The Arabidopsis homeobox gene, *ATHB16,* regulates leaf development and the sensitivity to photoperiod in Arabidopsis

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Abstract

This report describes the characterisation of *ATHB16,* a novel *Arabidopsis thaliana h*omeobox gene, which encodes a homeodomain-leucine zipper class I (HDZip I) protein. We demonstrate that ATHB16 functions as a growth regulator, potentially as a component in the light-sensing mechanism of the plant. Endogenous *ATHB16* mRNA was detected in all organs of Arabidopsis, at highest abundance in rosette leaves. Reduced levels of *ATHB16* expression in transgenic Arabidopsis plants caused an increase in leaf cell expansion and consequently an increased size of the leaves, whereas leaf shape was unaffected. Transgenic plants with increased *ATHB16* mRNA levels developed leaves that were smaller than wild-type leaves. Therefore, we suggest *ATHB16* to act as a negative regulator of leaf cell expansion. Furthermore, the flowering time response to photoperiod was increased in plants with reduced *ATHB16* levels but reduced in plants with elevated *ATHB16* levels, indicating that *ATHB16* has an additional role as a suppressor of the flowering time sensitivity to photoperiod in wild-type Arabidopsis. As deduced from the response of transgenic plants with altered levels of *ATHB16* expression in hypocotyl elongation assays, the gene may act to regulate plant development as a mediator of a blue light response.
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Introduction

The members of the HDZip family of plant transcription factors are characterized by the presence of a homeodomain, a DNA-binding sequence motif conserved between homeodomain proteins in different eukaryotes. A second conserved sequence motif, a leucine zipper, is shared among the HDZip proteins, but not present in other homeodomain proteins in plants, or in other eukaryotes (Bürglin, 1994).

The functions of the majority of the 42 members of the HDZip family in Arabidopsis (The Arabidopsis Genome Initiative, 2000) are unknown. The functional information available on HDZip genes of subclasses I and II (Sessa et

al., 1994) indicates that at least some of the genes may act to mediate the effects on plant growth and development of external factors, such as water availability (Söderman et al., 1996, 1999; Johannesson et al., 2003) and light (Carabelli et al., 1993, 1996; Steindler et al., 1999), or in response to internal metabolic signaling (Hanson et al., 2001). The most extensively studied HDZip gene; *ATHB2* (*Arabidopsis thaliana H*omeobox 2, also known as *HAT4*; Schena et al., 1993), has been demonstrated to act as a regulator of cell expansion, mediating the shading response (Carabelli et al., 1993, 1996; Steindler et al., 1999). In this report, we describe a novel member of the HDZip family, *ATHB16,* and demonstrate a role also for this gene in the plant growth response to light.

Light affects all aspects of plant growth and development, from germination and de-etiolation to leaf expansion, stem growth, floral initiation, and phototropism (Chory et al., 1996; Chory, 1997). Plants perceive light through photoreceptors. Two different types of photoreceptor have been

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identified (reviewed in Deng and Quail, 1999). The phytochromes absorb light primarily in the red and far-red regions of the visible spectrum, and the blue/UV-A light is absorbed mainly by cryptochromes and phototropins. The UV-B photoreceptor is as yet uncharacterized at a molecular level.

In Arabidopsis, five phytochrome genes, *PHYA-E* (Quail et al., 1995), and two cryptochrome genes, *CRY1* and *CRY2* (Ahmad and Cashmore, 1996; Lin et al., 1996a), have been identified. The different phytochromes and cryptochromes regulate either distinct responses or similar responses under different light conditions (light quantity, quality, and timing). Assays for the light-dependent inhibition of hypocotyl elongation have played a critical role in the genetic and functional studies of photoreceptors. The Arabidopsis cryptochrome gene, *CRY1,* was identified by the isolation of the *hy4* mutant, impaired in blue light inhibition of hypocotyl elongation (Koornneef et al., 1980; Ahmad and Cashmore, 1993). The second cryptochrome gene, *CRY2,* shares about 50% amino acid sequence identity to *CRY1.*

CRY1 mediates a range of blue light responses, including the accumulation of anthocyanin, the regulation of leaf and cotyledon expansion, the inhibition of hypocotyl elongation, and the expression of blue light-regulated genes (Short and Briggs, 1994; Ahmad and Cashmore, 1996; Lin et al., 1996b). CRY2 affects the timing of reproductive development more strongly than hypocotyl elongation, and the *cry2* mutant was found to be allelic to *fha* (Guo et al., 1998); a photoperiod insensitive late-flowering mutant that previously has been characterised by Koornneef (1991).

The transition from vegetative to reproductive growth in Arabidopsis is controlled by a complex set of regulatory mechanisms. Mutations at more than 20 loci have been reported to specifically affect the timing of flowering (reviewed in Levy and Dean, 1998; Mouradov et al. 2002), and additional mutations affect flowering time but also have pleiotropic effects on other aspects of development. A genetic analysis of these mutants has supported a model in which three major signaling pathways regulate the transition to reproductive growth. A photoperiodic promotion or long day promotion pathway operates in long days (LD), a second pathway is active primarily in short days (SD) and requires the phytohormone gibberellin, and a third, autonomous pathway is required both in LD and SD.

In this paper, we show that *ATHB16* regulates cell expansion in the leaf as well as the photoperiodic control of flowering, and provide evidence to suggest that the function of *ATHB16* is related to blue light signaling.

Materials and methods

Plant material and growth conditions

Wild-type and transgenic seeds of Arabidopsis ecotype Columbia (Col-0) were sterilized and cultured on growth medium consisting of 0.5× MS-medium (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with 0.8% (w/v) agarose and 1% (w/v) sucrose at 20°C under continuous warm white fluorescent light. After 8–10 days, the plantlets were transferred to a soil/vermiculite mixture in a culture room with long day photoperiod, LD (70–120 μmolm^{-2}s^{-1}), of cool white fluorescent light at 20°C.

For flowering time assays, seeds were cold treated for 4 days in the dark, spread on soil, and cultivated under cool white light at 20°C in culture room. The timing of transition to reproductive development was studied under long day (LD, 16 h light/8 h dark, 120 μmolm^{2}s^{-1}), equal day/night (EDN, 12 h light/12 h dark, 160 μmolm^{-2}s^{-1}), and short day (SD, 8 h light/16 h dark, 240 μmolm^{-2}s^{-1}) conditions.

For hypocotyl length measurements, seeds were spread evenly on growth medium, cold treated for 4 days in the dark, and exposed to white light (70 μmolm^{-2}s^{-1}) for 4 h to enhance germination. Following white light treatment, the seeds were moved to blue, red, or far-red light conditions and grown at 20°C for 4 days. Continuous red or far-red light was provided by Q-BEAM 2200 (Quantum Devices, INC.) with a light intensity of approximately 3 μmolm^{-2}s^{-1}. Continuous blue illumination was provided by white fluorescent light filtered through a blue plexiglass filter (2424 Blue; Polycast Technology Corp., Stamford, CT). Parallel batches of seeds were exposed for 4 days to cold treatment and transferred to 20°C in darkness for 4 days. Hypocotyl lengths were measured manually under a Leica-Wild-M10 stereomicroscope.

