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RESEARCH PAPER

A novel *Arabidopsis* MYB-like transcription factor, MYBH, regulates hypocotyl elongation by enhancing auxin accumulation

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Abstract

Critical responses to developmental or environmental stimuli are mediated by different transcription factors, including members of the ERF, bZIP, MYB, MYC, and WRKY families. Of these, *MYB* genes play roles in many developmental processes. The overexpression of one *MYB* gene, *MYBH*, significantly increased hypocotyl elongation in *Arabidopsis thaliana* plants grown in the light, and the expression of this gene increased markedly in the dark. The MYBH protein contains a conserved motif, R/KLFGV, which was implicated in transcriptional repression. Interestingly, the gibberellin biosynthesis inhibitor paclobutrazol blocked the increase in hypocotyl elongation in seedlings that overexpressed *MYBH*. Moreover, the function of MYBH was dependent on phytochrome-interacting factor (PIF) proteins. Taken together, these results suggest that hypocotyl elongation is regulated by a delicate and efficient mechanism in which *MYBH* expression is triggered by challenging environmental conditions such as darkness, leading to an increase in PIF accumulation and subsequent enhanced auxin biosynthesis. These results indicate that MYBH is one of the molecular components that regulate hypocotyl elongation in response to darkness.

Key words: *Arabidopsis*, auxin, hypocotyl elongation, MYBH, PIF, photomorphogenesis.

Introduction

Photomorphogenesis, which is the light-mediated regulation of plant development, integrates many genetic and environmental factors. Light-grown seedlings exhibit short hypocotyls, whereas dark-grown seedlings exhibit long hypocotyls. During plant growth, the intensity and characteristics of light determine the rate and extent of cell elongation, and thus these factors directly affect hypocotyl length. Numerous studies have identified the key factors that trigger photomorphogenesis (for reviews, see Chory, 1993; Deng, 1994; McNellis and Deng, 1995). The *hy1*, *hy2*, *hy3*/*phyB*, and *hy6* mutations are associated with phytochromes, whereas the *cry1* (*hy4*) mutation is associated with a blue light receptor (Koornneef *et al.*, 1980; Chory *et al.*, 1989; Parks and Quail, 1991; Ahmad and Cashmore, 1993; Reed *et al.*, 1993). In contrast, Constitutive

Abbreviations: 5-MT, Trp analogue 5-methyltryptophan; GA, gibberellic acid; GFP, green fluorescent protein; GUS, β-glucuronidase; IAA, indole-3-acetic acid; MS, Murashige and Skoog; PAC, paclobutrazol; PIF, phytochrome-interacting factor; qRT-PCR, quantitative reverse transcriptase-PCR.

Photomorphogenic/De-etiolated/Fusca (COP/DET/FUS) proteins play negative roles in photomorphogenesis (Wei and Deng, 1999). *COP1* encodes a RING-type E3 ubiquitin ligase that mediates the ubiquitination of positive regulators of photomorphogenesis in the dark, thereby targeting these regulators for degradation (Yi and Deng, 2005).

Plant photomorphogenesis is regulated by various hormone signalling pathways. In plants overexpressing auxin biosynthetic genes, hypocotyl elongation was found to be enhanced because of elevated auxin levels (Zhao *et al*., 2002). Furthermore, the expression of several auxin-inducible genes during hypocotyl elongation has been observed in *Arabidopsis thaliana* and *Glycine max* (soybean) (McClure and Guilfoyle, 1989; Li *et al*., 1991; Gil and Green, 1997). Recently, the overexpression of *A. thaliana SMALL AUXIN UP RNA63* was shown to promote hypocotyl elongation (Chae *et al*., 2012). Additionally, gibberellic acid (GA) appears to promote skotomorphogenic growth and repress photomorphogenesis. Thus, reductions in endogenous levels of GA cause a light-grown phenotype in seedlings that are grown in the dark (Alabadi *et al*., 2004). The same phenomenon was also observed in a *ga1* mutant, which cannot perform GA biosynthesis (Alabadi *et al*., 2004; Achard *et al*., 2007). Recently, GA responses were shown to be repressed by the DELLA protein, which interferes with the function of basic helix–loop–helix transcription factors called phytochrome-interacting factors (PIFs) (De Lucas *et al*., 2008; Feng *et al*., 2008). PIFs are degraded via proteasome-dependent pathways in response to interactions with light-activated phytochrome B (Bauer *et al*., 2004; Park *et al*., 2004; Shen *et al*., 2005). Thus, PIFs appear to play crucial roles in the integration of light and GA signalling, thereby contributing to the fine-tuning of plant development in response to various signals. In contrast, cytokinins have been found to promote de-etiolation in the absence of light (Chory *et al*., 1991). Moreover, an *amp* mutant, which expresses high levels of cytokinins, showed different de-etiolation phenotypes in the dark (Chin-Atkins *et al*., 1996). These observations indicate that hypocotyl elongation must be optimally modulated by both positive and negative regulators in response to environmental challenges.

Various transcription factors, including members of the ERF, bZIP, MYB, MYC, and WRKY families, are involved in regulating gene expression in response to developmental or environmental stimuli. The MYB protein family controls gene expression by binding to DNA via MYB domain repeat sequences. MYB proteins are associated with a diverse array of cellular responses, including plant secondary metabolism, as well as biotic and abiotic stress tolerance (Jin *et al*., 2000; Singh *et al*., 2002; Vom Endt *et al*., 2002; Taki *et al*., 2005; Agarwal *et al*., 2006; Cheng *et al*., 2009; Mandaokar and Browse, 2009). In particular, MYB proteins play positive or negative roles in the production of enzymes involved in the biosynthesis of phenylpropanoids (Legay *et al*., 2007; Bomal *et al*., 2008), flavonoids (Grotewold, 2005), and benzenoids (Verdonk *et al*., 2005). Although more than 100 R2R3-MYBs have been identified in *Arabidopsis*, the function of many MYB proteins remains largely unknown (Yanhui *et al*., 2006). Therefore, this study aimed to identify the *MYB* gene that plays a role in plant growth and development by using an ectopic expression approach. We showed that the MYB hypocotyl elongation-related gene, *MYBH*, is involved in the positive regulation of dark-induced hypocotyl elongation in *Arabidopsis*. However, a T-DNA knockout mutant of *MYBH* (*mybh*) showed no significant phenotypic differences in hypocotyl elongation. *MYBH* promoter activity in the hypocotyls was strong in the dark, which is consistent with the role of MYBH in regulating dark-induced hypocotyl elongation. Furthermore, MYBH increased the accumulation of PIFs, thereby leading to increased biosynthesis of auxin. Our results suggest that a delicate and efficient mechanism regulates hypocotyl elongation and show that MYBH may be one of the molecular components that regulate hypocotyl elongation in response to the dark.

Materials and methods

Plant materials and growth conditions

A. thaliana ecotype Columbia (Col-0) plants were used in this study. The seeds were surface-sterilized and sown in normal Murashige and Skoog (MS) agar medium supplemented with 2% sucrose. After 3 d of stratification at 4 °C, the seeds were allowed to germinate at 23 ± 1°C under normal light conditions, namely a 16 h light/8 h dark cycle (white light: 200 μmol m^{-2} s^{-1}). The T-DNA insertion mutant line *AT5G47390*, which is termed *mybh* (GK-783B02: NASC ID N365026), was obtained from the Nottingham *Arabidopsis* Stock Centre (http://arabidopsis.info). The presence of the insertion was confirmed by PCR using a combination of four primers: MYBH-F (5′-ATGACTCGTCGATGTTCTCACTGC-3′), MYBH-R (5′-GCGTGTATCACGCTTTTG-3′), GABI-F (5′-CGCCAGGGTTTTCCCAGTCACGACG-3′), and GABI-R (5′-GAAGGCGGGAAACGACAATCTG-3′). In the *mybh* mutant line, the T-DNA was inserted into the first exon of *AT5G47390*.

