A non-DNA-binding activity for the ATHB4 transcription factor in the control of vegetation proximity

Marçal Gallemi1, Maria Jose Molina-Contreras1*, Sandi Paulišić1*, Mercè Salla-Martret1, Céline Sorin1, Marta Godoy2, Jose Manuel Franco-Zorrilla2, Roberto Solano2 and Jaime F. Martínez-García1,3

1Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra, Barcelona 08193, Spain; 2National Centre for Biotechnology (CNB), CSIC, Campus University Autónoma, Madrid 28049, Spain; 3Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona 08010, Spain

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Summary
- In plants, perception of vegetation proximity by phytochrome photoreceptors activates a transcriptional network that implements a set of responses to adapt to plant competition, including elongation of stems or hypocotyls. In Arabidopsis thaliana, the homeodomain-leucine zipper (HD-Zip) transcription factor ARABIDOPSIS THALIANA HOMEBOX 4 (ATHB4) regulates this and other responses, such as leaf polarity.
- To better understand the shade regulatory transcriptional network, we have carried out structure–function analyses of ATHB4 by overexpressing a series of truncated and mutated forms and analyzing three different responses: hypocotyl response to shade, transcriptional activity and leaf polarity.
- Our results indicated that ATHB4 has two physically separated molecular activities: that performed by HD-Zip, which is involved in binding to DNA-regulatory elements, and that performed by the ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR-associated amphiphilic repression (EAR)-containing N-terminal region, which is involved in protein–protein interaction. Whereas both activities are required to regulate leaf polarity, DNA-binding activity is not required for the regulation of the seedling responses to plant proximity, which indicates that ATHB4 works as a transcriptional cofactor in the regulation of this response.
- These findings suggest that transcription factors might employ alternative mechanisms of action to regulate different developmental processes.

Introduction
Transcriptional control is at the core of how living organisms develop and adapt to their surroundings. In plants, deciphering transcriptional mechanisms that control gene expression is fundamental for understanding developmental plasticity. In crowded vegetation, light for photosynthesis might become limiting: in various species, growing in a high plant density environment activates the shade avoidance syndrome (SAS), a set of strategic responses to adjust growth that has a strong impact on plant development. SAS includes modulation of hypocotyl and stem elongation, leaf expansion, flowering time or levels of photosynthetic pigments. Plant proximity or shade is perceived as a light signal: the surrounding canopy, which absorbs red light (R) and reflects far-red light (FR), reduces the R to FR ratio (R:FR), a signal perceived by the phytochrome photoreceptors. Phytochromes exist in two photoconvertible forms, an inactive photo-equilibrium towards the active Pfr, whereas the low R:FR signal from crowded vegetation displaces the phytochrome photoreceptors with PHYTOCHROME INTERACTING FACTORS (PIFs), transcriptional regulators of the basic helix–loop–helix (bHLH) family, modulates the expression of PHYTOCHROME RAPIDLY REGULATED (PAR) genes. Shade-induced changes in PAR expression eventually activate the SAS (Martinez-Garcia et al., 2010; Leivar & Quail, 2011; Casal, 2013; Bou-Torrent et al., 2015).

Molecular analyses have revealed that PIFs positively regulate some PAR genes expressed during shade-induced hypocotyl elongation, such as ARABIDOPSIS THALIANA HOMEBOX 4 / HOMEBOX ARABIDOPSIS THALIANA 4 (ATHB2/HAT4, hereafter ATHB2), HYPOCOTYL IN FAR RED 1 (HFR1) and PIF3-LIKE 1 (PILI) (Lorrain et al., 2008; Li et al., 2012). Genetic analyses have also demonstrated the involvement of a diversity of non-PIF factors in the regulation of the SAS hypocotyl response that, nonetheless, also participate in the modulation of PAR gene expression, such as homeodomain-leucine zipper (HD-Zip) class III, CONSTITUTIVE SHADE AVOIDANCE 1 and DRACULA2 (a nucleopore complex

*These authors contributed equally to this work.

Key words: Arabidopsis thaliana, ARABIDOPSIS THALIANA HOMEBOX 4 (ATHB4), DNA-binding activity, ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR-associated amphiphilic repression (EAR) motif, homeodomain-leucine zipper, shade avoidance syndrome, transcription factors, transcriptional cofactors.

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Research
component) (Faigon-Soverna et al., 2006; Brandt et al., 2012; Gallemi et al., 2016), highlighting the complexity of the transcriptional network that implements the SAS responses. Further genetic studies of this response demonstrated roles for several PAR genes encoding transcriptional regulators of at least three different families: HD-Zip class II (ATHB2, ATHB4, HAT1, HAT2 and HAT3), B-BOX CONTAINING (BBX) and bHLH (e.g. BRASSINOSTEROID-ENHANCED EXPRESSION 1-3 (BEE1), BES1-INTERACTING MYC-LIKE 1-3 (BIMs), HFR1, PAR1, PAR2 and PIL1). BBX and bHLH family members appear to have negative (HFR1, PAR1, PIL1, BBX21 and BBX22) and positive (BEEs, BIMs, BBX24, BBX25, ATHB2, HAT1, HAT2 and HAT3) roles in the regulation of the SAS hypocotyl response (Steindler et al., 1999; Sessa et al., 2005; Roig-Villanova et al., 2007; Sorin et al., 2009; Crocco et al., 2010, 2015; Hortitschek et al., 2012; Cifuentes-Esquivel et al., 2013; Turchi et al., 2015). In the case of the identified members of the HD-Zip II family, their overexpression results in a phenotype in high R : FR that is reminiscent of that displayed by wild-type plants grown in low R : FR, and hence these factors have been proposed as SAS positive regulators (Ciabelli et al., 2008; Turchi et al., 2015). A deeper analyses of ATHB4 activity, however, indicated attenuated hypocotyl elongation in both loss- and gain-of-function mutants grown under low R : FR, which led to the proposal that ATHB4 is, rather than positive, an SAS complex regulator (Sorin et al., 2009).

Proteins of the HD-Zip family have an HD adjacent to the Zip motif, an association that is unique to plants. The HD is responsible for the specific binding to DNA, whereas the Zip acts as a dimerization motif: HD-Zip proteins bind to DNA as dimers and recognize pseudo-palindromic cis elements; the absence of the Zip absolutely abolishes their DNA-binding ability (Ariel et al., 2007). Overexpression of HD-Zip II transcription factors (TFs) has provided evidence that several of them function as negative regulators of gene expression (Steindler et al., 1999; Ohgishi et al., 2001; Sawa et al., 2002; Sorin et al., 2009) in contrast with other HD-Zip proteins, for example those of subfamily III (Brandt et al., 2012; Turchi et al., 2013; Xie et al., 2015), that act as transcriptional activators. Several of the HD-Zip II proteins contain in their conserved N-terminal region an ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR-associated amphipathic repression (EAR) motif, which may be involved in transcriptional repressor activity (Brandt et al., 2014).