Analyses of variance (ANOVA) using multiple comparison (Fisher's PLSD, Scheffe's and Bonferroni/Dunn) were conducted to test differences in flowering time and hypocotyl length. The calculations were performed by using the StatView 4.01 software (abacus Concepts Inc.).

cDNA amplification, genomic library screening, and mapping of the ATHB16 locus

ATHB16 cDNA was amplified by use of the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Total RNA was prepared according to the protocol of Chang et al. (1993), and the polyA+ mRNA was isolated by the PolyAT tract mRNA Isolation System (Promega, High-Tech-park, Mannheim, Germany). The gene-specific primer used in the initial PCR was HB16:R1 (5′-CACAAACTGTAA-GAAACTCCCGCCAG-3′), and the gene-specific nested primer was HB16:R2 (5′-GGTGGTTGCCGGAATATTC-CTCGATTAGTG-3′).

A 2.9-kb *Eco*RI fragment, including the *ATHB16* coding sequence, was isolated from a genomic library of the Arabidopsis Columbia ecotype (EMBL, Heidelberg, Germany) by use of the full-length *ATHB16* cDNA as a probe. The map position of *ATHB16* was obtained by using simple sequence length polymorphism, SSLP (Tautz, 1989), and

Arabidopsis Col/Ler-recombinant inbred (RI) lines (Lister and Dean, 1993). *ATHB16* gene-specific primers 5′-GAAC-CATTGCTCTCTAGA-3′ and 5′-CGAGGAGACTTTA-GAGGCTC-3′ were used.

Construction of transgenic plants

The full-length coding sequence of the *ATHB16* cDNA (930 nt) was used for construction of transgenic plants expressing *ATHB16* sense mRNA, whereas an *ATHB16* cDNA fragment from the 5′-end of the coding region (410 nt, corresponding to amino acids 1–135) was used for expression of *ATHB16* antisense mRNA. The 930-nt and 410-nt fragments were ligated into the expression vector pHTT202 (kindly provided by Teemu Teeri, Univ. of Helsinki, Finland) behind the CaMV 35S promoter. The two constructs were introduced into the *Agrobacterium tumefaciens* strain C58 and transformed into Arabidopsis Col-0 plants via vacuum infiltration (Bechtold et al., 1993).

The offspring of transformed plants (T$_1$) was selected on growth medium supplemented with 50 μg/ml kanamycin. Kanamycin-resistant T$_1$ plants were self-fertilized, and the T$_2$ seeds were screened for 3:1 segregation and used for further analysis.

RNA gel blot analysis

RNA was extracted from different organs of 4-week-old Col-0 or from 2-week-old *ATHB16* transgenic plants, according to Chang et al. (1993). Samples of total RNA (15 μg) were denatured, subjected to electrophoresis on 1.2% agarose gels with 17% formaldehyde in 1× running buffer (10× running buffer is 0.2 M Mops, pH 7.0, 80 mM NaOAc, and 5 mM EDTA, pH 8.0), and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech). Transfer and hybridizations were performed according to the manufacturer's instructions. Then 0.50-kb and 0.51-kb DNA fragments corresponding to the gene-specific 3′ ends of the *ATHB16* and *ATHB6* cDNAs, respectively, were used as double-stranded probes. Single-stranded DNA probes were generated by asymmetric PCR (Gyllensten and Eelich, 1988) with full-length *ATHB16* cDNA as template. The reverse (5′-TGCCCACTTCTCTGTTTTC-3′) and forward oligonucleotide (5′-TCATGAAGAGACTAAGCAGC-3′) in a ratio of 1:50 and 50:1 were used as the PCR primers. Quantitative data on hybridization were obtained by use of a BAS 2000II Bio-Analyzer (Fuji, Tokyo, Japan) image plate reader.

Cell size determination and anatomy analysis

For scanning electron microscopy (SEM) analyses, the fifth rosette leaf of each plant was dissected and immediately fixed by incubation for 24 h at +8°C in ethanol:acetic acid:formaldehyde (50:5:3.7%) and dehydrated through an ethanol series to 100% ethanol. After critical point drying and 22-nm gold sputter coating, samples were analyzed by scanning electron microscopy, using an XL30 microscope (Philips Technologies, Cheshire, CT) with a LaB6 filament operating at 5 kV. The brightness and contrast of images were adjusted by use of the PhotoShop software (Adobe Inc., CA, USA).

For measurement of leaf area, all expanded rosette leaves were scanned by using an AGFA Studioscan II scanner (Gevaert N. V., Mortsel, Belgium). Leaf areas and epidermal cell areas were measured by using the public domain NIH Image 1.61 program (National Institutes of Health, Bethesda, MD).

Hormone treatments

Seeds of wild-type and *ATHB16* transgenic plants were allowed to germinate on growth medium. After 1 week, seedlings were tranferred to fresh medium containing 10, 1.0, or 0.1 μM indole-3-acetic acid (Sigma), GA$_3$ (Duchefa, Haarlem, The Netherlands), kinetin (Sigma), or epibrassinolide (Sigma), respectively. The development of rosette leaves was documented continuously.

For flowering time experiments, SD grown wild-type and *ATHB16* transgenic plants were sprayed once a week, from day 21 after planting, with a solution containing 0.1 mM GA$_3$ and 0.02% Tween 20. Control plants were treated with a solution containing only 0.02% Tween 20.