Gene constructs

Transgenic *Arabidopsis* plants (Col-0) expressing full-length *AT5G47390* (*MYBH*) cDNA were generated. Full-length *MYBH* cDNA was amplified via PCR and cloned into *pBI121*, which contains the cauliflower mosaic virus (CaMV) 35S promoter. To obtain transgenic overexpression of *MYBH*, *Arabidopsis* plants were transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method. Homozygous T_4 transgenic plants were used for all of the experiments. For the construction of the *MYBHpro::GUS* plasmids, the *MYBH* promoter region was amplified by performing PCR and cloned using the pCR®8/GW/TOPO® TA Cloning® kit (Invitrogen). Additional information about the Gateway site-specific cloning protocols is provided online by the manufacturer (http://www.invitrogen.com/). The *MYBHpro::GUS* clones were created with LR Clonase reactions (Invitrogen) using MYBHpro/TOPO and the destination vector pMDC162.

Histochemical β-glucuronidase (GUS) staining

MYBHpro::GUS transgenic seedlings were grown on MS medium containing 2% sucrose and cultivated under normal light or dark conditions for 7 d. To observe GUS activity in the transgenic plants, whole seedlings were treated with GUS staining buffer [0.1 M $NaPO_4$ (pH 7.0), 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 1.9 mM X-glucuronide, and 0.1% Triton X-100] and incubated at 37°C overnight. GUS-stained seedlings were cleared overnight in 100% ethanol to remove their chlorophyll.

RNA gel blot analysis

Col-0 and transgenic plants were grown on MS medium (2% sucrose) in a growth chamber for 7 d. The seedlings were then exposed to the treatment conditions (normal light or continuous dark). Total RNA was purified by using the aurintricarboxylic acid and lithium chloride method (Lee *et al*., 2002). Total RNA (20 µg) was separated by performing electrophoresis on 1.5% (w/v) agarose gels that contained formaldehyde and then transferred onto a Hybond^{TM}-XL membrane (Amersham Biosciences) and left for over 24 h before being subjected to UV cross-linking. Radiolabeled probes were prepared using a random primer DNA Labeling System (Invitrogen). After pre-hybridization of the membrane for 1 h at 65 °C, hybridization was performed with ^{32}P-labelled probes for over 16 h. The RNA blot was then washed twice in 1× SSC, 0.1% SDS and once more in 0.1× SSC, 0.1% SDS at 42 °C. The RNA blot was then scanned using a Multiplex Bio-Imaging System (Fujifilm FLA-7000).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from whole seedlings using TRI Reagent (MRC Inc.). Briefly, 1 µg of total RNA was subjected to cDNA synthesis using oligo(dT) reverse transcriptase (Promega) and an RNase inhibitor (Intron Biotechnology). qRT-PCR was carried out in 20 µl reactions that contained 10 ng of diluted cDNA, 10 µl of 2× iQSYBR Green Supermix (Bio-Rad), and 0.2 mM gene-specific primers (Supplementary Table S1 at *JXB* online). The relative expression level of each transcript was analysed using Gene Expression Analysis for the iCycleriQ Real-Time PCR Detection System (Bio-Rad). Transcript levels were normalized to the expression of *ACTIN7*. Each experiment was repeated at least three times.

Plant hormone analysis

Arabidopsis plants were grown on MS medium for 8 d and then approximately 500 mg (fresh weight) of each sample (Col-0, *MYBH-OX*, and *mybh*) was collected. Whole seedlings were frozen and homogenized with liquid nitrogen. Plant hormones were extracted at 4 °C with 3 ml of 80% methanol that contained 1% acetic acid. The extraction was repeated twice with a 1 h incubation in the first extraction and a 10 min incubation in the second extraction. The supernatant was collected after centrifugation at 3000*g* for 10 min and then filtered with a 3 ml capacity RESERVOIR-2 FRITS (Varian). The filtrates were evaporated *in vacuo* using a centrifuge evaporator (SpeedVac). The dried sample was dissolved in 1 ml methanol, which was subsequently removed by evaporation. Phytohormones were quantified by liquid chromatography-tandem mass spectrometry by the Growth Regulation Research Group at RIKEN Plant Science Center. GA and indole-3-acetic acid (IAA) were analysed as described previously (Yoshimoto *et al*., 2009).

Subcellular localization of MYBH

The *CaMV35S::MYBH:GFP* construct was used for the subcellular localization of MYBH in onion epidermal cells. The plasmid DNA (5 µg per experiment) was precipitated onto gold microparticles. Onion (*Allium cepa*) epidermal cells were grown on MS agar prior to transfection by particle bombardment (Bio-Rad, http://www.bio-rad.com/). After bombardment, the cells were placed in the dark at 23 °C for 18–48 h. Green fluorescent protein (GFP) fluorescence was visualized via confocal lase- scanning fluorescence microscopy (LSM 5 Exciter; Carl-Zeiss, http://www.zeiss.com/).

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (Duncan's test) at a 95% confidence level. Differences were considered statistically significant when $P < 0.05$.

Accession numbers

Sequence data for this article can be found in the *Arabidopsis* Genome Initiative databases under the following *Arabidopsis* Information Resource accession numbers: *MYBH* (AT5G47390), *PIF4* (AT2G43010), *PIF5* (AT3G59060), *HY5* (AT5G11260), *CAB* (AT1G29910), *EXP3* (AT2G37640), *YUCCA8* (AT4G28720), and *SAUR-like* (AT2G21220 and AT2G45210).

Results

Enhanced hypocotyl elongation in Arabidopsis MYBH-OX *plants*

Although many *MYB* genes that play important roles in various cellular processes have been identified, many of them have not yet been characterized at the molecular level. Therefore, we decided to study the functions of *MYB* genes with respect to growth performance and stress tolerance. As most *MYB* genes exist in family groupings (Dubos *et al*., 2010), we thought that their knockout mutants may not show significant alterations in growth or stress tolerance because of redundancy. Therefore, we attempted to ectopically express various *MYB* genes in *Arabidopsis* and then recovered the *MYBH* gene that was involved in hypocotyl elongation. *MYBH-OX* plants showed significantly increased hypocotyl elongation under the light growth condition compared with wild-type plants (Fig. 1).

To understand the function of *MYBH* in hypocotyl elongation, a *mybh* knockout mutant (GK-783B02) was obtained for our experimental analysis. However, no significant differences in hypocotyl elongation were detected between the *mybh* mutant and Col-0 under the light or dark continuous conditions (Fig. 1A, B). In contrast, substantial differences in root growth were observed in the *mybh* line, and this defect could be recovered by the recombinant expression of wild-type *MYBH* (Fig. 1A and Supplementary Fig. S1 at *JXB* online). Additionally, the *MYBH-OX* seedlings exhibited longer hypocotyl cells than did Col-0 seedlings (Fig. 1C), and the *MYBH-OX* plants exhibited altered phenotypes when grown on MS medium under continuous light (Fig. 2). The *MYBH-OX* plants had darker leaves than the Col-0 plants. Moreover, leaf curling and increased root hair number were observed only in the transgenic plants (Fig. 2).