To understand how complex transcriptional networks control a process, in addition to identifying the components and elucidating their organization into functional modules, it is necessary to clarify the basic mechanism of transcriptional control of the individual components. In this study, we aimed to address this latter issue in ATHB4, a complex SAS regulatory component whose molecular and biological activity is not well understood (Sorin et al., 2009) and that emerges as a paradigm for understanding other HD-Zip II members. After analyzing its DNA-binding and transcriptional properties, we performed a detailed structure–function analysis of ATHB4 activity by overexpressing truncated and mutated derivatives in Arabidopis. As a result, we were able to establish the mechanistic duality of this transcriptional regulator in modulating different environmental and developmental responses.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh (Arabidopsis) plants were grown in the glasshouse to produce seeds. The line overexpressing ATHB4 fused to the rat glucocorticod receptor domain (GR) (35S:ATHB4-GR.05) (Sorin et al., 2009) and the new transgenic lines presented in this work were all in the Columbia (Col-0) background. Experiments were performed with surface-sterilized seeds sown on Petri dishes containing solid growth medium without sucrose (0.5xGM—) (Roig-Villanova et al., 2006), unless otherwise stated. For gene expression and chromatin immunoprecipitation (ChIP) analyses, seeds were sown on filter paper or nylon membranes placed on top of the 0.5xGM—. After stratification (for 3–5 d), plates were incubated in a growth chamber at 22°C under white light (W), which was provided by four cool-white vertical fluorescent tubes (25 μmol m−2 s−1) of photosynthetically active radiation (PAR; R : FR ratio 2.8–6.3). Simulated shade (W + FR) was generated by enriching W with supplementary FR provided by GreenPower LED module HF far-red (Philips; www.philips.com/horti) (25 μmol m−2 s−1) of photosynthetically active radiation; R : FR ratio 0.06. Fluence rates were measured using a Spectrosense2 meter associated with a four-channel sensor (Skye Instruments Ltd; www.skyeinstruments.com) (Martinez-Garcia et al., 2014).

Measurement of hypocotyl length

The National Institutes of Health IMAGEJ software (Bethesda, MD, USA; http://rsb.info.nih.gov/) was used on digital images to measure hypocotyl length as described previously (Sorin et al., 2009). At least 15 seedlings were used for each treatment and experiments were repeated three to five times; a representative experiment is shown. Statistical analyses of the data (t-test and two-way ANOVA) were performed using GraphPad PRISM v.4.00 for Windows (http://www.graphpad.com/).

Construction of transgenic lines

Details of the generation of the described transgenic lines are given in Supporting Information Methods S1. Sequences of the primer used can be found in Table S1. For each construct, > 10 independent transgenic lines were identified; from those, at least two independent transgenic lines showing detectable levels of transgene expression were selected and characterized, although only a representative line is shown.

Gene expression analysis

For microarray analysis, plant material was prepared and analyzed as indicated in Methods S2. For real-time qPCR analysis, triplicate samples were harvested. Total RNA was isolated from seedlings or...
Results

Overexpression of HD-Zip II family members similarly alters hypocotyl responses to simulated shade

First, we aimed to address whether overexpression of other HD-Zip II family members affected hypocotyl elongation in response to simulated shade (W + FR) in the same way as overexpression of ATHB4. We used available lines constitutively expressing ATHB2 (35S:ATHB2) and HAT2 (35S:HAT2) (Ciarelli et al., 2008), and newly prepared transgenic lines constitutively expressing HAT1 (35S:HAT1) and ATHB4-GFP (35S:ATHB4-GFP) (Methods S1). Nontransformed (Col-0) plants were used as controls. Under continuous W, constitutive overexpression of these HD-Zip II family members promoted hypocotyl elongation compared with wild-type seedlings, as expected. By contrast, under W + FR, hypocotyl length was inhibited in 35S:HAT1 and 35S:ATHB4-GFP seedlings and unaffected in 35S:ATHB2 and 35S:HAT2 seedlings (Fig. S1a–d). These analyses indicate that constitutive overexpression of HD-Zip II family members promotes hypocotyl elongation under W, whereas it had no effect or even inhibited this trait under simulated shade.

We next used an inducible transgenic line constitutively expressing a fusion between ATHB4 and the GR domain of the rat glucocorticoid receptor, which encodes the hormone-binding domain (35S:ATHB4-GR line) (Sorin et al., 2009). In the absence of glucocorticoids, the resulting protein is retained in the cytoplasm; upon the addition of the synthetic glucocorticoid dexamethasone (DEX), the fusion protein translocates to the nucleus, where it acts. When this transgenic line was used, increasing concentrations of DEX resulted in a progressive reduction of the hypocotyl length under W + FR, whereas it did not affect it under W (Fig. S1E), an effect not observed in Col-0 seedlings. When inducible transgenic 35S:HAT2-GR lines were used, DEX-induced HAT2 was also shown to strongly inhibit hypocotyl elongation under W + FR and to be ineffective in promoting hypocotyl elongation under W (Fig. S1F). These results showed that (1) different HD-Zip II family members inhibited hypocotyl elongation under simulated shade and (2) they promoted hypocotyl elongation under W only when constitutively expressed; and that (3) this latter promoting activity was absent in the GR fusions even when DEX was applied. Together, our results indicate that HD-Zip II proteins only promote hypocotyl elongation under limited conditions, and therefore argue against considering them as SAS positive regulators.

ATHB4 acts fundamentally as a transcriptional repressor

To obtain a deeper understanding of ATHB4 function, we identified its target genes on a genome-wide scale using the 35S:ATHB4-GR line (Sorin et al., 2009). To establish (1) a list of putative direct targets of ATHB4 activity, and (2) whether this HD-Zip II regulator acts as a repressor or an activator of gene expression, we performed triplicate microarray experiments (Affymetrix microarray platform; see Methods S2 for a full description) using the 35S:ATHB4-GR line. Transgenic

Accession numbers

Sequence data from this paper can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ATHB2 (At4g16780), ATHB4 (At2g44910), HAT2 (At5g47370) and SMALL AUXIN UPREGULATED 15 (SAUR15) (At4g38850). The full data set is available to download from the NASCarrays database (www.affymetrix.arabidopsis.info; reference: NASCARRAYS-545).
seedlings grown for 7 d under W were treated for 4 h with or without DEX in the absence or presence of the protein synthesis inhibitor cycloheximide (± CHX) (i.e. a total of four treatments; Fig. 1a). In the absence of CHX (−CHX), addition of DEX altered the expression of 433 genes (≥ 1.5-fold), whereas in the presence of CHX (+CHX), addition of DEX altered the expression of 1055 genes (Fig. 1b; Table S3). Comparison of the DEX-regulated genes in the absence and presence of CHX highlighted 104 ATHB4-regulated genes whose expression does not require de novo protein synthesis, probably reflecting direct targets of ATHB4 action. Among these 104 genes, were found HAT1, HAT2 and HAT22 (Fig. 1b; Table S3), previously shown to be repressed by ATHB4 (Sorín et al., 2009). Although ATHB2 was not found in this list, it is directly repressed by ATHB2 itself, HAT2 and the ATHB4 paralog HAT3 (Ohgishi et al., 2001; Sawa et al., 2002; Turchi et al., 2013), which suggested that it is also directly regulated by ATHB4 (see below, Figs 5, 6). From this list of 104 genes, 97 were repressed by DEX application (i.e. by ATHB4) and only seven were up-regulated (Fig. 1B), supporting the conclusion that ATHB4 acts fundamentally as a transcriptional repressor of gene expression.