Results

ATHB16 is a new HDZip I gene

A database search for sequences similar to the HDZip I genes resulted in the identification of an EST clone, 72B4T7 (GenBank Accession no. R 86816), which corresponds to a previously noncharacterized HDZip gene which we refer to as *ATHB16*. By use of RACE, a 1410-bp full-length cDNA was isolated. The cDNA encodes a protein of 294 amino acids and a calculated molecular mass of 33,392. The amino acid sequence deduced from the cDNA contains a stretch of 60 residues, which shows a distinct similarity to homeodomains from other proteins. In addition, the sequence contains a leucine zipper motif with 5 leucines and 1 isoleucine occurring in every seventh position, C-terminal to the homeodomain, in a position similar to those of previously known HDZip proteins (Fig. 1). Four classes of HDZip proteins, HDZip I to IV, have been distinguished on the basis of amino acid sequence similarities within the homeodomain (Sessa et al., 1994). The ATHB16 amino acid sequence shows extensive similarity over the homeodomain region specifically to HDZip I proteins (Fig. 1). Overall, the HDZip I proteins share approximately 60% amino acid identity in the homeodomain, the most highly conserved
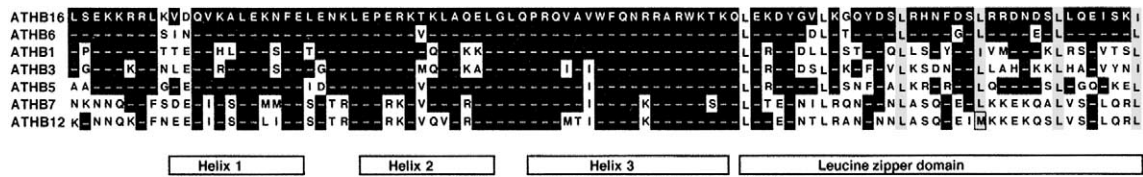
Fig. 1. Deduced amino acid sequences of the HDZip I proteins ATHB16, ATHB6, ATHB1, ATHB3, ATHB5, ATHB7, and ATHB12 in the region corresponding to the homeodomain and leucine-zipper domain. Amino acids conserved between ATHB16 and other proteins are displayed as white dashes. Leucines or isoleucines in the leucine-zipper domains are displayed in gray. The methionine in the leucine-zipper domain of ATHB12 is boxed.

part corresponding to the helix 3 region. In this comparison, ATHB16 shows a high degree of sequence similarity specifically to ATHB6 (Söderman et al., 1994); 93% amino acid identity over the homeodomain and 86% amino acid identity over the leucine zipper motif (Fig. 1).

A DNA sequence comparison of the full-length cDNA with a 2.9-kb genomic clone, including 1.3 kb of the *ATHB16* cDNA as well as 1.6 kb of upstream sequence (GenBank Accession no. AF076641), showed that the *ATHB16* open reading frame is split by two introns. One intron (120 bases in length, from nucleotide 1556 to 1676) was located upstream of the homeobox, and the second (90 bases in length, from nucleotide 2061 to 2151) downstream of the homeobox. Introns at identical positions in relation to the homeobox are found in most of the HDZip I genes (our unpublished observations).

By use of recombinant inbred lines (Lister and Dean, 1993), *ATHB16* was mapped to the bottom of chromosome IV. The distance to the nearest flanking marker, g3713 DHS1, is 1.9 cM. The position of *ATHB16* on chromosome IV was confirmed by the Arabidopsis Genome Initiative, 2000 (gene number At4g40060).

ATHB16 is expressed in most organs

In Northern blot experiments, *ATHB16* mRNA was detected in all organs examined, the mRNA level being relatively high in rosette leaves, intermediate in roots, cauline leaves, inflorescences, and buds, but low in the stem and in siliques, as shown in Fig. 2A. The *ATHB16* probe hybridized to a single band of an approximate size of 1400 nt, in agreement with the size of the amplified *ATHB16* cDNA.

Transgenic lines with altered levels of ATHB16 expression

To study the phenotypic effects of increased and reduced levels of *ATHB16* expression, the full-length coding sequence (930 bp) of *ATHB16* was transcriptionally fused to the constitutive CaMV 35S promoter in the sense (35S::*ATHB16*) orientation and a 410-bp fragment from the 5′-end of the coding sequence was fused to the same promoter in the antisense (35S::*antiATHB16*) orientation. The constructs were introduced into Arabidopsis by Agrobacte-

rium-mediated transformation. Forty 35S::*ATHB16* and 25 35S::*antiATHB16* independent primary transgenic lines were generated. In the T_2 generation, 15 and 13 of these transformant lines, respectively, segregated 3:1 for kanamycin resistance, indicating the presence of single insertions of the transgene in these lines. Homozygous plants, generated from these lines, were subjected to further analysis.

In Northern blot experiments, a single-stranded DNA probe complementary to antisense *ATHB16* RNA hybridized only to mRNA from the 35S::*antiATHB16* plants, not
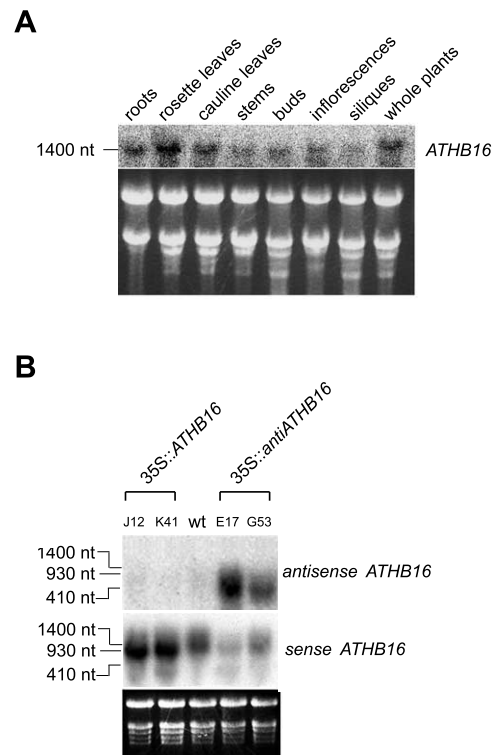

Fig. 2. Northern blot analysis of *ATHB16* mRNA levels. (A) The expression of *ATHB16* in different tissues of wild-type Arabidopsis. RNA was isolated from different tissues of plants grown in LD conditions for 4 weeks. (B) Accumulation of *ATHB16* sense and antisense mRNA in transgenic plants. RNA isolated from 35S::*ATHB16,* wild-type, and 35S::*antiATHB16* plants grown in LD for 2 weeks were blotted, and the same filter was probed with labeled antisense single-stranded *ATHB16* cDNA or sense single-stranded *ATHB16* cDNA, respectively. The lower panels in both (A) and (B) show the ethidium bromide stained gels prior to blotting.
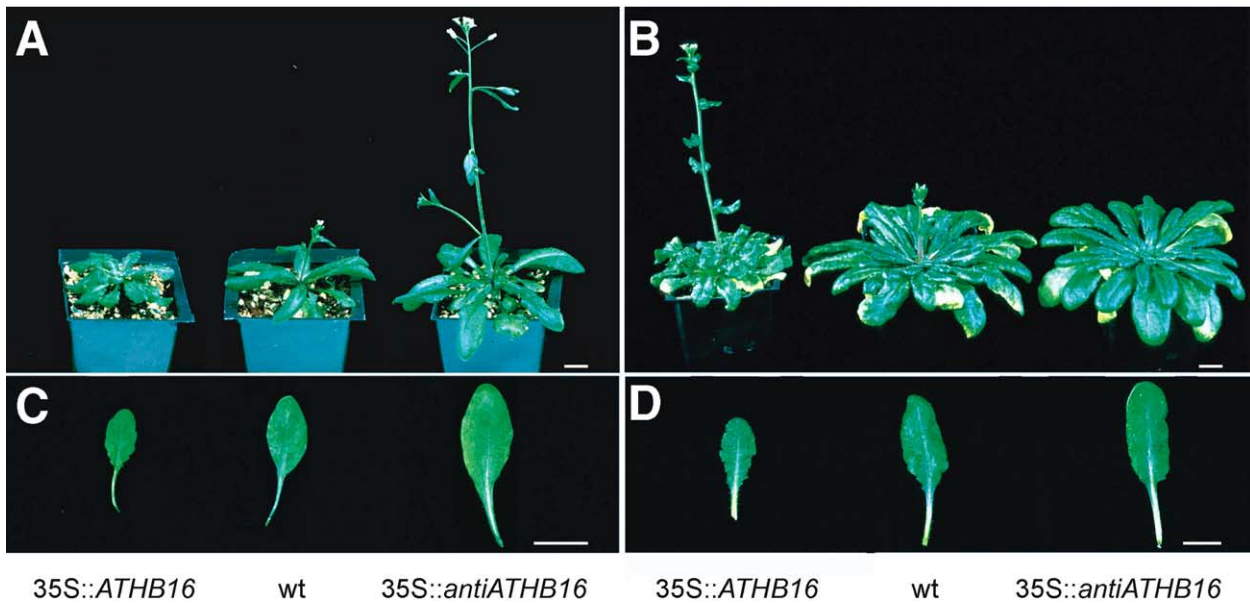
Fig. 3. Rosette leaf phenotype of *ATHB16* transgenic plants. Leaf rosette of homozygous of 35S::*ATHB16* plants (line J12), 35S::*antiATHB16* plants (line E17), and wild-type, cultured under LD (A), or SD photoperiod (B). (C) and (D) show the fifth rosette leaf of the plants in (A) and (B), respectively. The scale bars represent 1 cm.