A schematic representation of MYBH, including its putative functional domains, is shown in Supplementary Fig. S2 at *JXB* online. *MYBH* encodes a MYB-like transcription factor; thus MYBH was expected to localize to the nucleus. MYBH localization was assessed using a construct containing a fusion between *MYBH* and the *GFP* reporter gene. The *CaMV35S::MYBH:GFP* construct was transiently expressed in onion epidermis and stably transformed into *Arabidopsis*. As predicted, the MYBH–GFP fusion protein localized to the nucleus in both the onion and *Arabidopsis* (Supplementary Fig. S3 at *JXB* online).

MYBH promoter activity is regulated by light

As expected, *MYBH* transcript levels were increased in *MYBH-OX* seedlings; however, for both the *MYBH-OX* and Col-0 seedlings, the highest transcript levels were observed in

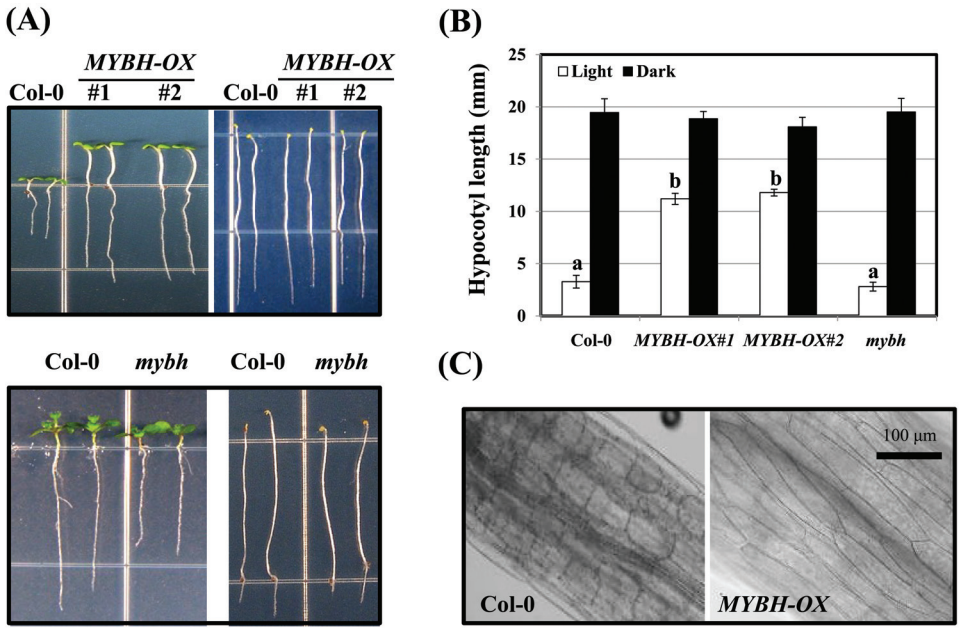

Fig. 1. Enhanced hypocotyl elongation in *MYBH-OX* seedlings. Hypocotyl elongation phenotype (A) and length quantification (B) of wild-type seedlings (Col-0), *MYBH-OX* (#1 or #2), and *mybh* at 7 d after germination under light or in the dark. In (A), the left and right panels show seedlings that were grown in the light and dark, respectively. Three independent experiments were conducted (n=100). One-way analysis of variance (Duncan's test) was conducted to indicate significant differences (P <0.05) in hypocotyl length between Col-0, *MYBH-OX* (#1 or 2), and *mybh*. Columns labelled above with the letter a are significantly different (P <0.05) from those labelled with b. Vertical bars represent standard error (n=300). (C) Hypocotyl cells in wild-type (Col-0) and *MYBH-OX* (#1) seedlings. Seven-d-old seedlings were grown under normal conditions and used for these photos. The samples were visualized using a microscope (LSM 5 Exciter, Carl-Zeiss). (This figure is available in colour at *JXB* online.)

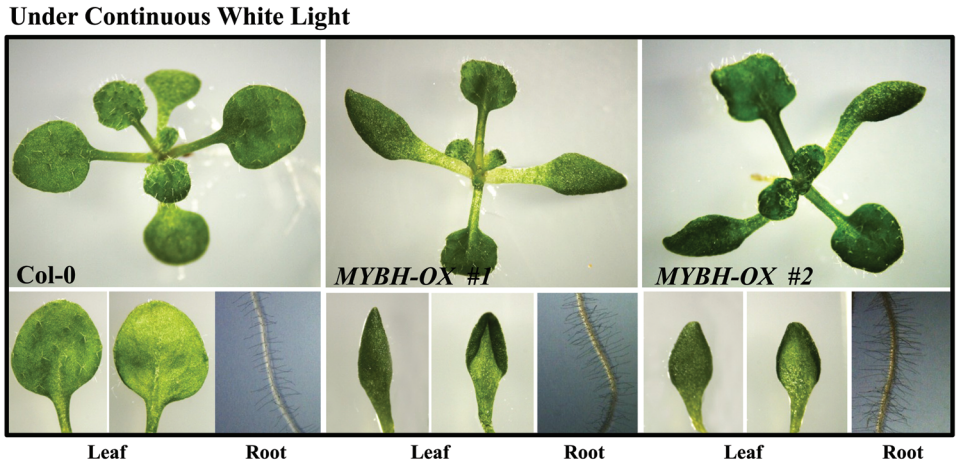

Fig. 2. Phenotypic changes in *MYBH-OX* seedlings grown under continuous light. Col-0 and two lines of *MYBH-OX* (#1 and #2) seedlings were allowed to germinate on MS medium (2% sucrose) under continuous light for 14 d prior to being transferred to soil. The root samples were visualized using a microscope. The leaves of *MYBH-OX* (#1 and #2) seedlings were darker in colour, more curled, and had increased root hair numbers compared with those of Col-0. (This figure is available in colour at *JXB* online.)

plants grown in continuous dark conditions (Fig. 3A). These results suggested the existence of post-transcriptional regulatory mechanisms that stabilize *MYBH* transcript levels. As altered hypocotyl elongation was observed in *MYBH-OX* plants grown under light conditions, the effect of light on promoter activity was examined by fusing ~1 kb of the sequence located upstream of the *MYBH* start codon to the *GUS* reporter gene. Surprisingly, *GUS* was only expressed at low levels in the light, whereas its expression increased strongly in the dark (Fig. 3B and Supplementary Fig. S4 at *JXB* online). *GUS* expression became apparent 1 h after *MYBHpro::GUS* seedlings were transferred from the light to the dark (Fig. 3C). Moreover, when dark-grown *MYBHpro::GUS* seedlings were exposed to light, *GUS* expression decreased gradually (Fig. 3C and Supplementary Fig. S4).