ATHB4 binds to specific DNA sequences

To test the ability of ATHB4 to specifically bind DNA, we next hybridized PBMs using recombinant ATHB4-MBP protein (produced as a fusion of ATHB4 with MBP; Methods S6) (Godoy et al., 2011). ATHB4 showed a strong preference for the 7-mer pseudopalindromic AAT(G/C)ATT sequence; binding of ATHB4 preferred the nucleotide A immediately 5' and 3' of this core sequence, resulting in the 9-mer consensus AAAT(G/C)ATTA sequence (Fig. 1c). Binding of ATHB4 to the 7-mer core sequence in vitro was poorly influenced by the central G/C nucleotides, in position 4, and strongly influenced by the nucleotides A and T, in positions 2 and 6, respectively (Fig. 1d). These results demonstrated that ATHB4 does possess a sequence-specific DNA-binding capacity that is similar to that shown for its close relatives HAT3 and ATHB2 (NAAT(G/C)ATTN) (Turchi et al., 2013). Combining the target sequence information with co-expression analyses allows the prediction of whether specific TFs act as activators or repressors through a particular DNA sequence (Franco-Zorrilla et al., 2014). A similar analysis for ATHB4 target sequence specificity showed that the promoters of negatively co-regulated genes were enriched in binding motifs for ATHB4 (Fig. S2), in agreement with our conclusion that ATHB4 acts mainly as a transcriptional repressor.

Apart from the HD and Zip domains, members of HD-Zip class II family show high conservation in two additional regions: the C-terminal end, which contains the conserved amino acids CPSCE sequence (proposed as responsible for sensing the redox cell state), and an N-terminal consensus sequence, of unknown
function (Ariel et al., 2007). Aligning the amino acid sequences of ATHB4 and ATHB2, which has been studied in some detail (Sessa et al., 1997), led to the division of ATHB4 into four regions: N-terminal (N, 1–142), HD (H, 143–219), Zip (Z, 220–263) and C-terminal (C, 264–318) (Fig. S3a). Using a yeast two-hybrid (Y2H) assay, we observed that the HZ region of ATHB4 interacts with itself (Fig. S3b,c), in agreement with the view that ATHB4 dimerizes via the Zip motif to bind to the DNA.

The HD region contains a functional nuclear localization signal (NLS)

Next, seven truncated constructs were generated (NHZ0, 0HZ0, 00Z0, 00ZC, 0HZC, NH00 and 0H00), fused to the green fluorescent protein reporter gene (GFP) reporter gene and placed under the control of the constitutive 35S promoter to study their subcellular localization in vivo in onion epidermal cells using microbombardment assays. Full-length ATHB4 fused to GFP (35S:ATHB4-GFP) and GFP alone (35S:GFP) constructs were used as controls. The individual constructs were co-transformed with a plasmid to constitutively express the DsRed protein (a Red Fluorescent Protein (RFP)), to identify the transformed cells. As expected, cytoplasmic and nuclear localization were observed for the GFP alone, whereas full-length ATHB4-GFP was mostly nuclear (Fig. S4a,b). Variants containing the HD region (NHZ0, 0HZ0, 0HZC, NH00 and 0H00) displayed GFP fluorescence quite restricted to the nuclei, whereas the remainder (00Z0 and 00ZC) showed a GFP fluorescence pattern similar to that of GFP alone (Fig. S4c–i). These results suggested that the HD contains a nuclear localization signal (NLS). Indeed, a web-based program (http://nls-mapper.iab.keio.ac.jp/) predicted the existence of a monopartite NLS (GDGK) within the HD, which is conserved in the HD of other members of the HD-Zip II family (Fig. S4j).

The DNA-binding activity of ATHB4 is not required for controlling seedling responses to simulated shade

Next, we aimed to address the function of the different regions of ATHB4 in transgenic plants. From the possible genetic backgrounds to obtain transgensics, we chose to transform wild-type plants rather than the double athb4 hat3 mutant for two main reasons. On the one hand, the latter genotype displays an important set of developmental defects at both vegetative (leaf and cotyledon polarity defects) and reproductive stages (severe flower structure alteration, and very low fertility) (Bou-Torrent et al., 2012; Reymond et al., 2012; Turchi et al., 2013) that might prevent plant transformation. More importantly, because the defective cotyledons of the athb4 hat3 seedlings might affect the hypocotyl response to shade by indirect mechanisms (Sorin et al., 2009), the possible complementation of the cotyledon and leaf defects by some ATHB4 derivatives might also interfere with the hypocotyl elongation response, preventing an adequate interpretation of the results. On the other hand, overexpression of ATHB4 derivatives in a wild-type background provides morphological and molecular phenotypes that can be easily compared with those of the full-length wild-type ATHB4 form (see below, Figs 3, 4). Therefore, to analyze the structure–function relationship of ATHB4, we overexpressed the truncated ATHB4 forms fused to the GR domain in wild-type plants (Fig. 2a).

Nontransformed (Col-0) and transgenic 35S:ATHB4-GR plants (Sorin et al., 2009) were used as controls. DEX application had little effect on Col-0, whereas it inhibited general growth in transgenic 35S:ATHB4-GR seedlings grown under either W or W + FR (Fig. S5A,B), in agreement with published information (Sorin et al., 2009). ATHB4 biological activity was easily scored by comparing the simulated shade-induced hypocotyl lengths of seedlings grown in medium supplemented with and without DEX (Fig. S5c). Therefore, in the following experiments, the biological activity of ATHB4 derivatives was estimated as the ratio of hypocotyl lengths of W + FR-grown seedlings in the presence and absence of DEX (Figs 2b, S5d). Among the different lines generated, only seedlings overexpressing NHZO-GR, 00ZC-GR, 0HZC-GR and NH00-GR displayed a significant DEX-dependent attenuated response to W + FR, similar to 35S: ATHB4-GR seedlings (Fig. 2c). Independently of the hypocotyl phenotype, all the analyzed lines had detectable levels of gene expression (Fig. S6). These results suggest that both the C-terminal region and, surprisingly, the DNA-binding activity of ATHB4 are dispensable for the modulation of the seedling responses to simulated shade.

