suggested that AtHB1 might be a PIF1 target involved in the regulation of hypocotyl growth.

To investigate whether *AtHB1* is regulated by other PIF proteins, its expression was quantified in single (*pif1-2*, *pif3-7*, *pif4-2* and *pif5-3*) and quadruple *pif* mutant (*pifq*) seedlings grown under short-day conditions. The results showed that *pif1-2*, *pif5-3* and *pifq* presented lower *AtHB1* transcript levels than WT plants, whereas *pif3-7* and *pif4-2* showed no significant differences (Fig. S2a). Furthermore, phenotypic analyses of 4-d-old WT and mutant seedlings grown in darkness, long-day and short-day conditions indicated that the *athb1-1* hypocotyl phenotype was similar to that observed in *pil5-2*, *pif1-2* and *pif5-3* single mutants (Fig. S2). These results suggested that both PIF1 and PIF5 can regulate *AtHB1* expression. Because PIF1 recognizes the *AtHB1* promoter region (Oh *et al.*, 2009), we continued studying this bHLH TF.

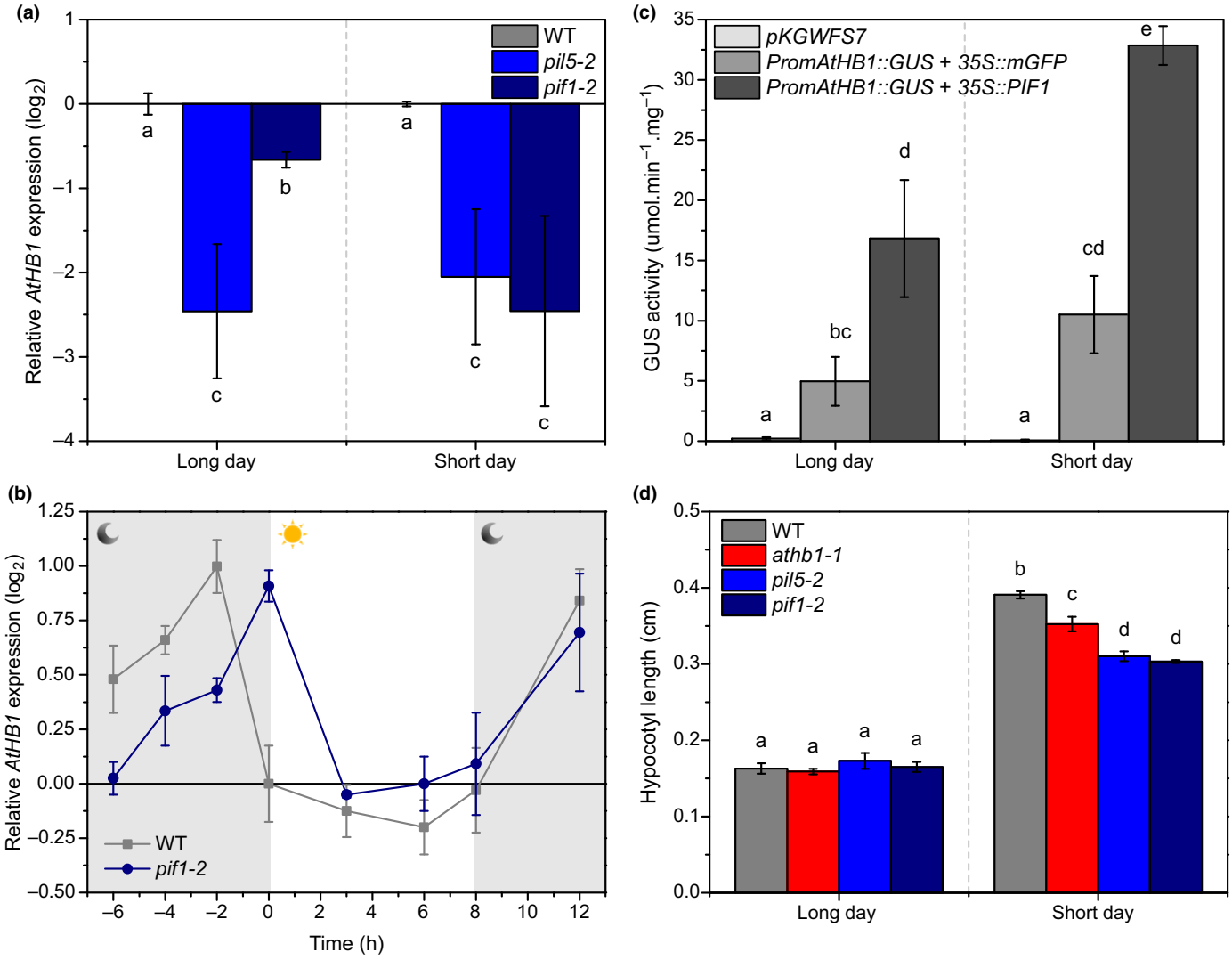

Fig. 3 PHYTOCHROME-INTERACTING FACTOR 1 (PIF1) induces *Arabidopsis thaliana HomeoBox 1* (*AtHB1*) expression. (a) Transcript levels of *AtHB1* in *A. thaliana* wild type (WT) and *PIF1* mutant (*pil5-2* and *pif1-2*) 4-d-old seedlings grown under either long- or short-day conditions. All the values were normalized with that of WT using the $\Delta\Delta C_t$ method. (b) Diurnal expression profile of *AtHB1* in WT and *pif1-2* mutant 4-d-old seedlings grown under short-day conditions. Expression levels were normalized with that of WT at 0 h using the $\Delta\Delta C_t$ method and *Actin* transcripts (*ACTIN2* and *ACTIN8*) as a reference. (c) β-glucuronidase (GUS) activity evaluated by fluorometry in protein extracts from 2-wk-old *Nicotiana benthamiana* transiently transformed leaves with *Agrobacterium tumefaciens* carrying *PromAtHB1::GUS* or *promoterless::GUS* (*pKGWFS7*) combined with *35S::mGFP* or *35S::PIF1*. (d) Quantification of hypocotyl lengths in 4-d-old WT, *athb1-1*, *pil5-2* and *pif1-2* long-day- or short-day-grown seedlings. Error bars represent the ± SD of (c) five independent biological replicates and (a, b, d) three independent biological replicates. Analysis of variance (ANOVA) was performed, and different letters denote significant differences with a Tukey *post hoc* test at $P < 0.05$.

To determine whether PIF1 acts upstream of AtHB1 to control hypocotyl elongation, WT and *athb1-1* plants were transformed with *35S::PIF1*. Three independent lines for each genotype (named A–C in the WT background and D–F in the *athb1-1* background) were obtained, and the *PIF1* expression levels were analyzed (Fig. 4a,b). Hypocotyl lengths of 4-d-old seedlings grown under long- or short-day conditions were measured and compared with the corresponding untransformed background. Under both photoperiodic regimes, *35S::PIF1* seedlings in a WT background exhibited longer hypocotyls than WT controls; however, in the *athb1-1* background, no significant differences were observed compared with the controls (Fig. 4c,d), indicating that AtHB1 is necessary for PIF1-mediated hypocotyl growth regulation.

Next, *pif1-2* mutants were transformed with *35S::AtHB1*, and three independent lines (named A–C) were obtained and grown under two different photoperiodic conditions. Four-day-old seedlings were analyzed, and the results indicated that *AtHB1* overexpression promoted hypocotyl growth even in the absence of a functional PIF1 in a photoperiod-independent manner (Fig. 4e).

Double *athb1-2/pif1-2* mutants were obtained by crossing *athb1-2* and *pif1-2* plants. A schematic representation of *AtHB1* and *PIF1* gene structures as well as the PCR performed to check

