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Shade avoidance responses are mediated by the ATHB-2 HD-Zip protein, a negative regulator of gene expression

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SUMMARY

The *ATHB-2* gene encoding an homeodomain-leucine zipper protein is rapidly and strongly induced by changes in the ratio of red to far-red light which naturally occur during the daytime under the canopy and induce in many plants the shade avoidance response. Here, we show that elevated ATHB-2 levels inhibit cotyledon expansion by restricting cell elongation in the cotyledon-length and -width direction. We also show that elevated ATHB-2 levels enhance longitudinal cell expansion in the hypocotyl. Interestingly, we found that ATHB-2-induced, as well as shade-induced, elongation of the hypocotyl is dependent on the auxin transport system. In the root and hypocotyl, elevated ATHB-2 levels also inhibit specific cell proliferation such as secondary growth of the vascular system and lateral root formation. Consistent with the key role of auxin in these processes, we found that auxin is able to rescue the ATHB-2 lateral root phenotype. We also show that reduced levels of ATHB-2 result in reciprocal phenotypes. Moreover, we demonstrate that ATHB-2 functions as a negative regulator of gene expression in a transient assay. Remarkably, the expression in transgenic plants of a derivative of ATHB-2 with the same DNA binding specificity but opposite regulatory properties results in a shift in the orientation of hypocotyl cell expansion toward radial expansion, and in an increase in hypocotyl secondary cell proliferation. A model of ATHB-2 function in the regulation of shade-induced growth responses is proposed.

Key words: Cell elongation, Lateral root formation, Secondary thickening, Phytochrome responses, Transcription factors, *ATHB-2*, *Arabidopsis thaliana*

INTRODUCTION

To grow and develop optimally, all organisms need to perceive and process information from their environment. As sessile organisms, plants are exquisitely sensitive to external stimuli and adapt their developmental pattern to changes in the natural environment thereby ensuring survival and reproduction. In particular, being photosynthetic, plants are extremely sensitive to their light environment, constantly monitoring light intensity, quality and duration to control diverse developmental processes. Photomorphogenic responses are especially evident at the seedling stage. Depending on whether germination occurred in darkness or in light, angiosperms choose between two distinct developmental pathways. In complete darkness, a seedling develops according to the skotomorphogenic pathway. In *Arabidopsis*, for example, the cotyledons remain closed and unexpanded, and the hypocotyl becomes extremely elongated. The seedling utilizes its energy resources to rapidly emerge from below the soil surface and reach the light. Under direct sunlight, the photomorphogenic pattern rapidly establishes the seedling as a photoautotrophic organism. The plant's energy is essentially used for leaf development, while longitudinal extension is minimised (von Arnim and Deng, 1996, and references therein). The transition from the heterotrophic etiolated seedling to the established photoautotrophic seedling coincides with the assumption of the capacity to respond to changes in the red:far-red (R:FR) ratio (Smith, 1995, and references therein). These changes in wavelength distribution mainly occur in the proximity of other vegetation. In fact, the light environment within a plant canopy is selectively depleted of red and blue light because of strong chlorophyll absorbance by the overlying leaves. The angiosperms have evolved impressive capacity to avoid shade by stimulation of extension growth. Many plants react within 5-10 minutes of exposure to reduced R:FR ratios by accelerating extension up to 3- or 4-fold. In dicotyledous plants such as *Arabidopsis*, elongation growth induced by canopy shade is often associated with a reduction of leaf expansion, a marked strengthening of apical dominance, and an acceleration of flowering (Smith, 1995, and references therein). Several lines of evidence indicated that the

shade avoidance reactions are all initiated by a single environmental signal, the reduction in the ratio of R to FR radiation, perceived by light-stable phytochromes (Smith and Whitelam, 1997, and references therein).