to that from 35S::*ATHB16* or wild-type plants (Fig. 2B). The transcript detected by this probe in 35S::*antiATHB16* plants is approximately 400 nt, corresponding to the anti-sense transgene. In contrast, a probe complementary to sense single-stranded ATHB16 mRNA hybridized to the endogenous *ATHB16* mRNA (approximately 1400 nt) in wild-type and transgenic plants, as well as to the transcripts corresponding to the introduced 35S::*ATHB16* constructs, in the 35S::*ATHB16* plants (approximately 900 nt; Fig. 2B).

In five out of 15 35S::*ATHB16* homozygous lines tested, the amount of *ATHB16* transcript was estimated to be in-creased by a factor of 4–15 as compared with wild-type. Fig. 2B shows plants derived from two lines, J12 and K41, in which the *ATHB16* mRNA levels were increased by a factor of 10 and 8, respectively, as estimated by quantitative measurements of the relative intensity of signals. The re-maining 10 35S::*ATHB16* lines had *ATHB16* transcript lev-els that were similar to that of the wild-type. In two 35S::*antiATHB16* lines out of the 13 lines tested, the *ATHB16* transcript levels were significantly lower than that of wild-type (Fig. 2B). The endogenous *ATHB16* mRNA level, quantified from Fig. 2B and from repeated Northern blot analyses (data not shown), was reduced to 5–7% (E17) and 14–15% (G53) of the wild-type level, respectively. In lines expressing both the sense and antisense constructs (F_1 offspring of a cross between J12 and E17), the level of *ATHB16* mRNA was further reduced to 3% of wild-type level (data not shown). Effective gene silencing resulting from simultaneous expression of sense and antisense RNA was shown previously by Waterhouse et al. (1998). To investigate whether the 35S::*antiATHB16* construct affected

the expression of genes closely related to *ATHB16* (Fig. 1), we studied the *ATHB6* mRNA levels in the transgenic lines. In the 35S::*ATHB16* lines J12 and K41, the *ATHB6* mRNA levels were 93 and 113%, and in the 35S::antiATHB16 lines E17 and G53, the *ATHB6* mRNA levels were 98 and 126% of the wild-type level (data not shown), indicating that the *ATHB16* sense and antisense constructs did not affect *ATHB6* mRNA levels.

ATHB16 expression levels determine leaf size

The most obvious phenotypic changes observed in the *ATHB16* transformants were alterations in flowering time, in leaf expansion, and in shoot elongation. The degree to which the plants differed from wild-type differed between transformant lines and the severity of the phenotypic devi-ations from wild-type largely correlated with the levels of *ATHB16* expression. All five lines with significantly in-creased *ATHB16* mRNA levels showed similar phenotypic deviations. In this paper, we present data from one of these, line J12. Similarly, the two antisense lines with significant reductions in *ATHB16* mRNA levels showed similar phe-notypes. The data presented derive from the line E17. Other transgenic 35S::*ATHB16* or 35S::*antiATHB16,* which did not differ from wild-type in the levels of *ATHB16* expres-sion, resembled wild-type plants in their growth and devel-opment (data not shown).

Alterations in the levels of *ATHB16* expression affected the size and shape of the rosette leaves. As shown in Fig. 3, 35S::*ATHB16* plants had rosette leaves with an increased serration of edges and a size smaller than that of wild-type

Table 1
Rosette leaf characteristics of 32 day-old 35S::*ATHB16* and
35S::*antiATHB16* plants grown in LD

Measurement	35S:: *ATHB16*	Wild-type	35S:: *antiATHB16*
Length of leaf blade (cm)[a]	1.6 ± 0.4*	3.4 ± 0.5	4.1 ± 0.3*
Width of leaf blade (cm)[a]	0.6 ± 0.3*	1.4 ± 0.3	1.6 ± 0.2*
Length/width ratio	2.7 ± 0.4	2.4 ± 0.5	2.6 ± 0.3
Average leaf area (mm²)[b]	98 ± 24*	141 ± 34	204 ± 27*
Average epidermal cell Area (X10⁻³ mm²)[c]	1.12*	1.80	2.57*

[a] Measurements taken from the fifth rosette leaf. Results shown are the average ± SD. n = 24.

[b] Measurements taken from all expanded rosette leaves. Results shown are the average ± SD. n = 24.

[c] Measurements taken from 50 epidermis cells of the fifth rosette leaf from five plants.

* Statistical significance of the difference to wild-type plants (*t* test P ≤ 0.001).

in plants grown under LD and SD, whereas the leaves of 35S::*antiATHB16* plants grown under LD were larger than wild-type leaves (Fig. 3A–D). SD-grown 35S::*antiATHB16* plants did not differ significantly from wild-type in leaf size (Fig. 3B and D).