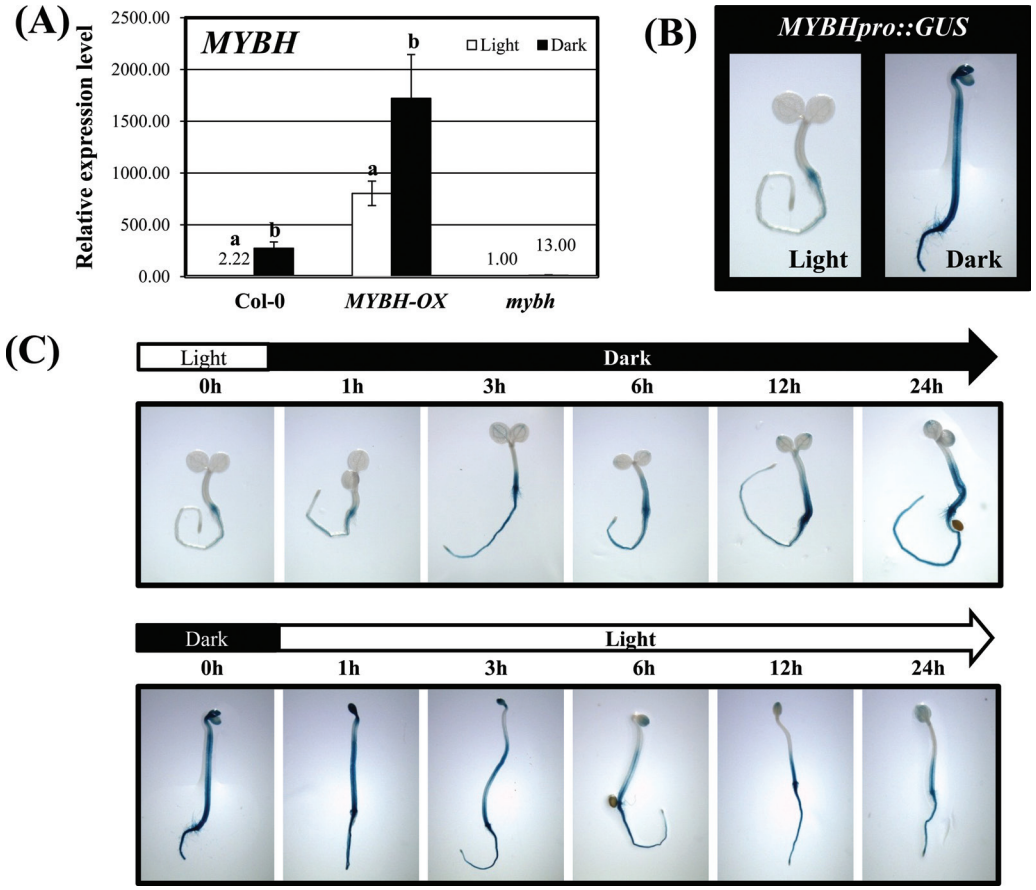

Fig. 3. The promoter activity of *MYBH* is regulated by light. (A) qRT-PCR was performed to determine *MYBH* transcript levels in wild-type (Col-0), *MYBH-OX*, and *mybh* plants that were grown in the dark or light. Three independent experiments were conducted. The error bars represent standard deviations. (B) Transgenic plants that harboured *MYBHpro::GUS* were grown on MS medium under normal light or continuous darkness for 3 d prior to histochemical GUS staining. (C) The *MYBHpro::GUS* seeds were sown on MS medium under light (top panel) or continuous darkness (bottom panel). After 3 d, the seedlings were transferred from light to dark (top panel) or from dark to light (bottom panel) for the indicated times. The *MYBHpro::GUS* transgenic seedlings were then collected for histochemical GUS staining at 0, 1, 3, 6, 12, and 24 h after exposure. Three independent experiments were conducted. (This figure is available in colour at *JXB* online.)

Hypocotyl elongation is suppressed by inhibition of GA biosynthesis in MYBH-OX *plants*

The presence of long hypocotyls in various *Arabidopsis* mutants revealed that phytohormones are important for the determination of this phenotype. In the present study, several inhibitors of hormone biosynthesis or activity were used to monitor the recovery of normal hypocotyl elongation in *MYBH-OX* seedlings. The GA biosynthesis inhibitor paclobutrazol (PAC) effectively restored hypocotyl length in *MYBH-OX* seedlings that were grown in the light (Fig. 4). In contrast, the Trp analogue 5-methyltryptophan (5-MT) was not able to suppress hypocotyl elongation in the *MYBH-OX* seedlings (Fig. 4). However, root development was strongly inhibited in *MYBH-OX* seedlings in response to high concentrations of 5-MT (Supplementary Fig. S5 at *JXB* online), indicating that the shoot and root tissues exhibit different sensitivities to 5-MT. The reason for this is not clearly understood. The *mybh* mutants were also observed to be highly sensitive to PAC and IAA (Fig. 4).

The sensitivity of *MYBH-OX* seedlings to PAC could be due to alterations in their GA levels. Therefore, hormone levels were determined in whole seedlings of Col-0, *MYBH-OX*, and *mybh* that were grown in MS medium for 8 d under normal light condition. Compared with Col-0 seedlings, *MYBH-OX* plants showed slightly elevated levels of auxin (IAA) but not GA (Fig. 5). The *mybh* mutants exhibited slightly decreased levels of auxin relative to Col-0. However, the *MYBH-OX* and *mybh* mutant seedlings did not show altered levels of the bioactive form GA_4 (Fig. 5A).

To examine the auxin sensitivity of the *MYBH-OX* seedlings, *MYBH-OX*/*DR5::GUS* F_3 progeny were obtained by crossing *MYBH-OX* with *DR5::GUS* plants that harboured a reporter system responsive to auxin levels. As shown in Fig. 6A, *GUS* expression that was driven by the *DR5* promoter was stronger in the *MYBH-OX* background than in the wild-type plants, which confirmed the result shown in Fig. 5B. Additionally, the *MYBH-OX* seedlings also produced more lateral roots than the wild-type seedlings (Fig. 6B), and the enhanced lateral root development was