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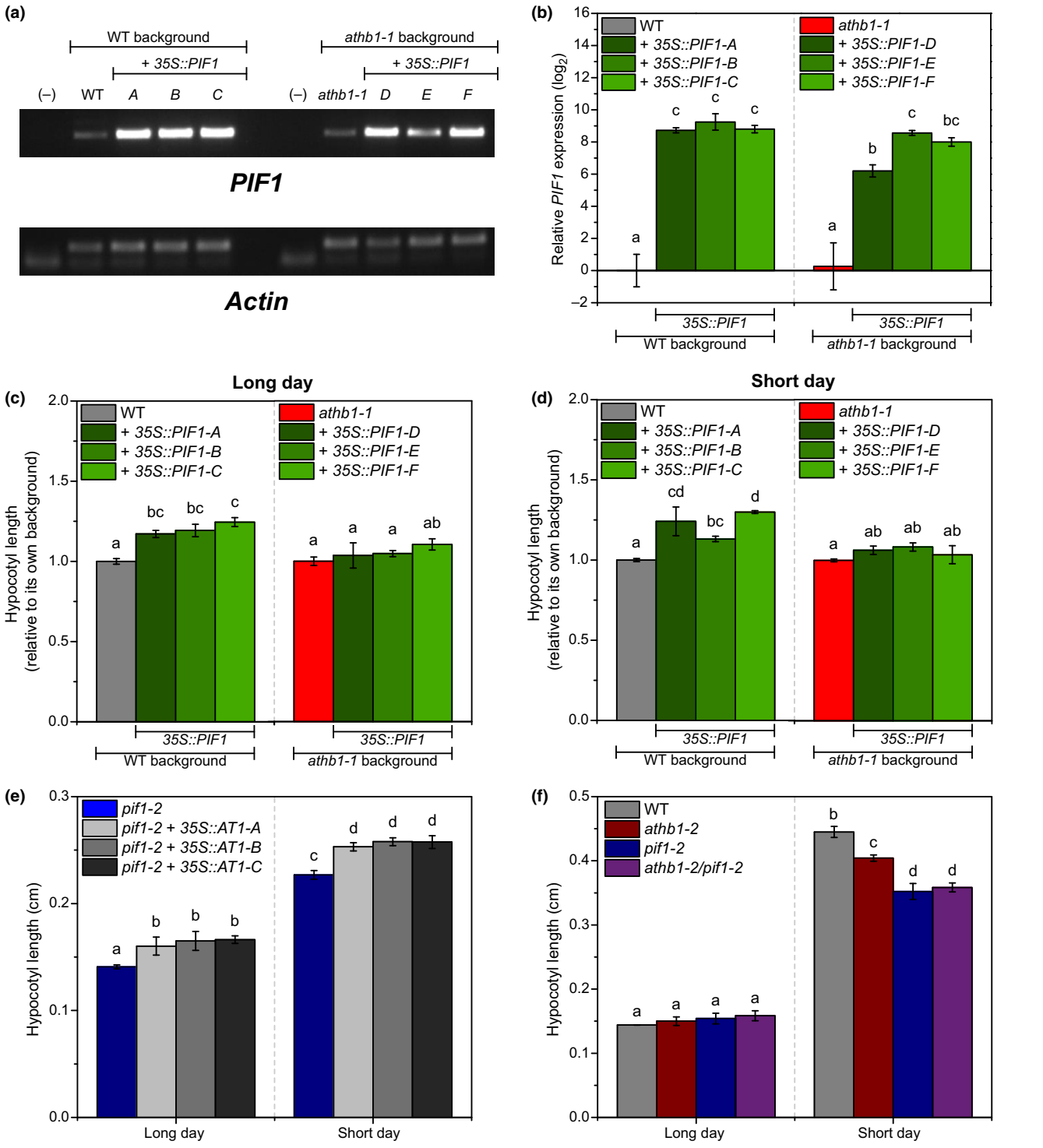

Fig. 4 *Arabidopsis thaliana* HomeoBox 1 (AtHB1) acts downstream of PHYTOCHROME-INTERACTING FACTOR 1 (PIF1) in hypocotyl length regulation. (a) Reverse transcription-PCR analysis showing the expression levels of *PIF1* in *A. thaliana* wild type (WT), *athb1-1* and three independent *35S::PIF1* transgenic lines in (A–C) a WT or (D–F) *athb1-1* background. RNA was extracted from 4-d-old seedlings grown under short-day conditions. (b) Transcript levels of *PIF1* in WT, *athb1-1* and *35S::PIF1* seedlings grown under a short-day regime. Values were normalized with that of WT using the $\Delta\Delta C_t$ method. *Actin* transcripts (*ACTIN2* and *ACTIN8*) were used as a reference. (c, d) Quantification of 4-d-old hypocotyl lengths of WT, *athb1-1* and *35S::PIF1* transgenic lines in WT or *athb1-1* backgrounds grown under (c) long- or (d) short-day conditions. Values are expressed as a proportion of that measured in each untransformed genotype, given an arbitrary unitary value. (e) Quantification of hypocotyl lengths of 4-d-old *pif1-2* mutants and three independent *35S::AtHB1* transgenic lines (*35S::ATHB1-A*, *35S::ATHB1-B* and *35S::ATHB1-C*) obtained on a *pif1-2* background. (f) Quantification of hypocotyl lengths of 4-d-old WT, *athb1-2*, *pif1-2* and *athb1-2/pif1-2* simple and double mutant seedlings. Error bars represent the ± SD of three independent biological replicates for each genotype. Analysis of variance (ANOVA) was performed, and different letters denote significant differences with a Tukey *post hoc* test at $P < 0.05$.