Next, we molecularly characterized those lines that showed different degrees of biological activity (35S:NHZ0-GR, 35S:00ZC-GR, 35S:0HZC-GR and 35S:NH00-GR). The effect of DEX on the expression of the shade-marker genes HAT2, SAUR15 (identified previously as putative direct targets of ATHB4 action; Table S3) and ATHB2 was investigated. As controls, we used 35S:ATHB4-GR and 35S:0HZ0-GR seedlings, which show full and no biological activity in the inhibition of shade-induced hypocotyl elongation, respectively. To better visualize the DEX-dependent repressor effect of ATHB4 on gene expression, seedlings were treated with W + FR for 1 h, which induces the expression of these genes, before harvesting the material (Fig. 3a). HAT2, SAUR15 and ATHB2 expression was reduced in DEX-treated 35S:ATHB4-GR and 35S:NHZ0-GR seedlings (+DEX) compared with those grown in the absence of DEX (−DEX), whereas it was unaffected in wild-type (Col-0) seedlings. Surprisingly, 35S:0HZ0-GR seedlings also presented a slight but significant DEX-dependent up-regulation of ATHB2 expression, whereas HAT2 and SAUR15 expression was unaffected. HAT2 expression was down-regulated only in DEX-treated 35S:NH00-GR seedlings, whereas SAUR15 was down-regulated in DEX-treated 35S:00ZC-GR and 35S:NH00-GR seedlings. By contrast, ATHB2 expression was unaffected in DEX-treated 35S:00ZC-GR and 35S:NH00-GR seedlings and slightly but significantly up-regulated in 35S:0HZC-GR seedlings (Fig. 3b–e). Despite the complexity of the observed expression profiles, our results clearly show that to repress gene expression neither the DNA-binding activity of ATHB4 nor protein dimerization via the Zip domain is required.
Fig. 2 Hypocotyl elongation of Arabidopsis thaliana seedlings overexpressing truncated derivatives of ARABIDOPSIS THALIANA HOMEBOX 4 (ATHB4). (a) Cartoon detailing the truncated ATHB4 derivatives overexpressed in plants. (b) Summary of growth conditions: seeds were sown in medium supplemented (+DEX) or not supplemented (−DEX) with dexamethasone. Seedlings were germinated and grown for 2 d in continuous white light (W) and then transferred to W enriched in far-red light (W + FR) for a further 5 d. (c) Ratio of hypocotyl lengths of seedlings grown in +DEX and −DEX media as an indicator of ATHB4 biological activity. Bars represent the mean ± SE. In each graph: white bars, the wild-type (Columbia (Col-0)); black bars, the 35S:ATHB4-GR line; light-gray bars, the ATHB4 (Columbia (Col-0)); black bars, the 35S:ATHB4 derivative line. Asterisks indicate significant differences (*, P < 0.05; **, P < 0.01) relative to the wild-type control.

To investigate the implications of this observation in more detail, a derivative of ATHB4 with impaired DNA-binding was generated by mutating Asn210, located in the HD, to Ala (ATHB4N210A) (Fig. 4a). This residue was selected because of the reported effect of the equivalent mutation on the DNA-binding activity of ATHB2 (ATHB2N176A) (Sessa et al., 1997). PBMs indicated that ATHB4N210A had no binding preference for any DNA sequence (Figs 4b, S7a–d), confirming that it was unable to bind DNA specifically. Seedlings overexpressing this mutant form fused to the GR (35S:ATHB4N210A-GR) displayed significant DEX-dependent attenuated hypocotyl elongation in response to W + FR, similar to 35S:ATHB4-GR seedlings (Fig. 4c, S7E,F). Molecularly, the effect of DEX on the expression of HAT2, SAUR15 and ATHB2 genes mirrored that observed in 35S:NH00-GR seedlings (Fig. 4e). Together, these results confirm that DNA-binding activity is neither necessary nor sufficient for the modulation of shade-induced hypocotyl elongation or for the control of gene expression by ATHB4 (at least for HAT2 and SAUR15). These results also indicate that ATHB2 expression is only affected by the DNA-binding activity of ATHB4 (Figs 3, 4e).

To address whether the analyzed genes are direct targets of the non-DNA-binding activity of ATHB4, we followed the strategy used to identify putative direct targets of full-length ATHB4 activity (see Fig. 1a) using 35S:NH00-GR and 35S:ATHB4N210A-GR lines. As controls, we employed Col-0 and 35S:ATHB4-GR lines. As in Fig. 1(a), seedlings were grown and treated with or without DEX in the absence or presence of the protein synthesis inhibitor cycloheximide (± CHX) for 4 h. As in Fig. 3, seedlings were treated with 1 h of W + FR before harvesting of the samples (Fig. 5a). In Col-0 and 35S:NH00-GR seedlings, addition of DEX had no significant effect on the expression of HAT2, SAUR15 and ATHB2 independently of the presence of CHX. In 35S:ATHB4-GR seedlings, HAT2, SAUR15 and ATHB2 expression was reduced by DEX application in the absence of CHX (−CHX); by contrast, only SAUR15 expression was also repressed by DEX in +CHX seedlings, further supporting our previous conclusion that SAUR15 is a direct target of ATHB4. In 35S:ATHB4N210A-GR seedlings, HAT2 and SAUR15 expression was reduced by DEX in −CHX samples, although only SAUR15 expression was also repressed by DEX in +CHX seedlings; by contrast, ATHB2 expression was promoted by DEX in both −CHX and +CHX samples (Fig. 5b). These results indicate an attenuated direct transcriptional activity of the truncated NH00 compared with the mutant ATHB4N210A derivative. In addition, they also suggest that SAUR15 and ATHB2 are direct targets of the non-DNA-binding activity of ATHB4 (i.e. provided by ATHB4N210A). Our results do not allow us to ascertain whether HAT2 also belongs to this group, at least when plants are growing in different light conditions.

ATHB2 and HAT2 promoters, but not the SAUR15 promoter, have several putative ATHB4 binding sites (Fig. 6a) (Brandt et al., 2012). To investigate whether ATHB4 regulates ATHB2 and HAT2 expression by physically interacting with their promoters, we performed ChIP. Our initial attempts using 35S:ATHB4-GFP seedlings failed, probably because the transgenic protein was virtually undetectable by immunoblot analysis (Fig. 8). We next used 35S:0HZC-GFP, in which GFP-fused protein levels were easily detectable (Fig. S8), and 35S:GFP seedlings as a control (Fig. 6b). Three fragments of the ATHB2 and HAT2 promoters (A1 to A3, and H1 to H3, all around putative ATHB4 binding sites) were over-represented in the immunoprecipitated chromatin from 35S:0HZC-GFP seedlings, in clear contrast with fragments in the CDS of ATHB2 (A4) and UBQ10 (U1) in the same samples, and all these fragments in the immunoprecipitated chromatin from 35S:GFP seedlings (Fig. 6). These results indicate that the DNA-binding domain of ATHB4 can physically interact with ATHB2 and HAT2 promoters in vivo. These findings, together with the rapid repression of ATHB2 and HAT2 expression upon ATHB4 induction (Figs 3–5), support the conclusion that these two genes are direct targets of ATHB4 DNA-binding activity.
The DNA-binding activity of ATHB4 is required for regulating leaf polarity in adult plants.