Table 1 describes the rosette leaf characteristics of 35S::*ATHB16,* 35S::*antiATHB16,* and wild-type plants grown in LD. Both the length and the width of rosette leaves were reduced in 35S::*ATHB16* but increased in 35S::*antiATHB16* plants, as compared with wild-type. The average rosette leaf area of 35S::*ATHB16* plants was approximately 30% smaller and that of 35S::*antiATHB16* plants 44% larger than that of the wild-type. Thus, the rosette leaf area differed between 35S::*ATHB16* and 35S::*antiATHB16* by a factor of two. The length/width ratio of the rosette leaves did not differ significantly between plants. Cross-sections prepared from the fifth rosette leaf of 35S::*ATHB16,* 35S::*antiATHB16,* and wild-type plants showed that altered levels of *ATHB16* expression did not affect the thickness or the anatomy of leaves (data not shown).

The difference in leaf area between wild-type, 35S::*ATHB16,* and 35S::*antiATHB16* plants may result from a difference in cell expansion, cell numbers, or both. To determine which parameter was affected by altered transgene expression, we examined the morphology of leaf epidermal cells of representative transgenic plants. As shown in Fig. 4 and Table 1, the leaf epidermal cells of 35S::*ATHB16* rosette leaves, on average, were 38% smaller than the corresponding wild-type cells. In contrast, 35S::*antiATHB16* leaf epidermal cells were 43% larger than wild-type cells. The relative difference in leaf epidermal cell size between the 35S::*ATHB16,* 35S::*antiATHB16* plants, and wild-type was similar to the relative difference in leaf size between the plants. These results indicate that *ATHB16*

affects rosette leaf growth mainly by regulating cell expansion.

At maturity, 35S::*ATHB16* plants exhibited a reduced shoot length and an increase in the number of lateral shoots, compared with wild-type, as shown in Table 2. Increased levels of *ATHB16* expression also significantly prolonged
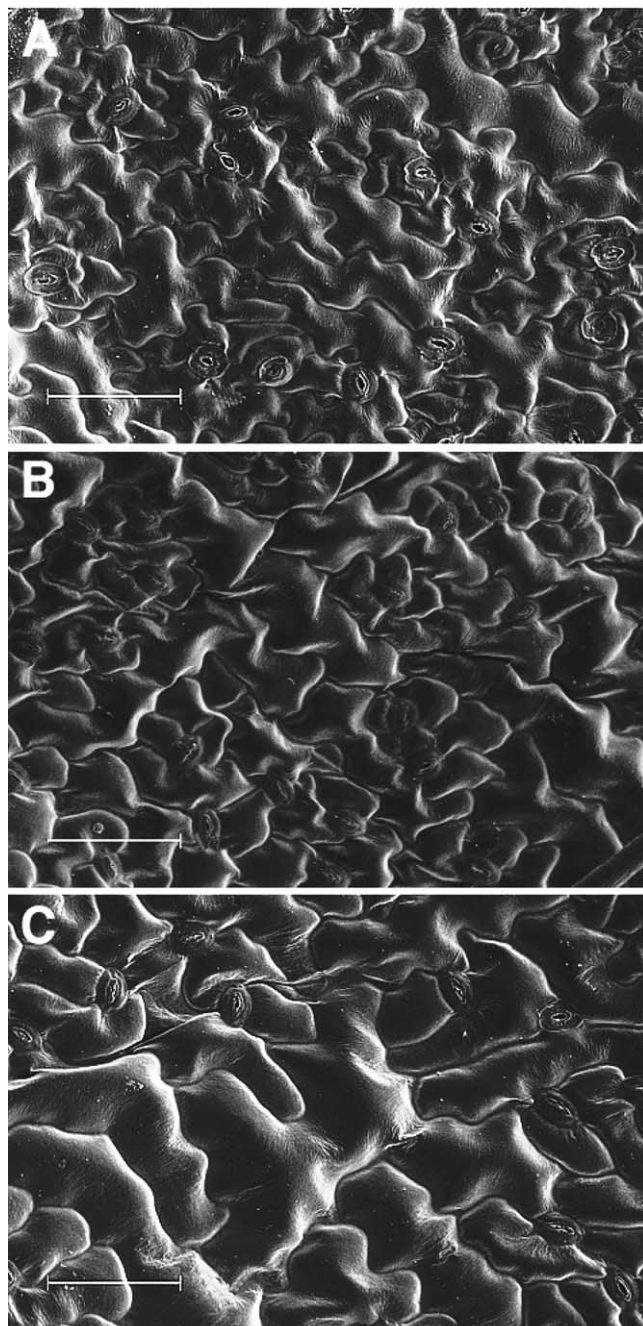

Fig. 4. Scanning electron micrographs of epidermal cells of the rosette leaves. (A) 35S::*ATHB16* plants. (B) Wild-type plants. (C) 35S::*antiATHB16* plants. The fifth rosette leaf of 4-week-old plants grown in LD were studied, and the panels show the adaxial epidermis oriented such that the proximal–distal axis of the leaf is running horizontally from left to right through the panels. Scale bars represent 50 μm.

Table 2
Shoot development of 35S::*ATHB16* and 35S::*antiATHB16* plants grown in LD

Measurement	35S:: *ATHB16*	Wild-type	35S:: *antiATHB16*
Days to maturity[a]	68.0 ± 4.3*	34.2 ± 2.0	30.0 ± 3.8
Primary shoot length (cm)[b]	16.9 ± 0.7*	30.5 ± 0.6	32.0 ± 1.2*
No. lateral shoots[b]	6.4 ± 1.5*	4.0 ± 0.2	4.0 ± 0.2
Total no of leaves[b]	46.7 ± 4.1*	16.2 ± 2.0	15.3 ± 1.4
Final no. of siliques[c]	156 ± 31*	114 ± 29	112 ± 39
Mature silique length (cm)[d]	0.65 ± 0.03*	1.25 ± 0.08	1.25 ± 0.08
No. seeds per silique[d]	23 ± 0.2*	44 ± 2.3	42 ± 4.6

[a] Maturity recorded as the time point at which the first silique became yellow. Results shown are the average $\pm$ SD. $n = 24$.

[b] Data documented at maturity. Results shown are the average $\pm$ SD. $n = 24$.

[c] Data documented at maturity. Results shown are the average $\pm$ SD. $n = 10$.

[d] Results shown are the average $\pm$ SD. Measurements taken from five siliques/plant. $n = 10$.

* Statistical significance of the difference to wild-type plants (*t* test $P \leq 0.001$)

the flowering phase. 35S::*ATHB16* plants matured at 68.0 days (approximately 28 days after the onset of flowering), while wild-type plants matured at 34.2 days (approximately 11 days after the onset of flowering). One result of this delay in senescence was that 35S::*ATHB16* plants produced significantly more leaves and siliques than the wild-type, but the total number of seed produced per plant was lower than that of wild-type plants (Table 2). 35S::*antiATHB16* plants displayed a slight increase in shoot length, but otherwise did not differ from wild-type in these aspects of development.