the mutants are shown in Fig. S3. Hypocotyls of the double mutant *athb1-2/pif1-2* were shorter than those of WT and *athb1-2* seedlings and similar to those of the *pif1-2* single mutant seedlings in short-day conditions (Fig. 4f). Under a long-day photoperiod, no significant differences were detected between the different genotypes.

Taken together, these results indicated that PIF1 regulates *AtHB1* expression, and both factors are involved in a pathway controlling hypocotyl elongation.

AtHB1 regulates hypocotyl growth by controlling the expression of genes involved in cellular elongation

To understand how AtHB1 affects hypocotyl growth in *A. thaliana*, an RNA-Seq transcriptomic analysis was performed using RNA from 4-d-old WT and *athb1-1* mutant seedlings grown in short-day conditions. A set of 426 genes was detected as differentially regulated with a fold change of at least two. However, only 22 genes were up-regulated and 24 genes were down-regulated with statistically significant read counts (Tables S2,S3). Among the differentially expressed genes, 15 genes exhibited elements in their promoter regions that partially matched the pseudopalindromic sequence, CAAT(A/T)ATTG, bound *in vitro* by the HD-Zip I TFs with maximal affinity (Sessa *et al.*, 1993; Palena *et al.*, 1999).

Some of the AtHB1-regulated genes have been shown to modulate cell elongation, particularly cell wall composition and elongation, or encode proteins that serve as a source of carbon, nitrogen, and sulfur for early seedling growth. These genes were *PATATIN-LIKE PROTEIN 4* (*PLP4*; At4g37050; Rietz *et al.*, 2010; Li *et al.*, 2011), *XYLOGLUCAN ENDOTRANS GLUCOSYLASE/HYDROLASE 26* (*XTH26*; At4g28850; Maris *et al.*, 2009) and *GALACTURONOSYLTRANSFERASE 12* (At5g54690, also known as *IRX8*; Persson *et al.*, 2007), which were up-regulated in *athb1-1* seedlings. In contrast, the 12S seed storage protein *CRUCIFERIN3* gene (*CRU3*; At4g28520) was down-regulated in the mutant plants.

The RNA-Seq results were validated by RT-qPCR for some of the differentially expressed genes (Fig. 5a). Four of these genes were analyzed in *AtHB1* overexpressor seedlings; three of the genes showed the opposite behavior to that observed in mutant plants, whereas the fourth did not change compared with WT (Fig. 5b). Furthermore, three genes regulated in *athb1* mutants, *ENHANCER OF CELL INVASION NO.10, CYTOCHROME P450 FAMILY 97 SUBFAMILY B POLYPEPTIDE 3* and *IRON-REGULATED TRANSPORTER 1*, were tested for their expression levels in *pif1-2*, *athb1-2* and *athb1-2/pif1-2* mutant plants. The results, shown in Fig. 5(c), indicated that, in the three simple mutants as well as in the double mutant, the three putative AtHB1 targets were regulated in the same manner, reinforcing the relationship between AtHB1 and PIF1.

A second strategy was applied to corroborate the regulation exerted by AtHB1, especially regulation of the genes involved in cell elongation. The promoter regions of *PLP4* and *IRT1* were cloned to direct the expression of *GUS* and were used to cotransform tobacco leaves together with *35S::AtHB1*. The results shown in Fig. S4 indicated that these two genes were differentially regulated by the presence of AtHB1.

Thus, AtHB1, which acts downstream of PIF1, seems to promote hypocotyl growth by regulating cell growth-related gene expression.