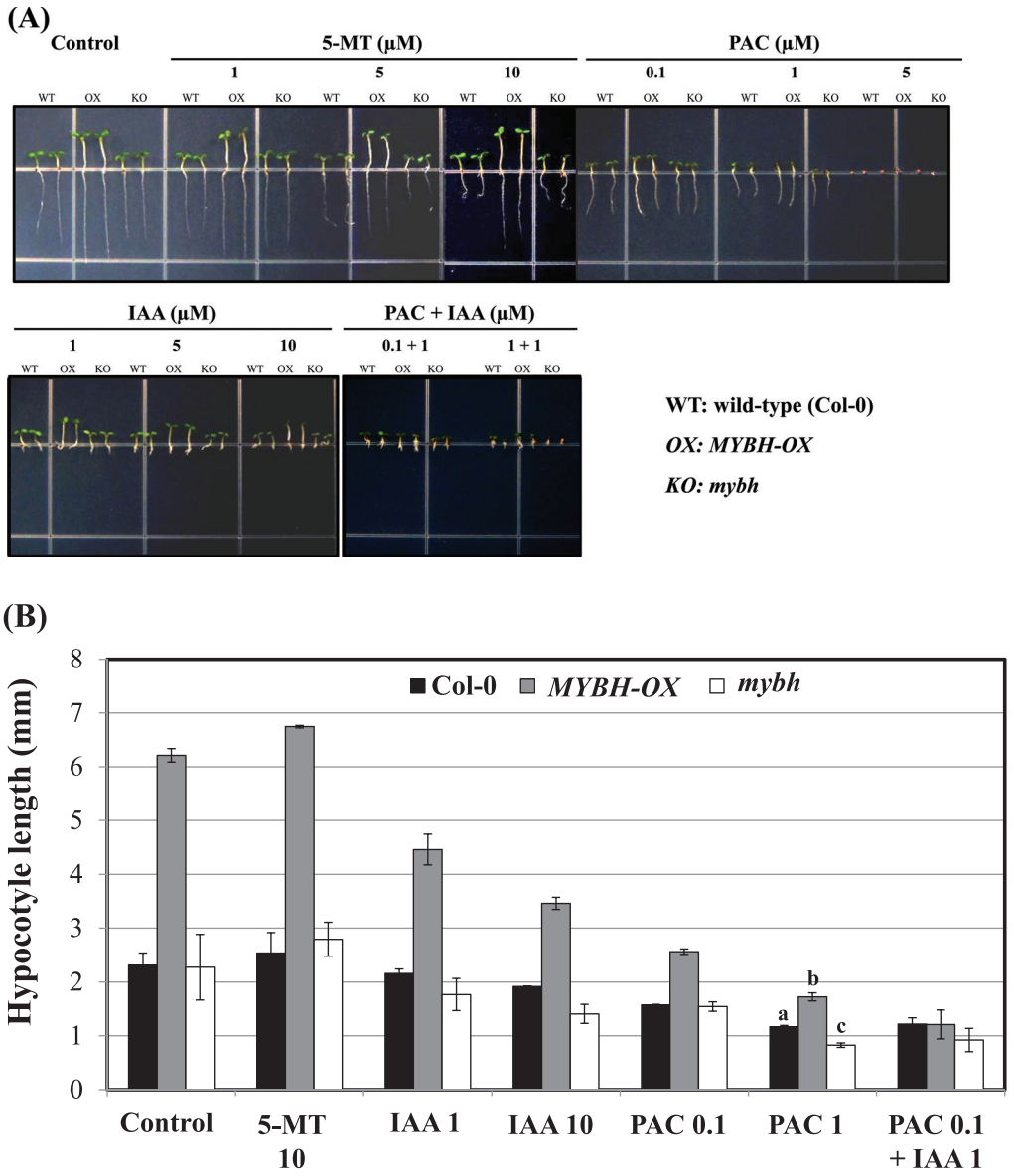

Fig. 4. Hypocotyl elongation of *MYBH-OX* seedlings in response to 5-MT, PAC, or IAA. Hypocotyl phenotype (A) and length quantification (B) of wild-type (Col-0), *MYBH-OX*, and *mybh* seedlings grown on MS medium that was supplemented with the indicated concentrations of 5-MT, PAC, or IAA. 5-MT did not affect hypocotyl elongation of *MYBH-OX*, while PAC and IAA showed inhibition hypocotyl elongation in all experimental samples. Three independent experiments were conducted. The letters a, b, and c above the columns represent statistically significant differences (P <0.05). Vertical bars show the standard error (n=30). (This figure is available in colour at *JXB* online.)

more obvious when the *MYBH-OX* seedlings were grown on MS medium that was supplemented with higher concentrations of sucrose (Supplementary Fig. S6 at *JXB* online). Furthermore, qRT-PCR was used to examine whether auxin-responsive genes or auxin biosynthetic genes were altered in the *MYBH-OX* seedlings. The transcript level of the auxin biosynthetic gene *YUCCA8* was increased in the *MYBH-OX* seedlings under both the dark and light conditions (Fig. 7A). In addition, other auxin-responsive marker genes such as the *SAUR* (small auxin responsive) genes were upregulated in the *MYBH-OX* seedlings, which could explain their increased auxin levels (Fig. 7B).

CHLOROPHYLL-BINDING PROTEIN (CAB) and EXPANSIN are up- and downregulated, respectively, in mybh *plants*

Although the *mybh* mutant did not exhibit shorter hypocotyls than Col-0, the expression of several genes differed among Col-0, *MYBH-OX*, and *mybh*. qRT-PCR was used to examine the transcript levels of the *CAB* and *EXPANSIN3* genes in these same plants. Transcription of *CAB*, which is involved in plant photomorphogenesis, was upregulated in the *mybh* mutant and downregulated in the *MYBH-OX* plants (Fig. 7C). Otherwise, the *MYBH-OX* seedlings showed

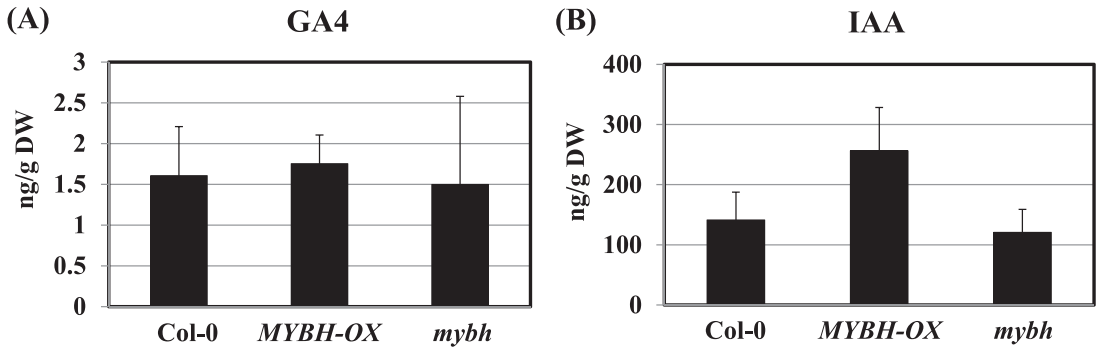

Fig. 5. Endogenous auxin and gibberellin (GA_4) accumulation in Col-0, *MYBH-OX*, and *mybh* seedlings. Wild-type (Col-0), *MYBH-OX*, and *mybh* seedlings were grown on MS medium for 8 d under normal light condition and used for the extraction and quantification of GA_4 (A) and IAA (B). No significant differences were observed. Vertical bars show the standard error (n=3).

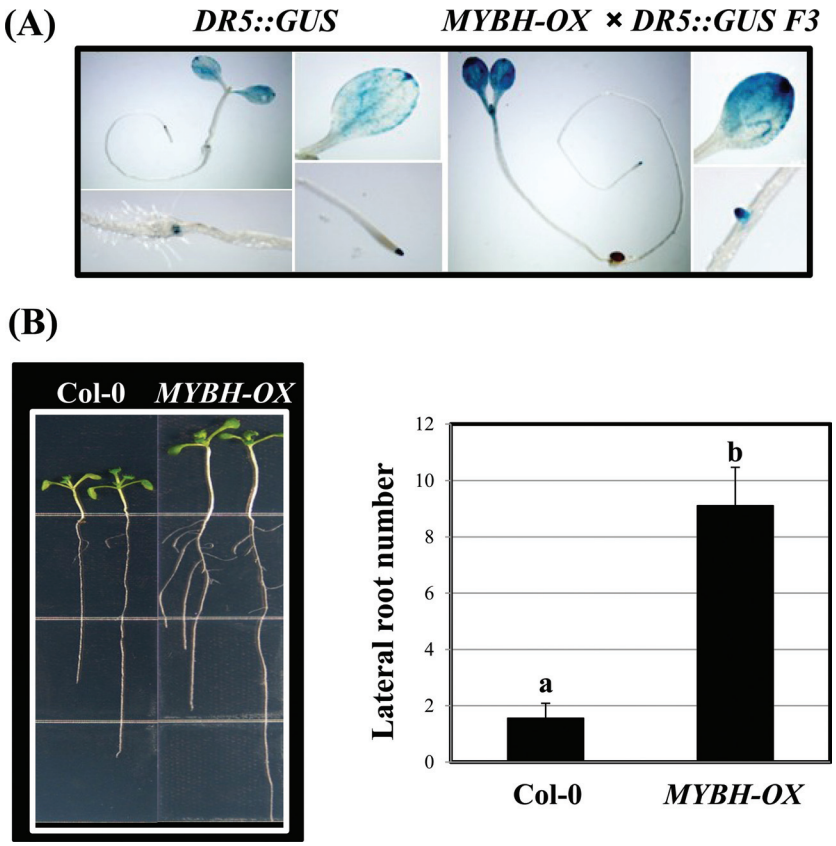

Fig. 6. Increased auxin sensitivity in *MYBH-OX* seedlings. (A) Histochemical GUS staining of *DR5::GUS* and *DR5::GUS*×*MYBH-OX* F3 seedlings. (B) Wild-type (Col-0) and *MYBH-OX* seedlings were grown on MS medium for phenotype observation and counting of the lateral roots. Three independent experiments were conducted. Columns labelled above with the letter a are significantly different (P <0.05) from those labelled with b. Vertical bars show the standard error (n=30). (This figure is available in colour at *JXB* online.)

elevated *EXPANSIN3* transcript levels, which may well explain the increased hypocotyl elongation that was detected in these plants (Fig. 7C). MYBH contains a MYB domain that could function in the transcriptional regulation of *HY5*, which is key player in the mediation of light signalling in plants. Thus, transcription of *HY5* in the Col-0, *mybh*, and *MYBH-OX* plants was assessed via Northern blotting. However, no significant difference in *HY5* expression was observed in these plants in response to light or dark conditions (Fig. 8A, B).