The immediate downstream target(s) of any of the phytochromes that regulate the shade avoidance responses is still unknown. However, the first gene (*ATHB-2*; also known as *HAT4*; Ruberti et al., 1991; Schena and Davis, 1992) specifically induced by changes in the R:FR ratio has been recently identified in *Arabidopsis* (Carabelli et al., 1993). In young seedlings and mature plants, *ATHB-2*, a gene encoding an homeodomain-leucine-zipper (HD-Zip) protein, is expressed at low levels under high R:FR ratios, but is rapidly and strongly induced by lowering the R:FR ratio. Returning the plants to a high R:FR ratio results in an equally rapid decrease in the *ATHB-2* mRNA levels (Carabelli et al., 1996).

The unique ability of the *ATHB-2* gene to respond to changes in the R:FR ratio in established plants suggested a direct involvement of ATHB-2 in the growth phenomena induced by neighbour detection and shade. In *Arabidopsis*, phytochrome B (PHYB) and at least one other light-stable phytochrome appear to play important roles in the regulation of these processes (Smith and Whitelam, 1997, and references therein). Consistently, in *phyB* mutants the *ATHB-2* gene is expressed at moderately higher levels in all organs both in the daytime and during the night, and it is still induced by low R:FR ratios (Carabelli et al., 1996; Steindler et al., 1997).

In the present report, we provide evidence that the product of the *Arabidopsis ATHB-2* gene is involved in the regulation of growth responses induced by neighbour detection and shade. We show that changes in the ATHB-2 levels affect cell elongation processes in hypocotyl and cotyledons. In the root and hypocotyl, these changes also affect specific cell proliferation such as secondary growth of the vascular system (secondary tickening) and lateral root formation. We also show that ATHB-2 interferes with auxin response pathways. Moreover, we show that ATHB-2 affects cell elongation and secondary thickening by acting as a negative regulator of gene expression.