To address whether other ATHB4-regulated responses did require its DNA-binding activity, we focused on leaf polarity. The role of ATHB4 in regulating this trait was first visualized by the abaxialization of leaf development in mutant plants deficient in both ATHB4 and its paralog HAT3 (Bou-Torrent et al., 2012). Consistently, constitutive overexpression of ATHB4 (35S:ATHB4-GFP) and DEX-treated 35S:ATHB4-GR plants resulted in upward curling of leaf blades (Fig. S9), a phenotype caused by the relative over-proliferation of abaxial-derived tissues compared with adaxial tissues in leaves (Wenkel et al., 2007; Bou-Torrent et al., 2012). We next analyzed
whether some truncated ATHB4 derivatives are functional in controlling leaf curling. Only DEX-induced 35S:NHZ0-GR plants showed upward curling of leaf blades, like 35S:ATHB4-GR plants. By contrast, DEX-treated 35S:NHZC-GR, 35S:NH00-GR and 35S:ATHB4N210A-GR plants, like Col-0, showed no obvious biological activity. The similar expression levels of these transgenes in seedlings and leaves for the lines analyzed (Figs 7a–d, S6, S7e–f) supports the conclusion that truncated NH00 and mutated ATHB4N210A forms truly have no wild-type activity in leaf polarity. The discrepancies between the activities of these two non-DNA-binding ATHB4 derivatives in the regulation of the shade-induced hypocotyl elongation and the modulation of leaf polarity (Figs 2, 4, 7a–d) suggested that the mechanism of action involved is different: whereas the DNA-binding activity of ATHB4 is required for the regulation of leaf polarity in adult plants, it is unnecessary (i.e. dispensable) for the control of seedling responses to simulated shade.

Fig. 4 Seedling responses of transgenic lines overexpressing ARABIDOPSIS THALIANA HOMEOBOX 4 (ATHB4) derivatives ATHB4N210A and ATHB4N210A. (a) Cartoon detailing the mutated ATHB4N210A and ATHB4N210A derivatives overexpressed in Arabidopsis thaliana plants. A black circle on top of a vertical line represents the location of the introduced point mutation. (b) Comparison of the box-plot enrichment scores (E-scores) with the 7- and 8-mer box consensus and the top scoring site-directed mutant variants for ATHB4 and ATHB4N210A. Boxes represent quartiles 25–75% and the black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1–25% (above) and 75–100% (below). Dots denote outliers of the distribution. (c, d) Ratio of hypocotyl lengths of seedlings grown in −DEX and +DEX media for the 35S:ATHB4N210A-GR (c) and 35S:ATHB4N210A-GR (d) lines. Seeds were sown in medium without dexamethasone (−DEX) or supplemented with dexamethasone (+DEX). Seedlings were germinated and grown for 2 d in continuous white light (W) and then transferred to W enriched in far-red light (W + FR) for a further 5 d. Bars represent the mean ± SE. White, black and light-gray bars correspond to the wild-type (Col-0), 35S:ATHB4-GR and derivative lines, respectively. Symbols indicate significant differences (*, P < 0.05; **, P < 0.01) relative to the control. (e, f) Transcript abundances of HOMEOBOX ARABIDOPSIS THALIANA 2 (HAT2), SMALL AUXIN UPREGULATED 15 (SAUR15) and ATHB2 genes, normalized to UBIQUITIN 10 (UBQ10), in seedlings of Col-0, 35S:ATHB4-GR, 35S:ATHB4N210A-GR (e) and 35S:ATHB4N210A-GR (f). Seedlings were germinated and grown for 7 d in medium supplemented (+DEX) or not (−DEX) with DEX under W. Samples were harvested after 1 h of W + FR treatment. Gene expression is presented relative to levels in Col-0 without DEX treatment. Values are means ± SE of three independent RT-qPCR biological replicates. White and blue columns correspond to −DEX and +DEX samples, respectively. Asterisks indicate significant differences in transcript levels (Student t-test: *, P < 0.05; **, P < 0.01) between −DEX and +DEX treatments.
The N-terminal region of ATHB4 has a protein–protein interaction domain that is needed for biological activity

These observations also indicate that the N-terminal region of ATHB4 is important for the control of gene repression in both the SAS seedling responses and leaf polarity. As a first step to understanding the molecular mechanism behind the functional regulatory duality of ATHB4, we performed Y2H screening using full-length ATHB4 as bait. From a total of 63 genes identified, we found HAT2, HAT3, HAT9 and HAT22, encoding HD-Zip II proteins, and TOPLESS (TPL) and TPL-RELATED 4 (TPR4), encoding related transcriptional corepressors (Table S4). The selected interaction domains (SIDs) of the HD-Zip proteins contained all or part of the Zip domain, required for dimerization between family members (Ariel et al., 2007; Brandt et al., 2014). The SIDs of TPL and TPR4 corresponded to the N-terminal region which contains a LisH dimerization domain, which has been proposed to interact with different proteins containing an EAR motif (Brandt et al., 2014). The N-terminal region of ATHB4 (N000) contains two EAR motifs (LxLxL type), one of which (residues 8–12) is conserved among members of the HD-Zip II subfamily (Kagale et al., 2010; Brandt et al., 2014). In Y2H assays, the N000 fragment of ATHB4 could interact with the LisH-containing N-terminal part of TPL (residues 1–242) (Fig. 8a). Bimolecular fluorescence complementation (BiFC) experiments in agroinfiltrated leaves of Nicotiana benthamiana confirmed that full-length ATHB4 binds full-length TPL in the nucleus (Fig. 8b). Together, these results suggested that the N-terminal region of ATHB4 can physically interact with TPL in vivo, probably via the EAR motifs. Although the biological relevance of this interaction is unknown, our results show that the N-terminal part of ATHB4 is involved in interacting with other proteins.

To establish whether EAR motifs within the N-terminal region of ATHB4 were important for its functionality, a truncated form without the first 52 amino acids (ATHB4\textsubscript{AN52}), lacking both EAR domains, was fused to the GR gene and overexpressed in plants (35S:ATHB4\textsubscript{AN52}-GR) (Fig. 4a). The resulting seedlings displayed more attenuated DEX-dependent inhibition of hypocotyl elongation in response to W + FR than 35S:ATHB4-GR seedlings, although levels of transgene expression were similar in the two cases (Figs 4d, S7f). At the molecular level, the expression of the HAT2 and SAUR15 genes was unaffected in DEX-treated 35S:ATHB4\textsubscript{AN52}-GR, whereas that of ATHB2 was up-regulated in 35S:ATHB4\textsubscript{AN52}-GR seedlings compared with those that were mock-treated (–DEX) (Fig. 4f). These results are consistent with those obtained with the truncated derivatives 00ZC and 0HZC, missing the whole N-terminal region (Figs 2, 3), and confirm that the EAR-containing N-terminal region of ATHB4 has an important role in the modulation of shade-induced hypocotyl elongation and the repression of gene expression of HAT2 and, to a lesser extent, SAUR15. By contrast, the expression of ATHB2 can be activated by DNA-binding derivatives of ATHB4 that lack the EAR motifs. When we analyzed the activity of ATHB4\textsubscript{AN52} in leaf polarity, DEX-treated plants showed no obvious upward-curling activity, as was observed for 0HZC. The
transgene is expressed in leaves to even higher levels than \textit{ATHB4-GR}, confirming that the mutated \textit{ATHB4}_{AN52} form has no wild-type activity in leaf polarity (Fig. 7e,f).