ATHB16 regulates the flowering time response to photoperiod

Fig. 5 presents data derived from wild-type, 35S::*ATHB16,* and 35S::*antiATHB16* plants on the timing of the transition from vegetative to floral development, measured both as the number of days to flowering (Fig. 5A) and as the number of rosette leaves at flowering (Fig. 5B). Under long day (LD, 16 h light/8 h dark) conditions, the flowering was significantly delayed in 35S::*ATHB16* plants, which flowered after 39.8 days (19.2 rosette leaves) as compared with wild-type plants which flowered after 22.8 days (8.6 rosette leaves, $P < 0.001$). When grown in equal day/night photoperiod (EDN, 12 h light/12 h dark), 35S::*ATHB16* plants flowered at 50.5 days (29.4 rosette leaves), as compared with 47.3 days (24.6 rosette leaves) for the wild-type. 35S::*ATHB16* plants grown under short day conditions (SD, 8 h light/16 h dark) showed a weak but significant shift to early flowering. 35S::*ATHB16* plants flowered after 74.2 days (36.7 rosette leaves) and wild-type plants after 79.3 days (40.8 rosette leaves, $P < 0.001$). These data show that 35S::*ATHB16* plants, like wild-type plants, display an increase in the time

to flowering as the day length decreases. However, 35S::*ATHB16* plants are less responsive to photoperiod than the wild-type.
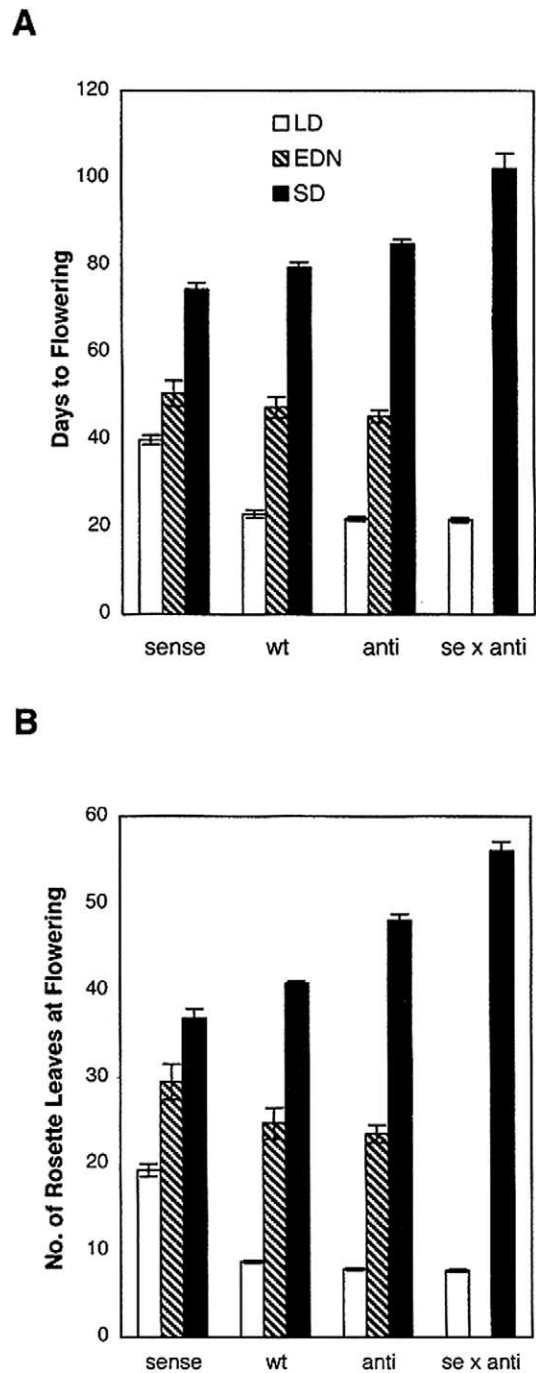
Fig. 5. Time to initiation of reproductive development in plants grown in different photoperiods. 35S::*ATHB16* (sense), wild-type (wt), 35S::*antiATHB16* (anti), and 35S::*ATHB16* $\times$ 35S::*antiATHB*16 (se $\times$ anti) plants were grown under cool white fluorescent light in LD (16 h light/8 h dark), EDN (12 h light/12 h dark), or SD (8 h light/16 h dark) photoperiods. (A) Days to flowering defined as the number of days from sowing until floral buds were visible in the center of rosettes. (B) The number of rosette leaves produced at the onset of flowering. Both histograms show the average value of 24 plants/experiment, from 3 experiments. Bars represent the standard error in both (A) and (B).

In contrast, the 35S::antiATHB16 plants showed an enhanced responsiveness to photoperiod, as compared with wild-type (Fig. 5). Under LD conditions, the timing of flowering of 35S::antiATHB16 plants did not differ significantly from wild-type plants, 21.7 days as compared with 22.8 days for the wild-type. Under SD conditions, 35S::antiATHB16 plants took 84.9 days (48 rosette leaves) to flower, whereas wild-type plants flowered slightly but significantly earlier, at 79.3 days ($P < 0.001$). Simultaneous expression of both the 35S::ATHB16 and 35S::antiATHB16 constructs in F_1 offspring from a cross between 35S::ATHB16 (line J12) and 35S::antiATHB16 (line E17) plants resulted in an even further delay in flowering in SD, to 102.2 days (Fig. 5A). This enhanced effect is likely a consequence of a further reduction of ATHB16 transcript levels in these plants as compared with the 35S::antiATHB16 plants.

ATHB16 regulates the duration of the adult growth phase

In Arabidopsis, the vegetative phase of growth consists of a juvenile and an adult phase, and plants acquire reproductive competence during the adult phase to undergo sexual reproduction (Poethig, 1997). One morphological characteristic that changes at the juvenile-to-adult transition is the presence and density of trichomes on the abaxial side of rosette leaves (Telfer et al., 1997). We monitored the presence of abaxial trichomes to determine whether adult traits were expressed differently in ATHB16 transformants than in wild-type plants. In wild-type plants, abaxial trichomes appeared on rosette leaf 4.2 ± 0.5 and 12.3 ± 0.7 under LD and SD, respectively, and increased in density on subsequent leaves. The timing of the appearance of trichomes was similar to that of wild-type in both 35S::ATHB16 (4.5 ± 0.2 and 13.5 ± 1.2 under LD and SD, respectively) and 35S::antiATHB16 plants (3.9 ± 0.7 and 12.9 ± 1.4 under LD and SD, respectively). This observation indicates that the effect of ATHB16 on flowering time is not attributed primarily to a change in the duration of the juvenile phase, but to an alteration in the duration of the adult phase prior to flowering.

ATHB16 does not affect plant sensitivity to phytohormones

To determine whether the effect of alterations in ATHB16 expression on leaf development were related to changes in the sensitivity to plant hormones known to affect this process, we subjected wild-type, 35S::ATHB16, and 35S::antiATHB16 plants to treatments with indole-3-acetic acid (IAA), gibberellin (GA3), kinetin (kin), and epibrassinolide (BL) at different concentrations. Neither of the hormones tested restored leaf size or shape of either type of ATHB16 transformant plant to those of the corresponding wild-type (data not shown).