MATERIALS AND METHODS

DNA constructs

The plant expression vectors ATHB-2 and αATHB-2 were constructed by excising the *HD-Zip-1-VP16* sequence from a derivative of pMON 721 (Aoyama et al., 1995) using *Bgl*II and *Bam*HI, and inserting the *ATHB-2* cDNA sequence (Carabelli et al., 1993) in either the sense (ATHB-2) or antisense orientation (αATHB-2). The *ATHB-2* coding sequence was obtained by PCR amplification using ATHB-2 5′ and ATHB-2 3′ primers. The *ATHB-2* sequences were preceded by the –343 to +1 region of the CaMV 35S promoter (Odell et al., 1985) and flanked by the poly(A) addition sequence of pea ribulose 1,5-bisphosphate carboxylase small subunit rbcS-E9 (Coruzzi et al., 1984). The ATHB-2 construct was also used as a transacting plasmid in particle bombardment experiments; the other transacting genes were cloned in the same vector. HD-Zip-2-V was constructed by excising the *HD-Zip-1* sequence from the HD-Zip-1-VP16 construct (Aoyama et al., 1995) using *Bgl*II and *Xho*I, and inserting a DNA fragment encoding the HD-Zip-2 domain obtained by PCR amplification with primers HD-Zip-2 5′ and HD-Zip-2 3′. The HD-Zip-2-V plasmid DNA was cut with *Xho*I and *Sal*I and recircularized to generate HD-Zip-2. mATHB-2 was obtained by site-directed mutagenesis. Two DNA fragments corresponding to the N- and C-terminal portions of ATHB-2 (ATHB-$2_{3\text{-}179}$ and ATHB-$2_{173\text{-}284}$, respectively) in which Asn176 (corresponding to position 51 of the homeodomain) was replaced by alanine were obtained by PCR reactions as described by Horton et al. (1989). The fragment corresponding to ATHB-$2_{3\text{-}179}$ was obtained using the oligonucleotides ATHB-2 5′ and ATHB-2 N51A 3′; the fragment corresponding to ATHB-$2_{173\text{-}284}$ was generated using ATHB-2 N51A (Sessa et al., 1997) and ATHB-2 3′. The primers ATHB-2 N51A and ATHB-2 N51A 3′ were designed so that the ends of the two PCR products contained complementary sequences. The PCR products were mixed and amplified to generate the recombinant fragment. To construct the HD-Zip-2-V-G coding DNA, three DNA fragments were sequentially assembled on the vector plasmid pHSG397 (Takeshita et al., 1987) in an in-frame manner. These DNA fragments were a synthesized double-stranded oligo-DNA containing the initiation codon (5′-CTAGAACAATGGCG-3′ and 5′-GATCCGCCATTGTT-3′), a fragment encoding the HD-Zip-2 domain obtained by PCR amplification with primers HD-Zip-2 5′ and HD-Zip-2 3′, and a fragment encoding the V-G region obtained by PCR amplification of the *GVG* gene (Aoyama and Chua, 1997) with primers VP16 5′ and GR 3′. The *Hin*dIII site of pBI121 (Jefferson et al., 1987) was changed into a *Sal*I site by fill-in ligation with a *Sal*I linker. The β-glucuronidase coding region encompassed by the *Xba*I and the *Sst*I sites in the modified pBI121 was replaced with the DNA fragment encoding HD-Zip-2-V-G. The resulting plasmid was named pMO-2. ATHB-2-G was constructed by excising the HD-Zip-2-V sequence from the pMO-2 construct using *Bam*HI, and inserting a DNA fragment encoding ATHB-2 obtained by PCR amplification with primers ATHB-2 5′ and ATHB-2-G 3′. A derivative of pGEM (Aoyama et al., 1995) was used as a vector for the reporter constructs. The *Hin*dIII-*Bgl*II fragment containing six copies of the ATHB-1 binding sequence was replaced with a DNA fragment containing either six copies of the ATHB-2 binding sequence (5′-CAATGATTG-3′) or a mutant sequence (5′-CAATGGTTG-3′). The sequences of the PCR oligonucleotide primers are as follows. ATHB-2 5′ 5′-CCCGGGATCCCTTCGAGAAAGACGATCTG-3′ (the underlined sequence corresponds to nucleotides 364-381 of the *ATHB-2* genomic sequence; Carabelli et al., 1993), HD-Zip-2 5′ 5′-CCCGGGAT-CCAGATGGTGATAACTCCA-3′ (nucleotides 826-842), ATHB-2 N51A 3′ 5′-TGCTCGTCT**AGC**CTGAAACCA-3′ (nucleotides 1245 to 1265; the triplet in bold corresponds to the substitution), HD-Zip-2 3′ 5′-GGCGCTCGAGGTAGTGGGTGGGCTCATG-3′ (nucleotides 1494-1511), ATHB-2-G 3′ 5′-CCCGGGATCCGCGGACCTAGGA-CGAAGAGC-3′ (nucleotides 1651-1668), ATHB-2 3′ 5′-CCCGG-GATCCTTAGGACCTAGGACGAAG-3′ (nucleotides 1654-1671), VP16 5′ 5′-TTTTCCTAGCTCGAGCGCC-3′ (nucleotides 213-231 of the *GVG* gene; Aoyama and Chua, 1997), GR 3′ 5′-GAACAAGCTAGCTTACTCAG-3′ (nucleotides 1313-1332 of the *GVG* gene).

Transient expression assay

Mature *Arabidopsis* leaves were bombarded using a Biolistic PDS 1000/HE particle delivery system (Bio-Rad) as previously described (Sessa et al., 1998a). Each sample was bombarded with 0.5 mg of 1.6 μm gold particles coated with a DNA mixture including 0.225 and 0.9 μg of transacting and reporter plasmids, respectively, and 0.375 μg of vector DNA carrying the GUS gene transcribed from the –343 CaMV 35S promoter. For competition experiments two transacting plasmids in a 3:1 ratio (0.225 μg DNA) were co-bombarded with a reporter plasmid (0.9 μg DNA). To the control, performed with a single transacting plasmid, was added vector DNA. The initial pressure of bombardment was 1100 p.s.i., and the travelling distance of particles to leaves was 10 cm. Bombarded leaves were incubated at 21°C for 20 hours (12 hours light/8 hours dark) in a growth chamber. After incubation, total cell extracts were assayed for their specific activities of luciferase and β-glucuronidase, as described by Millar et al. (1992) and Jefferson et al.

(1987), respectively. Each relative transactivation level was calculated by normalizing LUC activity with respect to GUS activity.