**Discussion**

In Arabidopsis seedlings, perception of plant proximity by phytochromes alters a pre-established transcriptional network organized in functional modules that collectively boost elongation growth. Genetics has helped to identify several of the constituents of the functional modules with both positive and negative activities in promoting hypocotyl elongation. Overall, components of the functional modules have the ability to modulate gene expression by at least two major mechanisms: binding to DNA regulatory sequences (i.e. as TFs) and binding to other transcriptional regulators and altering their transcriptional activity (i.e. as transcriptional cofactors) (Wray \textit{et al.}, 2003). A well-known group of SAS components are heterodimer-forming bHLH proteins, in which the positively acting SAS components are TFs (e.g. PIFs, BEEs and BIMs) and the negative components are transcriptional cofactors (e.g. HFR1 and PAR1) (Galstyan \textit{et al.}, 2011; Hornitschek \textit{et al.}, 2012; Cifuentes-Esquível \textit{et al.}, 2013).

In this study, we addressed the molecular mechanism of action of \textit{ATHB4}, a member of the HD-Zip II subfamily with a complex role in SAS hypocotyl elongation. Previous information about other HD-Zip II family members suggested a positive role in SAS regulation for \textit{ATHB2}, \textit{HAT1} and \textit{HAT2}; this conclusion was based mostly on constitutive overexpression phenotypes only under high R:FR light (Steindler \textit{et al.}, 1999; Sawa \textit{et al.}, 2002; Ciarbelli \textit{et al.}, 2008). Our analyses using various overexpressing and inducible lines showed that the increased activity of these factors does not promote hypocotyl growth under simulated shade but either had no effect (35S:\textit{ATHB2} and 35S:\textit{HAT2}) or even inhibited it (35S:\textit{ATHB4-GFP}, 35S:\textit{HAT1}, 35S:\textit{ATHB4-GR} and 35S:\textit{HAT2-GR}) (Fig. S1). In contrast, the promotion of hypocotyl elongation under W displayed only by the constitutive lines (Fig. S1) might reflect the increased activity of these HD-Zip II proteins in the mother plants and/or embryo development, a kind of preconditioning effect that is absent in the inducible GR-fusion lines, in which DEX is applied from the moment of germination (once the embryo is already formed). The embryo defects observed in the \textit{athb4 hat3} double mutant (Bou-Torrent \textit{et al.}, 2012) support this possibility. Despite the differences observed under W, our results indicate that these factors do not act as SAS positive regulators. In addition, these
observations suggested that HD-Zip II members have a similar molecular mechanism of action. Therefore, our analyses suggest that ATHB4 can be used as a paradigm for understanding the molecular mechanism of action of HD-Zip II family members with a similar role in SAS regulation.

Transcriptional regulation by TFs is one of the most investigated mechanisms, as it is generally accepted to be the primary level of regulation (Wray et al., 2003). The emergence of very powerful techniques, such as ChIP combined with next-generation sequencing approaches (e.g. ChIP-Seq) or PBMs of recombinant TF proteins combined with bioinformatics analyses, has allowed the experimental characterization of DNA-binding motifs and putative target genes bound and regulated by a specific TF. These analyses contribute to unravelling the biological role of the TF through the functional analysis of its targets. By employing PBMs, we were able to establish that the DNA-binding specificity of ATHB4 does not differ greatly from that of other HD-Zip II proteins studied. The absence of DNA-binding activity of the mutant ATHB4N210A protein indicates that the HD is required for this function, as expected, and that no other region of ATHB4 (such as the conserved N-terminal region of unknown function) has DNA-binding activity (Fig. 4). Transcriptome analyses performed using the inducible 35S:ATHB4-GR line showed that ATHB4 acts fundamentally as a transcriptional repressor, as described for other HD-Zip II family members (Steindler et al., 1999; Ohgishi et al., 2001; Sawa et al., 2002) and in contrast to HD-Zip III proteins that act as transcriptional activators (Xie et al., 2015). Surprisingly, only a small proportion of the ATHB4 putative target genes are also rapidly shade regulated, which supports other roles for ATHB4 in plant development (e.g. leaf polarity).

Our structure–function analyses have furthered current understanding of this type of transcriptional regulator. On the one hand, we have shown that truncated ATHB4 fragments containing only the HD-Zip domains (e.g. 0HZ0 and 0HZC) are not sufficient to confer full activity in the repression of shade-induced hypocotyl elongation and specific gene expression, and that the missing N-terminal region contains...
information relating to interaction with other proteins (Fig. 8) that is required for the wild-type biological activity of ATHB4. This conserved region of unknown function is very probably functionally necessary for all the members of the HD-Zip II subfamily. On the other hand, ATHB4 derivatives unable to bind DNA (truncated NH00 and ATHB4N210A) are fully functional in inhibiting both shade-induced hypocotyl elongation and up-regulation of HAT2 and SAUR15. As SAUR15 is directly regulated by ATHB4 even when it has lost its ability to bind DNA (Table S3; Fig. 5), these results suggest the unexpected possibility that a part of the ATHB4 mechanism of action involves modulating gene expression without direct binding to regulatory regions of the DNA; that is, ATHB4 acts also as a transcription cofactor (Fig. 9). This possibility is supported by our transcriptome analyses which show that the majority of the ATHB4 putative direct target genes (69 out of a total of 104 genes, i.e. c. 66%) lack 9-mer DNA-binding motifs for ATHB4 in their promoter regions, among which SAUR15 can be found (Table S3). The cofactor activity requires that ATHB4 either accesses promoters via proteins that can themselves bind DNA or impedes the binding of other TFs to their cis-regulatory targets. In either case, this activity is probably dependent on the protein-protein interaction abilities conferred by the N-terminal region (e.g. via the EAR domain), which is conserved between the members of this subfamily.

In light of these analyses, one might wonder why ATHB4 has a DNA-binding activity that seems dispensable for its function in vivo. One possibility is that other ATHB4-dependent functions do require its DNA-binding activity. Indeed, our analyses indicate that ATHB4 acts as a bona fide TF in controlling leaf polarity, an activity shared with other HD-Zip II family
members (Bou-Torrent et al., 2012; Turchi et al., 2013). Leaf flattening is also promoted by end-of-day FR, a treatment that mimics plant proximity (Kozuka et al., 2013). Recently it has been suggested that ATHB4, together with its paralog HAT3, might be part of the mechanisms regulating this SAS response in adult leaves (Roig-Villanova & Martinez-Garcia, 2016). As in the modulation of the SAS hypocotyl elongation response, the control of leaf polarity requires the EAR-containing first 52 residues of ATHB4; but, by contrast, the DNA-binding activity is indispensable (Fig. 7).