Discussion

HD-Zip I transcription factors were first identified in *A. thaliana* (Ruberti *et al.*, 1991; Schena & Davis, 1992) and, since then, members of this subfamily have been characterized in a wide range of plant species and are associated with developmental processes related to environmental changes. In a few cases, the characterization of mutant plants displaying abnormal phenotypes has indicated that these TFs also participate in developmental processes not necessarily associated with stress conditions. In this regard, *A. thaliana LATE MERISTEM IDENTITY 1* (Saddic *et al.*, 2006), garden pea (*Pisum sativum*) *Tendril-less* (Hofer *et al.*, 2009) and barley (*Hordeum vulgare*) *SIX-ROWED SPIKE 1* (Komatsuda *et al.*, 2007) are good examples of key players in developmental events.

This work has shown that *AtHB1* is mainly expressed in hypocotyl and root tissues and is involved in a developmental process connected with illumination conditions. More precisely, this gene promotes hypocotyl elongation, especially in short-day conditions. *athb1* mutants displayed shorter hypocotyls than WT plants under short-day conditions, whereas *AtHB1* overexpressors presented the opposite phenotype (longer hypocotyls) in both short- and long-day regimes.

AtHB1 has been previously described as conferring a constitutive de-etiolated phenotype when overexpressed in tobacco plants grown in darkness (Aoyama *et al.*, 1995). By contrast, *A. thaliana 35S::AtHB1* did not show such a de-etiolated phenotype, and *athb1* mutants did not show the opposite phenotype. These observations suggest that the effect of *AtHB1* on tobacco gene regulatory networks that lead to the constitutive de-etiolated phenotype could be an artifact caused by heterologous expression. Although these observations did not indicate a function for AtHB1 in etiolated seedlings, the possibility that it may have a function in these conditions cannot be excluded. The lack of a phenotype in both knock-down *athb1* mutants could be explained by the putative action of another HD-Zip I. Moreover, the overexpression of a positive regulator of growth, such as AtHB1, does not always produce differential phenotypes in skotomorphogenic seedlings. In support of this idea, four *pif* genes must be mutated to obtain a clear differential phenotype (Shin *et al.*, 2009). Furthermore, PIF1 overexpression in a *pif1-2* background did not result in an elongated phenotype when seedlings were grown in the dark (Shen *et al.*, 2005, 2008). However, no AtHB1 paralogs have been identified thus far, making the redundancy hypothesis rather weak. Notably, the experiments performed with overexpressors in tobacco provided the first evidence of the involvement of *AtHB1* in light responses. Because light and water availability are the most important environmental factors affecting plant development and HD-Zip I TFs are related to the response to environmental changes, these TFs probably act