PIF4 or PIF5 is required for increased hypocotyl elongation in MYBH-OX *plants under light conditions*

PIFs are key players in the regulation of skotomorphogenesis in plants; therefore, qRT-PCR was used to examine *PIF* transcript levels in the Col-0, *mybh*, and *MYBH-OX* plants. *PIF4* and *PIF5* expression levels were upregulated in the *MYBH-OX* plants (Fig. 8C, D). To determine whether *MYBH* is involved in the PIF-dependent light signalling

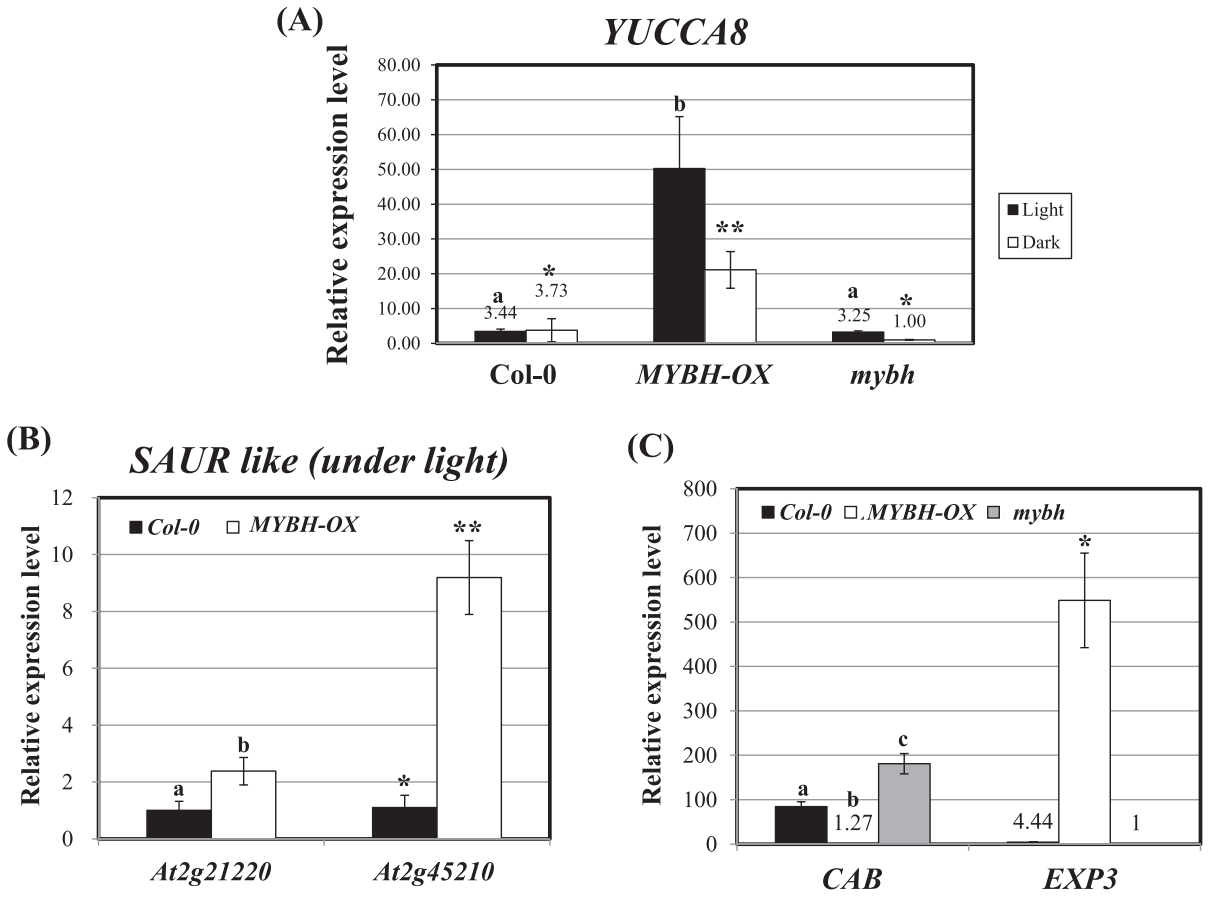

Fig. 7. Expression level of genes related to auxin and light signalling in *MYBH-OX* plants. qRT-PCR was performed to evaluate the level of expression of several genes: *YUCCA8* (A), *SAUR-like* (B) and *CAB* and *EXP3* (C). Seven-d-old seedlings of wild-type (Col-0), *MYBH-OX*, and *mybh* grown in light or continuous darkness were used for RNA extraction and qRT-PCR. The experiments were performed three times with 25 cycles of PCR amplification. Values are expressed as the means of three replicates. Columns labelled with the letter a are significantly different (P <0.05) from those labelled with b; columns labelled with a single asterisk are significantly different (P <0.05) from those labelled with a double asterisk. Vertical bars show the standard error (n=3).

pathway, *MYBH-OX* plants were crossed with *pif5*, and *pif4pif5* mutants. The *MYBH-OX*/*pif5* F_3 and *MYBH-OX*/*pif4pif5* F_3 seedlings exhibited inhibition of hypocotyl elongation in the light, suggesting that the function of MYBH is PIF dependent (Fig. 9B–E). We also generated *MYBH-OX*/*hy5* F_3 seedlings and found that hypocotyl elongation was not additive (Fig. 9A).

As the phenotype of the *mybh* mutant seedlings was not significantly different from that of Col-0 plants, *mybh* antisense (*mybh-AS*) lines were also generated. Although the *MYBH* transcript was not detected in most of the antisense lines, truncated bands were observed in several lines (Supplementary Fig. S7 at *JXB* online). Under light conditions, the Col-0 and *mybh-AS* seedlings exhibited similar hypocotyl lengths (Fig. 10A, B). However, the *mybh-AS* lines developed shorter primary roots than those of the Col-0 plants (Fig. 10A). In contrast, when grown in the dark, the hypocotyls of the *mybh-AS* plants were significantly shorter than those of the Col-0 plants (Fig. 10A, B). On the other hand, cotyledon structures were observed in Col-0, *mybh-AS*, *mybh*, and *MYBH-OX* seedlings after germination in the continuous dark condition. While the *MYBH-OX* seedlings had a folded apical hook, the apical hook of the *mybh-AS* seedlings was fully open, indicating that MYBH plays a crucial role in light signalling (Fig. 10D). As shown in Supplementary Fig. S8 at *JXB* online, the *MYBH* gene has some close homologues in *Arabidopsis*; thus, effects on the expression of these genes in the *mybh-AS* plants may explain the phenotypic differences between *mybh-AS* and *mybh* plants.