It seems therefore that ATHB4 has two activities, one DNA-binding dependent and the other DNA-binding independent (Fig. 9). Although, in physiological conditions, the two activities cannot be uncoupled, it is reasonable to postulate that both activities are not working at the same cellular levels. Genetics suggests that the leaf polarity phenotype (ATHB4 DNA-binding dependent) requires very low levels of ATHB4 expression (e.g. even single athb4 and hat3 loss-of-function mutants display a wild-type phenotype) (Sorin et al., 2009). In this framework, the DNA-binding-dependent activity of ATHB4 might act as a molecular switch to trigger normal cotyledon and leaf development: when there is no ATHB4 (and HAT3) activity, the switch is off, seedling cotyledons (the site of shade perception) are defective, and shade-induced hypocotyl elongation is impaired. When cotyledons are developing properly, the additional transcriptional cofactor activity has a role in SAS regulation. This activity would probably take place when cellular levels of ATHB4 are higher. Indeed, expression of ATHB4 (and that of many other PAR genes) is strongly promoted by shade exposure in an R:FR-dependent manner: the lower R:FR, the higher ATHB4 expression (Roig-Villanova et al., 2006). Shade-induced hypocotyl length is also strongly dependent on R:FR and, when R:FR is very low (precisely when ATHB4 expression is highest), hypocotyl elongation is inhibited by the antagonistic effect of phytochrome A (Martinez-Garcia et al., 2014). These very low R:FR conditions mimic the natural situation of deep shade, when canopy closure occurs (Yanovsky et al., 1995). Therefore, the non-DNA-binding activity of ATHB4 can contribute to the optimal hypocotyl elongation response under conditions of deep shade.

Is this dual molecular activity of ATHB4 a unique case among TFs? There are few reports indicating that this is not the case. Functional analysis of three closely related bHLH members, SPEECHLESS (SPCH), MUTE, and FAMA, which have distinct functions regulating sequential steps of stomatal development, provided surprising evidence that, despite deep sequence conservation in their DNA-binding domains, both SPCH and MUTE do not require DNA-binding activity for their in vivo activities in regulating this response (Davies & Bergmann, 2014). A mutated PIF3 form unable to bind DNA was shown to fully complement the pif3 mutation in terms of the hypocotyl response to monochromatic R, whereas the mutated form unable to bind to the phyB photoreceptor was inactive in complementing this trait (Al-Sady et al., 2008). These results indicated that, like in ATHB4, PIF3 DNA-binding was unnecessary to modulate hypocotyl elongation in response to specific light conditions (monochromatic R). Like ATHB4, PIF3 also contains an additional region involved in protein—protein interactions (i.e. the APB domain involved in interacting with the phyB photoreceptors). Our results suggest that all these proteins might be involved in regulating other responses as TFs: in the case of PIF3, it might also be involved in the control of carotenoid biosynthesis by directly regulating PHYTOENE SYNTHASE (PSY) gene expression (Toledo-Ortiz et al., 2010; Bou-Torrent et al., 2015).

A level of genome plasticity resides in the ability of a single gene to produce different protein isoforms via alternative splicing regulated by external stimuli (Pose et al., 2013; Nicolas et al., 2015). The functional duality of these HD-Zip II regulators acting as either a transcriptional factor or cofactor in the regulation of different developmental responses reveals an additional level of genome plasticity, in this case in different developmental processes. What the determinants are that drive the use of the possible molecular mechanisms is currently unknown, although we envisage that
cellular levels and the spatial and temporal expression of additional partners (via the N-terminal region) might be important.

In summary, we have functionally analyzed the mechanism of action of ATHB4, a specific factor that regulates the SAS and leaf polarity. Our results indicate that the DNA-binding activity of ATHB4 is not required for the regulation of SAS seedling responses (Fig. 9). These findings suggest that, when working with a protein containing canonical and functional DNA-binding domains, it cannot be assumed that the studied protein acts essentially as a TF, that is, that the basic mechanism of control of target gene expression action involves only binding to specific cis-acting regulatory sequences. Therefore, our results expand our current view and understanding of the function of TFs as mere DNA-binding proteins with transcriptional activity.

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Author contributions

M.Gallemi, M.J.M-C., S.P., M.S-M., C.S., M.Godoy, J.M.F-Z., R.S. and J.F.M-G. conducted the experiments, analyzed data and designed the experiments; M. Gallemi, M.J.M-C., S.P., J.M.F-Z. and J.F.M-G. wrote the paper.

References

Al-Sady B, Kikis EA, Monte E, Quail PH. 2008. Mechanistic duality of transcription factor function in phytochrome signaling. Notes Acad Natl Sci USA 105: 2232–2237.
Ariel FD, Manavella PA, Dezar CA, Chan RL. 2007. The true story of the HD-Zip family. Trends Plant Sci 12: 419–426.
Bou-Torrent J, Salla-Martret M, Brandt R, Musielak T, Palaquí JC, Martinez-Garcia JF, Wenkel S. 2012. ATHB4 and HAT3, two class II HD-ZIP transcription factors, control leaf development in Arabidopsis. Plant Signaling & Behavior 7: 1382–1387.

Bou-Torrent J, Toledo-Ortiz G, Ortiz-Alcaide M, Cifuentes-Esquível N, Halliday KJ, Martínez-García JF, Rodríguez-Concepción M. 2015. Regulation of carotenoid biosynthesis by shade relies on specific subsets of antagonistic transcription factors and cofactors. Plant Physiol 169: 1584–1594.
Bou-Torrent J, Cabezo M, Xie Y, Wenkel S. 2014. Homeodomain leucine-zipper proteins and their role in synchronizing growth and development with the environment. Journal Integrative Plant Biology 56: 518–526.
Brandt R, Salla-Martret M, Bou-Torrent J, Musielak T, Mølgaard T, Stahl M, Lanz C, Ott F, Schmid M, Greb T, Schwarz M et al. 2012. Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses. Plant Journal 72: 31–42.
Casal JJ. 2013. Photoreceptor signaling networks in plant responses to shade. Annual Review Plant Biology 64: 403–427.
Ciarbelli AR, Cioffi A, Salvucci S, Ruzza V, Possenti M, Carabelli M, Fruscalzo A, Sessa G, Morelli G, Ruberti I. 2008. The Arabidopsis homeodomain-leucine zipper II gene family: diversity and redundance. Plant Molecular Biology 68: 465–478.
Cifuentes-Esquível N, Bou-Torrent J, Galstan Y, Galleli M, Sessa G, Salla Martret M, Roig-Villanova I, Ruberti I, Martínez-García JF. 2013. The bHLH proteins BEE and BIM positively modulate the shade avoidance syndrome in Arabidopsis seedlings. Plant Journal 75: 989–1002.
Crocco CD, Holm M, Yanovsky MJ, Botto JF. 2010. AtBBX21 and COP1 genetically interact in the regulation of shade avoidance. Plant Journal 64: 551–566.
Crocco CD, Locascio A, Escudero CM, Abadini D, Blazquez MA, Botto JF. 2015. The transcriptional regulator BBX24 impairs DELLA activity to promote shade avoidance in Arabidopsis thaliana. Nature Communications 6: 6202.
Davies KA, Bergmann DC. 2014. Functional specialization of stomatal bHLHs through modification of DNA-binding and phosphoregulation potential. Proceedings National Academy of Sciences, USA 111: 15585–15590.
Faigon-Soverna A, Harmon FG, Storani L, Karayekov E, Staneloni RJ, Gassmann W, Mas P, Calas JJ, Kay SA, Yanovsky M. 2006. A constitutive shade-avoidance mutant implicates TIR-NBS-LRR proteins in Arabidopsis photomorphogenetic development. Plant Cell 18: 2919–2928.
Franco-Zorrilla JM, Lopez-Vidriero I, Carrasco JL, Godoy M, Vera P, Solano R. 2014. DNA-binding specificities of plant transcription factors and their potential to define target genes. Proceedings National Academy of Sciences, USA 111: 2367–2372.
Galleli M, Galstan Y, Paulisic S, Then C, Ferrandez-Ayela A, Lorenzo-Orts L, Roig-Villanova I, Wang X, Micol JL, Ponc MR et al. 2016. DRACULA2 is a dynamic nucleoporin with a role in regulating the shade avoidance syndrome in Arabidopsis. Development 143: 1623–1631.
Galstan Y, Cifuentes-Esquível N, Bou-Torrent J, Martinez-Garcia JF. 2011. The shade avoidance syndrome in Arabidopsis: a fundamental role for atypical basic helix-loop-helix proteins as transcriptional cofactors. Plant Journal 66: 258–267.
Godoy M, Franco-Zorrilla JM, Perez-Perez J, Oliveros JC, Lorenzo O, Solano R. 2011. Improved protein- binding microarrays for the identification of DNA-binding specificities of transcription factors. Plant Journal 66: 700–711.
Hornitscheck P, Kohnen MV, Lorrain S, Rougeman J, Ljung K, Lopez-Vidriero I, Franco-Zorrilla JM, Solano R, Trevisan M, Pradervand S et al. 2012. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant Journal 71: 699–711.
Kagale S, Links MG, Rozwadowski K. 2010. Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiology 152: 1109–1114.
Kozuka T, Suetogu N, Wada M, Nagataki A. 2013. Antagonistic regulation of leaf flattening by phytochrome B and phototropin in Arabidopsis thaliana. Plant Cell Physiology 54: 69–70.
Leivar P, Quail PH. 2011. PFs: pivotal components in a cellular signaling hub. Trends Plant Science 16: 19–28.
Li L, Ljung K, Breton G, Schmitz RJ, Pruneda-Paz J, Cowing-Zitrón C, Cole BJ, Ivens IJ, Pedmale UV, Jung HS et al. 2012. Linking photoreceptor excitation to changes in plant architecture. Genes Development 26: 785–790.
Lorrain S, Allen T, Duck PD, Whitelam GC, Fankhauser C. 2008. Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant Journal 53: 312–323.
Matteo Galleri, M, Molina-Contreras MJ, Llorente B, Bevilacqua MR, Quail PH. 2014. The shade avoidance syndrome in Arabidopsis: the antagonistic role of phytochrome a and B differentiates vegetation proximity and canopy shade. *PLoS ONE* 9: e109275.