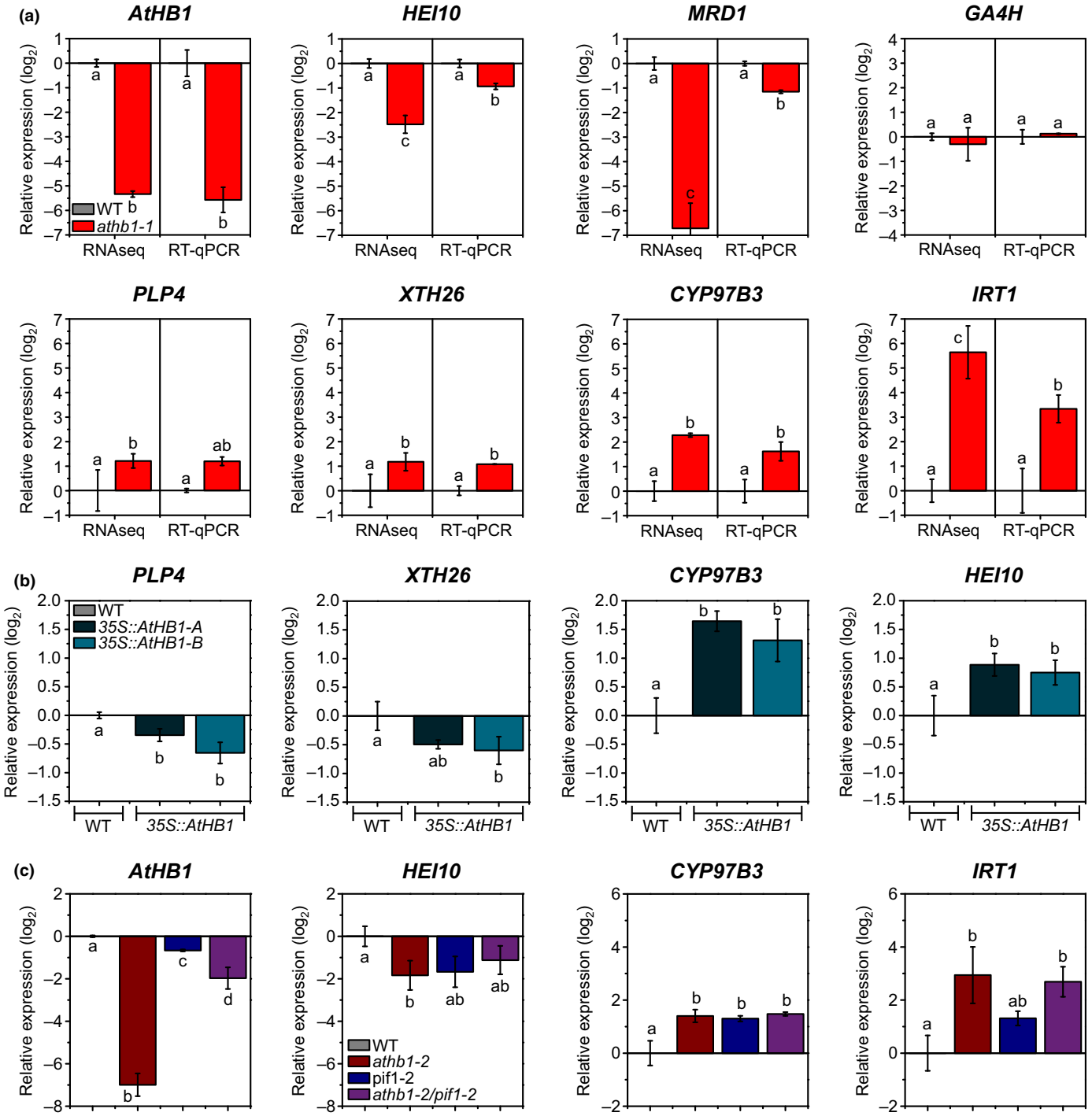

Fig. 5 *Arabidopsis thaliana* HomeoBox 1 (AtHB1) modulates hypocotyl growth by regulating genes involved in cell elongation. (a) Transcript levels of several selected *A. thaliana* genes detected as differentially regulated by RNA sequencing. Validation was performed using quantitative real-time PCR with RNAs isolated from wild type (WT) and *athb1-1* 4-d-old seedlings grown under short-day conditions. (b) Transcript levels of *PATATIN-LIKE PROTEIN 4* (*PLP4*; At4g37050), *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 26* (*XTH26*; At4g28850), *ENHANCER OF CELL INVASION NO.10* (*HEI10*; At1g53490) and *IRON-REGULATED TRANSPORTER 1* (*IRT1*; At4g19690) in WT and transgenic *35S::AtHB1-A* and *35S::AtHB1-B* seedlings grown as detailed under short-day conditions. (c) Transcript levels of *AtHB1*, *HEI10*, *CYTOCHROME P450 FAMILY 97 SUBFAMILY B POLYPEPTIDE 3* (*CYP97B3*; At4g15110) and *IRT1* in WT, *athb1-2*, *pif1-2* and *athb1-2/pif1-2* mutant seedlings grown under short-day conditions. All the values were normalized with that of the WT plant using the $\Delta\Delta C_t$ method. *Actin* transcripts (*ACTIN2* and *ACTIN8*) were used as a reference. Error bars represent the ± SD of three independent biological replicates. Analysis of variance (ANOVA) was performed, and different letters denote significant differences with a Tukey *post hoc* test at $P < 0.05$. *MRD1*, *MTO 1 RESPONDING DOWN 1* (At1g53480); *GA4H*, *GIBBERELLIN 3-OXIDASE 2* (At1g80340).

as a link between environmental sensing and plant development. Moreover, other members of the HD-Zip I family were previously related to light responses and include AtHB16 and AtHB23, which belong to clades II and V, respectively (Arce *et al.*, 2011). Transgenic *A. thaliana* plants overexpressing *AtHB16* or *antiAtHB16* showed longer and shorter hypocotyls, respectively, than WT seedlings grown under blue light (Wang *et al.*, 2003). Furthermore, *AtHB23* conferred the opposite phenotype (shorter hypocotyls) in response to red light and acted as a component of the phyB-mediated signaling pathway. The phenotype of *athb23* mutants includes longer hypocotyls, smaller cotyledons and a lower germination rate than WT plants when seedlings are grown in continuous red light conditions (Choi *et al.*, 2014). Although reports have related HD-Zip I TFs to illumination conditions, the function of AtHB1 was unknown, and the experiments presented here indicate a role in hypocotyl growth regulation.