Discussion

In this study, the molecular mechanisms that underlie increased hypocotyl elongation due to *MYBH* overexpression were characterized. Three lines of evidence were presented that indicate *MYBH* involvement in the regulation of skotomorphogenesis. First, the elongation of hypocotyls was enhanced in *MYBH-OX* plants. Increased hypocotyl elongation is a known phenotype of plants that are grown in the dark. However, although no significant differences in hypocotyl length were observed between the Col-0 plants and *mybh* mutants, the mutant plants exhibited much shorter

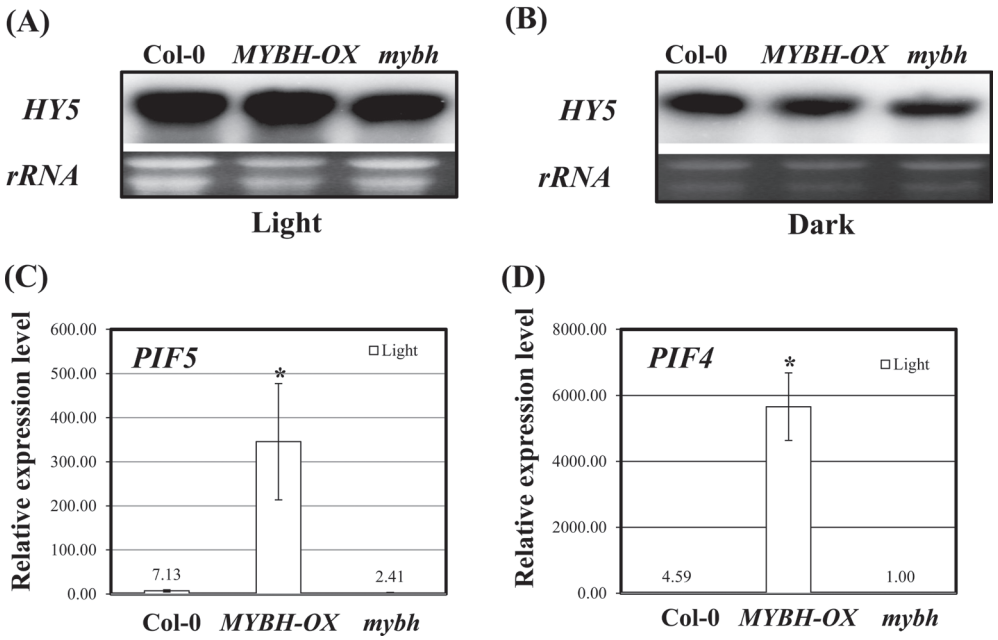

Fig. 8. Expression level of *HY5*, *PIF5*, and *PIF4* in *MYBH-OX* plants. Northern blotting and qRT-PCR were performed to evaluate the level of expression of several genes: *HY5* (A and B), *PIF5* (C) and *PIF4* (D). Samples were collected from 7-d-old wild-type (Col-0), *MYBH-OX* and *mybh* seedlings for total RNA preparation. The experiments were performed three times with 25 cycles of PCR amplification. Values are expressed as the means of three replicates. Columns labelled with an asterisk are significantly different (P <0.05) from those without an asterisk. Vertical bars show the standard error (n=3).

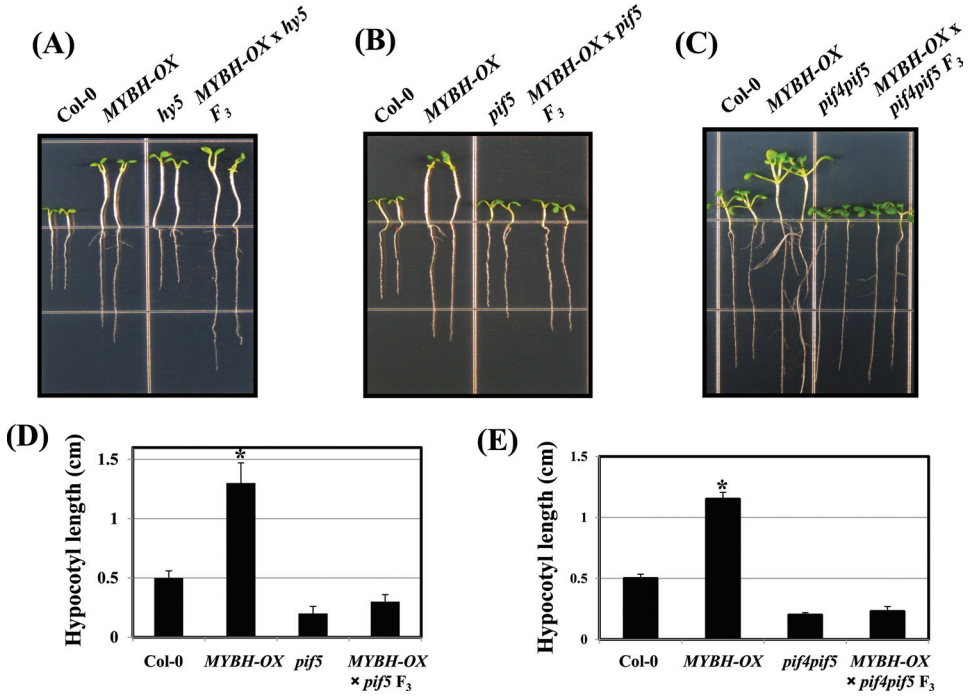

Fig. 9. Hypocotyl elongation phenotypes of F_3 progeny of *MYBH-OX*×*hy5*, *MYBH-OX*×*pif5*, and *MYBH-OX*×*pif4pif5*. The DNA for genotyping was obtained from F3 progeny seedlings of *MYBH-OX*×*hy5* (A), *MYBH-OX*×*pif5* (B), and *MYBH-OX*×*pif4pif5* (C). After germination under light, 7-d-old seedlings from Col-0, *MYBH-OX*, and each homozygous mutant were selected for quantification of hypocotyl elongation (D, E). Three independent experiments were conducted. Columns labelled with an asterisk are significantly different (P <0.05) from those without an asterisk. Vertical bars show the standard error (n=30). (This figure is available in colour at *JXB* online.)

roots and smaller leaves than the Col-0 plants (Fig. 1), indicating that MYBH may play an additional crucial role in the light, although this possibility was not examined in the current study. Secondly, using a GUS reporter system and qRT-PCR, we observed increased *MYBH* expression during growth in the dark. Stronger *GUS* expression was observed