Martinez-Garcia JF, Galstyan A, Salla-Martet M, Cifuentes-Esquível N, Galleri M, Bou-Torrent J. 2010. Regulatory components of shade avoidance syndrome. *Advances in Botanical Research* 53: 65–116.

Nicolas M, Rodríguez-Buey ML, Franco-Zorrilla JM, Cubas P. 2015. A recently evolved alternative splice site in the *BRANCHED1a* gene controls potato plant architecture. *Current Biology* 25: 1799–1809.

Ohgishi M, Oka A, Morelli G, Ruberti I, Aoyama T. 2001. The *HAT2* gene, a member of the HD-Zip family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in Arabidopsis. *Plant Physiology* 126: 4756–4767.

Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martínez-García JF. 2006. Identification of primary target genes of phytochrome signaling. Early transcriptional control during shade avoidance responses in Arabidopsis. *Plant Physiology* 141: 85–96.

Reymond MC, Brunoud G, Chauvet A, Martínez-García JF, Martin-Magniette ML, Menger F, Scott CP. 2012. A light-regulated genetic module was recruited to carpel development in Arabidopsis following a structural change to *SPATULA*. *Plant Cell* 24: 2812–2825.

Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martínez-García JF. 2006. The *HAT2* gene, a member of the HD-Zip gene family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in Arabidopsis. *Plant Journal* 32: 1011–1022.

Sessa G, Carabelli M, Sassi M, Giorfi A, Possenti M, Mittempergher F, Becker J, Morelli G, Ruberti I. 2005. A dynamic balance between gene activation and repression regulates the shade avoidance response in Arabidopsis. *Genes & Development* 19: 2811–2815.

Sessa G, Morelli G, Ruberti I. 1999. DNA-binding specificity of the homeodomain-leucine zipper domain. *Journal of Molecular Biology* 274: 303–309.

Sorin C, Salla-Martet M, Bou-Torrent J. 2015. Arabidopsis HD-ZIP II transcription factors control apical embryo development and meristem function. *Development* 140: 2118–2129.

Wenkel S, Emerj J, Hou BH, Evans MM, Barton MK. 2007. A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. *Plant Cell* 19: 3379–3390.

Xie Y, Straub D, Eguen T, Brandt R, Stahl M, Martínez-García JF, Wenkel S. 2015. Meta-analysis of Arabidopsis KANADI1 direct target genes identifies a basic growth-promoting module acting upstream of hormonal signaling pathways. *Plant Physiology* 169: 1240–1253.

Yanovsky MJ, Casal JJ, Whitelam GC. 1995. Phytochrome A, phytochrome B and HY4 are involved in hypocotyl growth responses to natural radiation in Arabidopsis: weak de-etiolation of the phyA mutant under dense canopies. *Plant, Cell & Environment* 18: 788–794.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Phenotypic analyses of seedlings over-expressing *ATHB2*, *HAT2*, *HAT1*, *HAT2-GR*, *ATHB4-GR* and *ATHB4-GR*.

**Fig. S2** Evaluation of the biological relevance of DNA motifs using data on co-regulation.

**Fig. S3** Analysis of the homodimerization activity of the HD-Zip region of *ATHB4*.

**Fig. S4** Subcellular localization of *ATHB4* and its derivatives.

**Fig. S5** Phenotypic analyses of seedlings over-expressing *ATHB4-GR*.

**Fig. S6** Levels of gene expression in *ATHB4*-derivative truncated lines.

**Fig. S7** *ATHB4* is impaired in DNA binding.

**Fig. S8** Detection of GFP-fused proteins in two transgenic *ATHB4* derivative lines.

**Fig. S9** *ATHB4* promotes upward leaf curling.

**Table S1** Primers used for amplifying and cloning genes used in this work

**Table S2** Primers used for qPCR analyses

**Table S3** Overlap of bioset of DEX-regulated genes in –CHX and +CHX treated 35S:ATHB4-GR (pCS19) seedlings; BH < 0.05; FC > 1.5

**Table S4** *ATHB4*-interacting proteins identified in the Y2H screen

**Methods S1** Generation of GFP- and GR-fused constructs for plant transformation.

**Methods S2** Transcriptomic analyses.

**Methods S3** RNA blot analysis of gene expression.

**Methods S4** Yeast two-hybrid (Y2H) assays.

**Methods S5** Subcellular localization analyses.
Methods S6 Expression of recombinant ATHB4-MBP for protein binding microarrays (PBMs).

Methods S7 Chromatin immunoprecipitation (ChIP).

Methods S8 Protein extraction and immunoblot analyses.

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