Previous reports indicated that PIF1 (also called PIL5), belonging to bHLH subfamily 15, repressed light-induced seed germination and chlorophyll biosynthesis (Huq *et al.*, 2004; Oh *et al.*, 2004). Moreover, PIF1 promotes hypocotyl elongation under short-day conditions and interacts with VQ MOTIF-CONTAINING PROTEIN29 and *CONSTITUTIVE PHOTOMORPHOGENIC 1-SUPPRESSOR OF PHYA-105* complexes to repress photomorphogenesis in *A. thaliana* (Li *et al.*, 2014; Soy *et al.*, 2014; Xu *et al.*, 2014). Nevertheless, the signaling pathway through which PIF1 regulates hypocotyl growth is not well characterized. Here, we showed that PIF1 induces *AtHB1* expression, and this HD-Zip I TF promotes hypocotyl elongation. *AtHB1* overexpression in a *pif1-2* background induced hypocotyl elongation, whereas *PIF1* overexpression was not able to rescue the *athb1* phenotype, indicating that PIF1 acts upstream of *AtHB1*. Furthermore, *athb1-2/pif1-2* double mutants showed the same phenotype as *pif1-2* single mutants in all conditions tested, reinforcing this idea. Consistent with these observations, a ChIP-chip assay performed with imbibed seeds, and more recently a ChIP-Seq analysis with etiolated seedlings, showed that the promoter region of *AtHB1* is one of the PIF1 binding sites. Nevertheless, no differential expression of *AtHB1* was detected in *pif1* mutants compared with WT at those developmental stages (Oh *et al.*, 2009; Pfeiffer *et al.*, 2014). Moreover, although previous reports have indicated that PIF1 recognizes the *AtHB1* promoter, such binding was not confirmed in these studies and could be happening in other tissues/organs or growth conditions. Additional experiments should be performed to determine whether the induction of *AtHB1* expression by PIF1 is the result of direct binding (Oh *et al.*, 2009; Pfeiffer *et al.*, 2014). We cannot rule out the possibility that PIF1, together with other PIF proteins, also regulates hypocotyl elongation through AtHB1-independent pathways, which would explain why *pif1* mutant plants are shorter than *athb1* mutants.

Hypocotyl growth is a complex event in which PIF1, AtHB1 and other proteins participate. Otherwise, a more pronounced phenotype (inability to germinate or grow) would be expected in athb1 or *athb1-2*/*pif1-2* double mutants if hypocotyl growth would only depend on AtHB1 or PIF1. In this regard, *AtHB2*, which belongs to the HD-Zip II TF family, has been reported to be a gene acting downstream of PIFs in hypocotyl growth regulation. This gene is directly induced by PIF4 and PIF5 to regulate the photoperiodic control of hypocotyl elongation (Kunihiro *et al.*, 2011).

As expected for a positive regulator of growth, *AtHB1* transcript abundance was at a maximum 2 h before the end of the night when WT seedlings were grown under short-day conditions. Furthermore, the expression of *AtHB1* was lower in *pif1-2* than in WT during the night and peaked at the end of the night, although 2 h later than in WT. This delay could be explained by the action of unknown factors, deregulated in *pif1-2* mutants. It is tempting to speculate that, in the absence of PIF1, another PIF might be able to replace it to regulate *AtHB1* expression; for example, PIF5 is an interesting candidate as its expression has been reported to peak at the end of the night (Niwa *et al.*, 2009; Soy *et al.*, 2014). Interestingly, *AtHB1* transcript levels were down-regulated in *pif5-3* mutant seedlings (Fig. S2a).