Arabidopsis transformation

Expression plasmids ATHB-2, αATHB-2, ATHB-2-G and HD-Zip-2-V-G were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90RK (Koncz and Schell, 1986) by electroporation using a Gene Pulser (Bio-Rad). *Arabidopsis* lines (Wassilewskija or Columbia) were transformed using the vacuum infiltration method (Bechtold et al., 1993). Seeds of T0 plants were harvested and sown on agar medium containing 0.5× Murashige and Skoog salt mixture, Gamborg's B5 vitamins, and 50 μg/ml kanamycin**.** 7-day-old kanamycin-resistant seedlings were transplanted to soil and grown at 21°C with a 16-hour-light and 8-hour-dark cycle to obtain their seeds. Seeds of T1 plants were plated on selection agar medium and incubated in a growth chamber for 7 days; T2 seedlings exhibiting a 3:1 segregation of kanamycin resistance were grown in soil to obtain their seeds. Finally, T3 seeds were sown on selection agar medium to identify homozygous lines (1:0 segregation of kanamycin resistance).

Growth and phenotypic analysis

To analyse hypocotyl and cotyledons, seeds were sterilized and plated in 9×1.5 cm Petri dishes containing 28 ml of germination medium (GM; Valvekens et al., 1988). For induction experiments, a stock solution of 30 mM dexamethasone (Sigma) in ethanol was prepared before use, and appropriate dilutions were added to the agar medium. The same volume of ethanol was added to each negative control. Dishes were stored in darkness for 2 days at 4°C. Germination was induced by placing the plated seeds in white light for 2 hours at 21°C, and proceeded in darkness at 21°C for 36 hours. Seedlings were grown in a light:dark cycle of 16:8 hours in growth chambers under fluorescent light (Osram L58 W31, 8.9×10^3 lux, $blue_{450}$:red_{655}:$far\text{-}red_{725}$ equal to 330:506:26 μW cm^{-2}). After 7 days of growth, seedlings were either analysed or moved to phytatrays containing 50 ml of GM and grown for other 7 days. To analyse the root, seeds were sterilized and plated on squared 9 cm plates containing 30 ml of GM. Vernalization was as described for the analysis of the hypocotyl. Plates were set in a vertical position and incubated for either 9 or 20 days in growth chambers at 21°C under a light:dark cycle of 16:8 hours. For low R/FR experiments, seedlings were grown in a light:dark cycle of 16:8 hours in growth chambers under fluorescent light (Osram L58 W31) supplemented with incandescent light (Osram Krypton qw3 60 W). Fluence rates were as follows: $blue_{450}$, 246 μW cm^{-2}; red_{655}, 790 μW cm^{-2}; $far\text{-}red_{725}$ 514 μW cm^{-2}. Measurements of fluence rate in the spectral regions were performed by using the Photometer IL 150 (International Light, Newburyport, MA).

Microscopy

For the histological analysis of transverse sections, hypocotyls and roots were fixed overnight in 1% glutaraldehyde – 4% formaldehyde in 50 mM sodium phosphate buffer pH 7.2. After washing for 30 minutes in the same buffer, the samples were dehydrated through a graded series of ethanol (from 30% to 100%, 30 minutes each step), and then infiltrated and embedded in Technovit 7100 (Kulzer, Hereaus) as indicated by the manufacturer. 5 μm sections were made on a Microm HM 330 microtome, then let dry at 37°C, and stained in 0.1% toluidine blue. For the histological analysis of longitudinal sections, hypocotyls were fixed overnight in 4% formaldehyde in 50 mM sodium phosphate buffer pH 7.2. After washing for 30 minutes in the same buffer, the samples were dehydrated through a graded series of ethanol (from 30% to 100%, 30 minutes each step), then passed through xylene (1 hour), and finally embedded in paraffin. 7 μm sections were made on a Microm HM 330 microtome, mounted on water, and let dry on slides overnight at 37°C. After removing wax in xylene, the tissue sections were hydrated through an ethanol series and stained in 0.1% toluidine blue. For the observation of size and shape of epidermal cells, prints of cotyledons surfaces were prepared using clear nail polish. Images of sections and prints were taken using a COHU 2252-1000 camera (ScienceWare s.r.l., Italy) mounted on a Leitz Dialux 22 Microscope. For the hypocotyl and root measurements, images of seedlings were taken under a Leica wild MZ8 binocular microscope, using the COHU camera, and analyzed in the image-analysis software PC–Image 2.2.02 (Forster Findlay Associates, UK). The number of lateral roots were counted under the binocular microscope. The number of individual epidermal cells in a single cell file of the hypocotyl was determined on seedlings fixed in Herr's liquid (Herr, 1982) as described by Gendreau et al. (1997) using the binocular microscope.