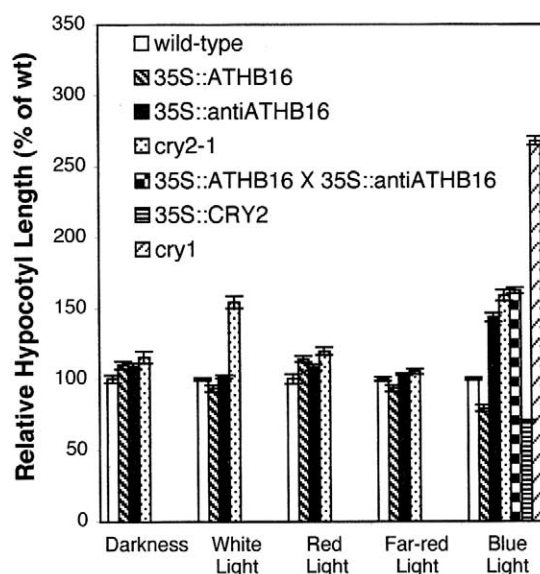To determine whether ATHB16 influenced flowering

Fig. 6. Hypocotyl length of seedlings grown under light of different spectral qualities. Seedlings were grown under different light conditions for 4 days. The hypocotyl length of the 35S::ATHB16 (line J12), 35S::antiATHB16 (line E17), and mutants were normalized to the hypocotyl length of the wild-type grown under the same light conditions. The average hypocotyl length of the wild-type seedlings was 9.30, 1.01, 3.95, 0.93, and 1.73 mm under darkness, white, red, far-red, and blue light, respectively. Each measurement was performed with 40 seedlings. Bars indicate standard error. The data presented here represent one time point from one experiment. Similar result was obtained from two additional time points and in two independent experiments, with two additional 35S::ATHB16 lines (K41 and B27) and one 35S::antiATHB16 line (G53).

time by affecting GA signaling, we examined the flowering time response of wild-type, 35S::antiATHB16 plants, 35S::ATHB16 plants, and, as controls, the GA biosynthesis mutant (ga1-5) and the GA insensitive mutant (gai) after application of GA (GA_3) under SD. Application of GA_3 caused an accelerated flowering of wild-type plants, 35S::antiATHB16, as well as 35S::ATHB16 plants (data not shown). GA did not affect the flowering time of the gai mutant, but restored the late flowering time of ga1-5 to that of wild-type. Thus, the ATHB16 effects on flowering time are not due to an effect on GA signal transduction or on the responsiveness of the plants to GA.

ATHB16 affects the blue-light control of hypocotyl elongation

The morphological changes observed in plants with altered levels of ATHB16 expression are reminiscent of the effects of light on plant development. To test whether changes in ATHB16 expression influenced photomorphogenic responses, we investigated the light-dependence of hypocotyl development in wild-type, 35S::ATHB16, and 35S::antiATHB16 plants. Fig. 6 shows that the hypocotyl length of seedlings grown in darkness, white light, red light,

or far-red light at 4 days after germination did not differ significantly between 35S::ATHB16, 35S::antiATHB16, and wild-type. When grown in blue light for 4 days, 35S::antiATHB16 seedlings developed significantly longer hypocotyls, and 35S::ATHB16 seedlings had significantly shorter hypocotyls than wild-type control seedlings. Additionally, the hypocotyls of the seedlings of plants simultaneously expressing both the 35S::ATHB16 and 35S::antiATHB16 constructs were even longer than 35S::antiATHB16 seedlings when grown in blue light. Like the 35S::antiATHB16 plants, the blue light photoreceptor mutants cry1 and cry2 developed longer hypocotyls than wild-type when germinated in blue light (Fig. 6). Under the same conditions, the 35S::CRY2 transgenic seedlings developed hypocotyls that were shorter than wild-type. These data indicate that ATHB16 may act as a mediator of blue light signaling effects on hypocotyl elongation.

To test whether ATHB16 might act as a direct regulator of the expression of the blue light receptors, we analyzed CRY1 and CRY2 transcript levels in 12-day-old seedlings grown in constant light, by Northern blot experiment. We found no difference in CRY1 or CRY2 expression levels between wild-type, 35S::ATHB16, and 35S::antiATHB16 seedlings (data not shown). To test the alternative possibility, that ATHB16 expression might be under the control of the blue light receptors, we analyzed ATHB16 transcript levels in cry1 and cry2 mutant plants and in plants overexpressing CRY2. In white light growth conditions, no reproducible change could be detected in ATHB16 transcript levels in cry1 or cry2 mutant plants or in plants overexpressing CRY2 (data not shown). ATHB16 mRNA levels also did not differ between wild-type seedlings grown under white or blue light or in darkness (data not shown).

Discussion

In this report, we describe the identification and functional characterization of ATHB16, a new member of the HDZip gene family in Arabidopsis. As judged by sequence criteria and intron exon organization data, ATHB16 is closely related to the previously characterized HDZip I gene ATHB6. ATHB6 is known to be upregulated in response to water-deficit conditions and to treatment of the plant with abscisic acid, and has been proposed to function as a regulator of growth and development in response to limiting water conditions (Söderman et al., 1999; Himmelbach et al., 2002). Similar functions in the drought response have also been proposed for two other members of HDZip I, ATHB7 and ATHB12 (Söderman et al., 1996; Lee and Chun, 1998; Hjellström et al., 2003). ATHB16 is expressed primarily in leaves, but like other characterized members of HDZip I, also in most or all other organs of the plant. We now present data on the phenotypic effects on the plants of altered levels of ATHB16 expression, derived from transgenic plants ex-

pressing an ATHB16 cDNA or an antisense ATHB16 cDNA under the control of the constitutive CaMV 35S-promoter. The data indicate that ATHB16 has a role in the control of leaf cell expansion.

Our results further show that ATHB16 acts to regulate the transition to flowering as part of the photoperiod pathway. This conclusion is based on the observation that transgenic Arabidopsis plants with elevated levels of ATHB16 expression show a reduced flowering time response to photoperiod as compared with wild-type, whereas plants with reduced levels of ATHB16 expression show the reverse phenotype, an enhanced responsiveness to photoperiod. Further, the timing of the transition to flowering was unaffected by GA in the transgenic plants.