To understand which genes were acting downstream of AtHB1 in promoting hypocotyl growth in response to illumination conditions, we performed a comparative transcriptome analysis using 4-d-old *athb1-1* seedlings. The *AtHB1* mutation affected the expression of genes involved in cell wall composition and elongation. Among these genes, *PLP4*, also known as *AtPLAIVC*, is a patatin-related phospholipase A that hydrolyzes phospholipids and galactolipids (Rietz *et al.*, 2010; Li *et al.*, 2011). Changes in *PLP4* expression caused lipid composition alterations, resulting

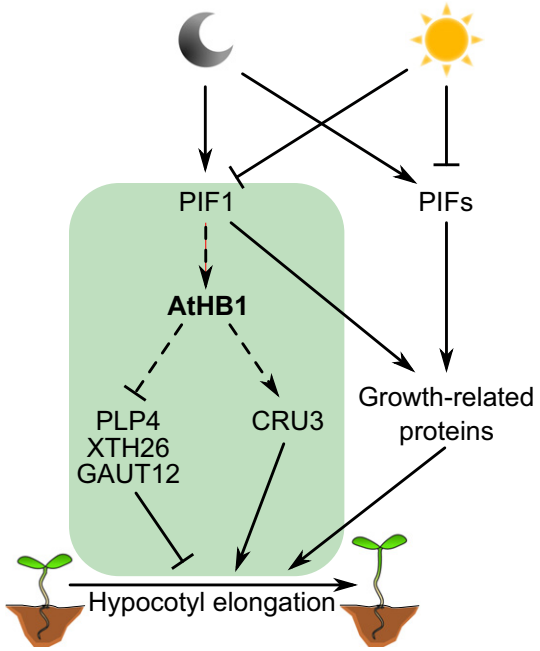

Fig. 6 Proposed model for the role of *Arabidopsis thaliana* HomeoBox 1 (AtHB1) in hypocotyl elongation in *A. thaliana*. During the night, PHYTOCHROME-INTERACTING FACTOR 1 (PIF1) accumulates in the nucleus and induces *AtHB1* expression. This expression contributes to the regulation of several genes involved in cell wall composition and cell elongation to promote hypocotyl growth. PIF1 and other PIFs (such as PIF3, PIF4 and PIF5) also regulate the expression of other growth-related proteins that are involved in hypocotyl cell elongation in AtHB1-independent pathways.

in cell elongation modifications; *plp4* mutant seedlings showed longer hypocotyls than WT plants, whereas *PLP4* overexpressors had the opposite phenotype (Rietz *et al.*, 2010; Li *et al.*, 2011). This result is consistent with our results because AtHB1 repressed *PLP4* expression according to the *athb1-1* transcriptome analysis. However, some results obtained in the RNA-Seq analysis were harder to interpret. For example, the up-regulation of *XTH26* in *athb1-1* mutants was difficult to interpret. This gene encodes an enzyme described as playing a role in the loosening of cell walls (Maris *et al.*, 2009). Another AtHB1-down-regulated gene is *GAUT12* (also known as *IRX8*), which belongs to the *GAUT1*-related gene family, a subgroup of glycosyltransferase family 8 (Brown *et al.*, 2005; Persson *et al.*, 2005). This gene has been suggested to positively affect secondary cell wall integrity (Persson *et al.*, 2007).

Taken together, the results presented here led us to propose the model presented in Fig. 6, which schematizes how AtHB1 regulates hypocotyl elongation in *A. thaliana*. According to the experimental data obtained in this work, PIF1 regulates the hypocotyl growth pathway by the direct or indirect induction of *AtHB1* expression, and this HD-Zip I protein represses *PLP4*, *XTH26* and *GAUT12* expression and induces *CRU3* and other genes that play a role in this important developmental event.

In summary, *AtHB1* is expressed in hypocotyls, root vasculatures and tips. This TF is positively regulated by PIF1 to promote hypocotyl elongation, especially in response to short photoperiod conditions, and *AtHB1* regulates genes involved in cell elongation. Hence, *AtHB1* represents a new component in the PIF1-mediated regulation of hypocotyl growth.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 AtHB1 does not affect hypocotyl elongation in skotomorphogenesis in *Arabidopsis thaliana*.

Fig. S2 *Arabidopsis thaliana* hypocotyl length is affected in several *pif* mutants and *athb1-1* seedlings.

Fig. S3 PCR genotyping of *athb1-2*|*pif1-2* double mutant plants.

Fig. S4 AtHB1 inhibits GUS expression directed by *IRT1* and *PLP4* promoters in transiently transformed tobacco leaves.

Table S1 Oligonucleotides used for cloning and RT-qPCR assays

Table S2 List of genes induced in *athb1-1* mutant seedlings

Table S3 List of genes repressed in *athb1-1* mutant seedlings

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