RESULTS

Phenotypes of *Arabidopsis* seedlings expressing altered levels of ATHB-2

To examine the role of ATHB-2 in plant development, the ATHB-2 coding sequence transcribed from the –343 cauliflower mosaic virus (CaMV) 35S promoter (ATHB-2), the same sequence cloned in reverse orientation relative to the promoter (αATHB-2) and the ATHB-2 coding sequence fused to a DNA fragment encoding the glucocorticoid receptor domain (amino acids 519-795; Miesfeld et al., 1986) transcribed from the CaMV 35S promoter (ATHB-2-G) were introduced into *Arabidopsis* using the vacuum infiltration method (Bechtold et al., 1993). More than 15 independent primary transgenic plants for each construct were obtained. T3 plants derived from 8 primary transgenic lines for each transgene were analysed. Consistently with a previous report (Schena et al., 1993), ATHB-2 lines (six out of eight) had longer hypocotyls, smaller cotyledons and leaves, and a thinner root system; αATHB-2 lines (five out of eight) exhibited roughly reciprocal phenotypes (see Fig. 1E,I showing representative ATHB-2 and αATHB-2 lines). Moreover, in the presence of dexamethasone (DEX) several ATHB-2-G lines (three out of eight) showed the same phenotype as that of ATHB-2 lines (data not shown). Expression levels of transgenes were examined by RNA gel blot hybridization. The expression level of the transgenes varied with different T3 transgenic lines (5-50 times that of the *ATHB-2* gene) and correlated well with the severity of the phenotype in each series of lines carrying the same transgene (data not shown). Hybridization analysis with an antisense RNA probe and RT-PCR experiments revealed a 3-fold reduction of *ATHB-2* steady state mRNA level in αATHB-2 lines expressing the transgene at high levels (data not shown). One ATHB-2 line, one αATHB-2 line, and one ATHB-2-G line in which the expression level of the transgene was 40-, 50-, and 20-fold that of the *ATHB-2* gene in wild-type plants, respectively, were analysed in more detail. The cotyledons, the hypocotyl and the root were used as models for studying the effects of altered ATHB-2 expression on plant growth because of their simple and well described cellular architecture (Bowman, 1994; Dolan et al., 1993; Gendreau et al., 1997).

Altered ATHB-2 expression affects cell elongation in cotyledons

Cotyledon expansion was significantly reduced in ATHB-2 transgenic seedlings compared to controls (Fig. 1B,F). Conversely, it was increased in αATHB-2 seedlings (Fig. 1J). We found that the elongation of epidermal cells in the cotyledon-