As compared with plants that are mutant for some of the well-characterized genes of the photoperiodic pathway, like CO (Putterill et al., 1995), the quantitative effects on flowering time we have observed in the ATHB16 transgenic plants are relatively limited. This might imply that the role of ATHB16 as a regulator of this process might be only indirect. We note, however, that the difference in the expression levels of ATHB16 between our transgenic plants and wild-type is also quite limited. Therefore, we expect that the observed effects are only partial, and that a complete loss of ATHB16 activity might cause more severe effects on flowering time. Our finding that the effects on flowering time of elevated levels of ATHB16 expression are reciprocal to the effects of a reduction in expression levels in itself strongly suggests that ATHB16 indeed functions as a negative regulator of the flowering time sensitivity to photoperiod also in the wild-type plants.

Several lines of evidence indicate that the activity of ATHB16 may be directly related to the response of the plant to light, specifically to blue light. In a general assay for light sensitivity, a hypocotyl elongation assay, plants with reduced levels of ATHB16 expression are impaired in their response specifically to blue light. This effect is quantitatively dependent on the degree to which gene expression is reduced, since plants with severely reduced transcript levels as a result of the simultaneous expression of both an ATHB16 cDNA and an ATHB16 antisense cDNA also display an enhanced phenotypic effect. A similarly altered response to blue light is observed in plants that are mutant for the CRY2 gene, which encodes a blue light receptor, and to an even larger extent in a mutant for a second blue light receptor, CRY1. A reverse hypocotyl response, a hypersensitivity specifically to blue light, was recorded for plants expressing ATHB16 at elevated levels, as well as in plants with elevated levels of CRY2 as previously documented (Guo et al., 1999). These data suggest that ATHB16 acts as a positive regulator of blue light-dependent inhibition of hypocotyl growth, by mediating CRY2 and/or CRY1 signaling.

Even though our data indicate that ATHB16 expression is unaffected in cry1 and cry2 mutant plants grown in white

light, ATHB16 may act in the blue light signaling mechanism downstream of CRY2 and/or CRY1, since in genome-wide expression profile analyses of Arabidopsis grown under different light regimes, *ATHB16* expression has been shown to be impaired in *cry1 cry2* double mutant plants grown in blue light, and a reverse effect on *ATHB16* expression was detected in seedlings overexpressing *CRY1* in blue light (Ma et al. 2001, supplementary data). Together with the data presented in this report, these data support the notion that ATHB16 acts as a regulator of hypocotyl elongation in response to blue light, downstream of CRY1 and/or CRY2.

The effects of altered levels of *ATHB16* expression on flowering time, however, are not consistent with this simple interpretation, since plants with elevated levels of *ATHB16* expression flowered late in LD, like the loss-of-function mutant for *CRY2*. This might be interpreted as the blue light effects on flowering time requiring the interaction of CRY2 with other cellular components, for example, the red light receptor PHYB, as previously suggested by Guo et al. (1998).

In addition to the effect on flowering time, *ATHB16* also affects leaf development, by acting as a negative regulator of cell expansion. Plants with reduced levels of *ATHB16* expression have larger rosette leaves and longer shoots, whereas plants with increased levels of *ATHB16* expression displayed smaller rosette leaves and shorter shoots, as compared with wild-type. The reciprocal character of the effects of reduced and elevated levels of *ATHB16* on organ size indicates that the gene acts as a regulator of cell size in leaves also in the wild-type plant. Plants that express *ATHB16* at elevated levels also showed other phenotypic differences from wild-type, including a suppression of stem elongation. In these aspects of development, however, transgenic plants with reduced levels of expression of *ATHB16* differed little from wild-type. Therefore, we cannot exclude the possibility that the ATHB16 effects on stem elongation in the transgenic overexpressor plants are due to ectopic expression of the gene, rather than being a reflection of the wild-type gene function.

Altered levels of *ATHB16* expression have similar effects on leaf size in LD and SD, whereas the effects on flowering time differ between these conditions. Therefore, the possibility that the *ATHB16* effect on flowering time is an indirect consequence of the difference in leaf size is unlikely. Instead, our data on hypocotyl development suggest that *ATHB16* may function to mediate the effect of light, potentially blue light, on cell expansion. This conclusion is consistent with the fact that organ size in transgenic plants with elevated levels of the *CRY1* gene expression have been reported to be severely reduced (Lin et al., 1996b). Further, plants with elevated levels of *ATHB16* expression showed a general increase in sensitivity to light (our unpublished observations). Taken together, our data

indicate that *ATHB16* in the wild-type acts to mediate light effects on organ development as well as on flowering time.

The *ATHB16* function in the regulation of cell expansion has an interesting parallel in the role of another HDZip gene, the HDZip II gene *ATHB2,* in Arabidopsis. This gene has been concluded to act as a mediator of the red/far-red light effects on leaf cell expansion in the shading response. As for *ATHB16,* increased levels of *ATHB2* expression resulted in a decrease in cotyledon cell expansion, and a reverse effect on cell expansion was recorded in plants with reduced levels of *ATHB2* expression (Steindler et al., 1999). This raises the interesting possibility that *ATHB16* and *ATHB2* may have roles in the plant that are similar in that they both act as negative regulators of cell expansion by similar mechanisms, as mediators of light responses, albeit their activity would depend on different light signaling pathways. The fact that the sequence specificity of DNA binding of ATHB16 is quite similar, although nonidentical to that of ATHB2 (Johannesson et al., 2001), further suggests that the similarity in function may result from the interaction of the two proteins with similar sets of target genes.

Evidence for functions relating to the regulation of cell expansion has also been presented for other members of HDZip I, which in total includes 17 members. The four closely related genes, *ATHB3,* −13, −20, and −23 (Hanson, 2000), all cause an inhibition of lateral cell expansion in leaves, when ectopically expressed, and *ATHB7* has been shown to inhibit cell expansion in stems when expressed at high level (Hjellström et al., 2003). This is particularly interesting, since the DNA binding specificity of ATHB16 is highly similar or identical to those of the HDZip I proteins examined (Johannesson et al. 2001). Therefore, it appears likely that ATHB16 may share downstream target genes with other HDZip I proteins. Further, ATHB16 has been demonstrated to heterodimerize with ATHB5 in vitro (Johannesson et al., 2001) and with ATHB6 and ATHB7 in yeast (Wang, 2001). This raises the possibility that ATHB16 may functionally interact with other HDZip I proteins in the plant. Potentially such interactions may provide a mechanism by which the plant could integrate different input signals, like light of different spectral qualities and water availability, in the regulation of growth.

A second consequence of the similarities in DNA binding properties, and also in primary sequence, of the HDZip I proteins, is that different proteins may have partly overlapping functions in the plant. In this perspective, phenotypic effects caused by ectopic expression of a gene might be difficult to interpret per se, in relation to the wild-type function of the gene. Our data on *ATHB16* show reciprocal phenotypic effects to be caused by increased and decreased levels of the gene expression, both on day-length sensitivity and on cell expansion. These results provide a strong indication that these effects directly reflect the function of the gene in the wild-type plant, rather than being an indirect

effect caused by *ATHB16* artificially interfering with the function of a second HDZip gene.

Acknowledgments

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