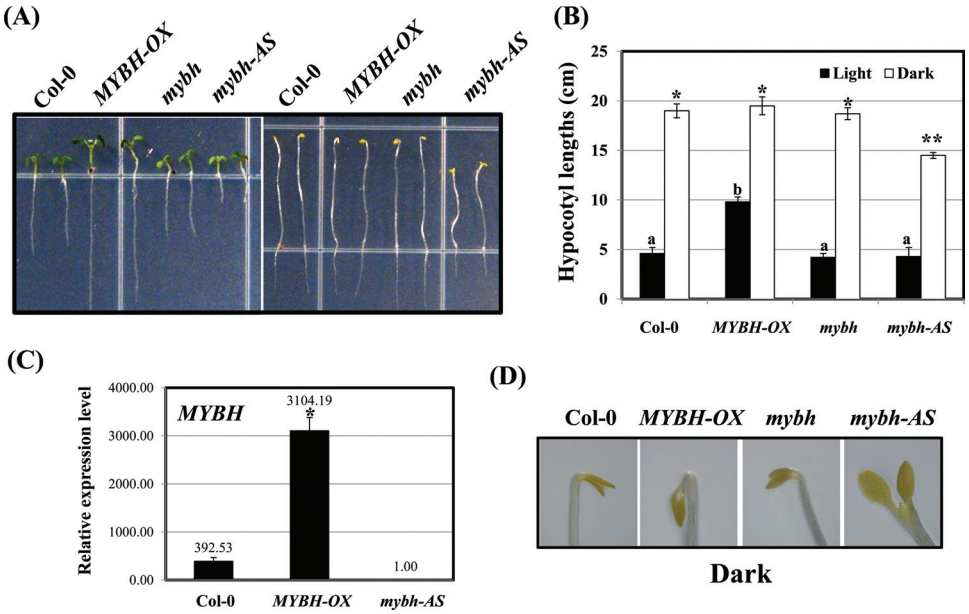

Fig. 10. *mybh-AS* plants exhibit shorter roots than wild-type seedlings in the light. Wild-type (Col-0), *MYBH-OX*, and *mybh-AS* seedlings were germinated on MS medium under light or continuous darkness for 7 d. The hypocotyl elongation phenotypes (A) and quantification (B) of each line of seedlings were compared with Col-0. Three independent experiments were conducted. Columns labelled above with the letter a are significantly different (P <0.05) from those labelled with b; columns labelled above with a single asterisk are significantly different (P <0.05) from those labelled with a double asterisk. Vertical bars show the standard error (n=100). (C) The expression level of *MYBH* was determined by qRT-PCR. The column labelled above with an asterisk is significantly different (P <0.05) from those without an asterisk. Vertical bars show the standard error (n=3). (D) The cotyledons of wild-type (Col-0), *MYBH-OX*, *mybh*, and *mybh-AS* seedlings that were grown in the dark for 7 d are shown. (This figure is available in colour at *JXB* online.)

in the hypocotyls, and positive staining was detected in the leaves and roots (Fig. 3), indicating that *MYBH* is expressed locally and is important for hypocotyl elongation. Moreover, *MYBH* promoter activity was reduced rapidly upon exposure to light (Fig. 3B). It remains unclear whether the reduction in promoter activity was due to repression by the light or activation by the dark. Thirdly, the *MYBH-OX* plants exhibited reduced expression of *CAB*, which is a marker of photomorphogenesis (Fig. 7C). The fact that the *mybh-AS* seedlings developed much shorter hypocotyls in the dark suggests that the function of *MYBH* can be replaced by homologous genes, as the expression of these genes may be downregulated in the *mybh-AS* seedlings (Fig. 10).

GA has been implicated in cell expansion in the hypocotyl (De Lucas *et al.*, 2008). Because the hypocotyl lengths of the *MYBH-OX* plants were comparable to those of the Col-0 seedlings in the presence of PAC, we thought that *MYBH-OX* plants may have increased levels of GA. However, the levels of active GA_4 were not different in the *MYBH-OX* plants and Col-0 plants that were grown under normal conditions (Fig. 5A). Instead, the auxin levels were slightly enhanced in the *MYBH-OX* plants, and altered auxin sensitivity in the *MYBH-OX* plants was detected in the *DR5::GUS*×*MYBH-OX* F3 seedlings (Fig. 6A). Moreover, the *MYBH-OX* plants produced more lateral roots than the Col-0 plants during normal growth, suggesting that overexpression of the *MYBH* gene led to increased auxin biosynthesis (Figs 5B and 6B). This notion was further supported by the observation that the auxin biosynthetic gene (*YUCCA8*) and auxin marker genes (*SAUR*s) were upregulated in the *MYBH-OX* plants (Fig. 7A, B).

PIF transcription factors function in promoting skotomorphogenic growth in the dark by controlling downstream gene expression (Leivar *et al.*, 2008). *MYBH* expression was enhanced in the dark, which suggests that MYBH might function in a PIF-dependent manner in hypocotyl elongation. Moreover, *PIF4* and *PIF5* transcript levels were significantly elevated in the *MYBH-OX* plants, which led us to examine the role of PIFs in the enhancement of hypocotyl elongation in the *MYBH-OX* seedlings (Fig. 8C, D). As illustrated in Fig. 9, MYBH appears to require PIF proteins to enhance hypocotyl elongation. PIF4 is known to play an essential role in high temperature-mediated morphological adaptation (Koini *et al.*, 2009), and binds to genes that are responsible for auxin biosynthesis, such as *YUCCA8* and *CYP79B2* (Franklin *et al.*, 2011; Sun *et al.*, 2012). An increase in accumulation of *YUCCA8* transcripts was also observed in the *MYBH-OX* plants (Fig. 7A). Thus, it appears that the increased auxin levels were due to the increased accumulation of PIF4 in the *MYBH-OX* seedlings, which led to increased hypocotyl elongation (Supplementary Fig. S9 at *JXB* online). Recently, the *Arabidopsis MYB* gene *REVEILLE 1* (*RVE1*) was shown to be involved in auxin biosynthesis and to promote hypocotyl elongation (Rawat *et al.*, 2009). *RVE1* was shown to mediate cell elongation independently of PIF4 and PIF5. Therefore, although auxin biosynthesis is regulated by both RVE1 and MYBH, their effects on hypocotyl elongation clearly occur through different mechanisms. Recently, it was also suggested that PIF4 and/or PIF5

may regulate hypocotyl elongation via control of the auxin signalling pathway (Nozue *et al*., 2011). It was shown that *pif4pif5* mutants are less sensitive to auxin in terms of the growth inhibition response to high auxin concentrations, whereas plants that overexpress *PIF5* are more sensitive. Because exogenous auxin does not recover the short-hypocotyl phenotype of the *pif4pif5* mutants, PIF5 and/or PIF4 do not seem to regulate auxin levels (Nozue *et al*., 2011). It was speculated in these studies that PIF5 and/or PIF4 may transcriptionally regulate one or more master regulator(s) of auxin sensitivity. However, the precise roles of auxin- or GA-regulated molecular components of hypocotyl elongation have not been established.

The auxin-related phenotype of the *MYBH-OX* plants observed in this study could be due to the altered levels of PIF4 and/or PIF5 interfering with the auxin signalling pathway (Supplementary Fig. S9). Compared with Col-0 plants, the *MYBH-OX* seedlings did not show significant differences in expression of the bZIP transcription factor *HY5*. This result indicates that enhanced hypocotyl elongation is not due to a reduction in HY5 activity. It appears that EXPANSIN is also involved in the enhanced hypocotyl elongation of *MYBH-OX* plants. *EXP3* expression was upregulated in the *MYBH-OX* seedlings and reduced in the *mybh* plants, while the photomorphogenesis marker gene *CAB* showed the opposite pattern of gene expression. The fine modulation of hypocotyl elongation in response to light is very important for seedling development. When the amount of light is insufficient, seedlings must continue to grow by elongating their hypocotyls. Otherwise, they will remain in the shade and be unable to perform photosynthesis. Therefore, the proper regulation of hypocotyl elongation requires a very delicate and efficient mechanism that must be responsive to changes in environmental conditions. The findings of this study support the possibility that the regulation of hypocotyl elongation may be achieved via a complex web of molecular components that regulate auxin levels.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Complementation assay.

Supplementary Fig. S2. Schematic of the *MYBH* gene and protein.

Supplementary Fig. S3. Subcellular localization of MYBH.

Supplementary Fig. S4. Light-dependent activity of the *MYBH* promoter.

Supplementary Fig. S5. Effect of the auxin biosynthesis inhibitor 5-methyltryptophan on *MYBH-OX* seedling growth.

Supplementary Fig. S6. Increase in lateral root number of *MYBH-OX* in response to sucrose.

Supplementary Fig. S7. *MYBH* transcript levels in Col-0, *MYBH-OX*, *mybh-AS*, and *mybh* plants.

Supplementary Fig. S8. *MYBH* homologues in *A. thaliana.*

Supplementary Fig. S9. Working model of MYBH activity during dark-induced hypocotyl elongation.

Supplementary Table S1. Sequences of the primers used in this study.

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