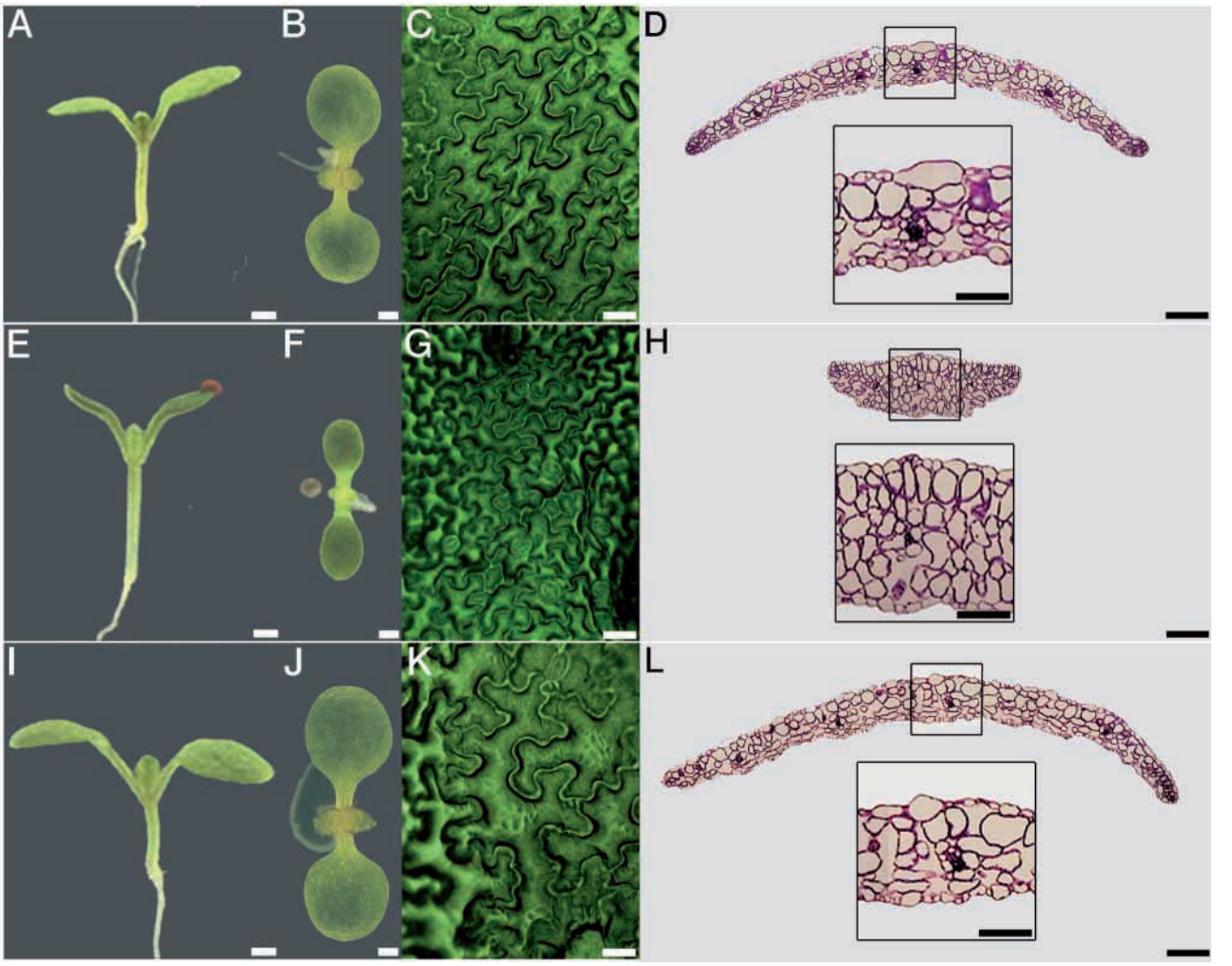

Fig. 1. Cellular organization in the cotyledons of ATHB-2 and αATHB-2 transgenic seedlings. (A-D) Wild-type Wassilewskija ecotype. (E-H) ATHB-2. (I-L) αATHB-2. (A,E,I) Side view of seedlings grown for 7 days on agar plates in a 16:8 hour light:dark cycle. (B,F,J) Top view of the same seedlings. (C,G,K) Prints of cotyledon surfaces showing epidermal cells. (D,H,L) Transverse sections of cotyledons from seedlings grown for 7 days on agar plates in a 16:8 hour light:dark cycle. The boxed region of each section is shown at a higher magnification in the inset. Scale bar: (A,B,E,F,I,J) 500 μm, (C,G,K) 20 μm, (D,H,L) 200 μm, (insets) 100 μm.

length and cotyledon-width direction was reduced in ATHB-2 cotyledons compared to controls (Fig. 1C,G) whereas it was increased in αATHB-2 cotyledons (Fig. 1C,K). To investigate whether altered levels of ATHB-2 also affect cell elongation in the cotyledon-thickness direction, we analysed transverse sections of wild-type and transgenic seedlings. Compared to those of wild-type cotyledons, palisade cells and, more significantly, spongy mesophyll cells of ATHB-2 cotyledons exhibited restricted elongation in the cotyledon-width direction and enhanced elongation in the cotyledon-thickness direction (Fig. 1D,H). Transverse sections of αATHB-2 cotyledons revealed an increase in cell elongation in the cotyledon-width direction (Fig. 1L). From these data we concluded that ATHB-2 affects the cell elongation processes in cotyledons.

Altered *ATHB-2* expression affects cell elongation in the hypocotyl

Hypocotyls in ATHB-2 transgenic seedlings were significantly longer than those in wild-type controls (Fig. 1A,E). Conversely, the hypocotyl length was reduced in αATHB-2 seedlings (Fig. 1A,I). Moreover, DEX-treated ATHB-2-G seedlings showed a significant increase in hypocotyl length compared to controls (Fig. 4A,D). Consistently with the finding that elongation growth of the hypocotyl does not involve significant cortical or epidermal cell divisions (Gendreau et al., 1997), we found that the apical to basal length of hypocotyl epidermal cells was increased in ATHB-2 and DEX-treated ATHB-2-G seedlings compared to controls (Figs 2A,D, 4B,E). Conversely, the length of the hypocotyl epidermal cells was significantly reduced in αATHB-2 transgenic seedlings (Fig. 2A,G). To confirm that no change in epidermal cell divisions occurred in the hypocotyl of seedlings expressing altered levels of ATHB-2, we determined the number of individual epidermal cells in a single cell file of wild-type and transgenic hypocotyls. We observed 18.89±0.63 (n=10), 18.80±0.47 (n=10), and 19.18±0.33 (n=10) cells in wild-type, ATHB-2 and αATHB-2 epidermal files, respectively. To exclude that changes in epidermal cell length may be a consequence of altered cell division in internal cell layers, we

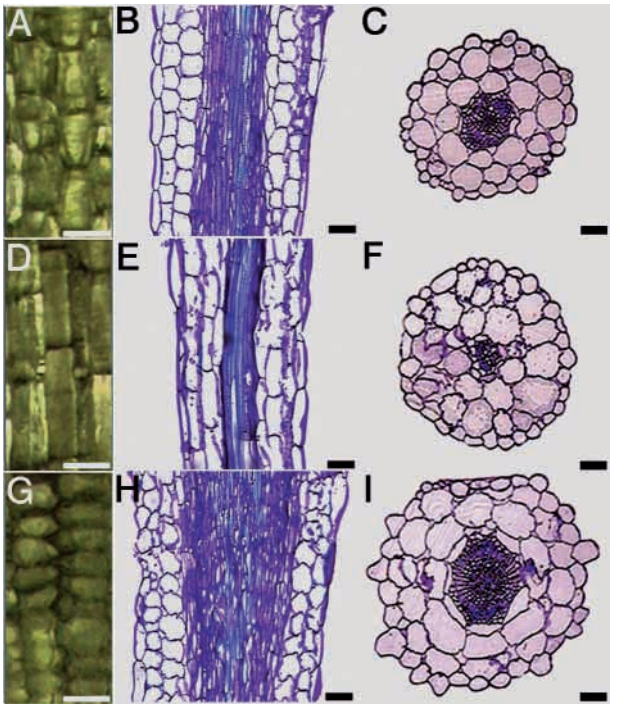

Fig. 2. Cellular organization in the hypocotyls of ATHB-2 and αATHB-2 seedlings. (A-C) Wild type. (D-F) ATHB-2. (G-I) αATHB-2. All plants were grown for 14 days on agar plates in a light:dark cycle of 16:8 hours. (A,D,G) Detail of hypocotyl epidermal cells. (B,E,H) Longitudinal sections of hypocotyl. (C,F,I) Transverse sections of hypocotyl from the same seedlings. Scale bar (A-I) 50 μm.

analysed longitudinal sections of wild-type and transgenic hypocotyls. The longitudinal length of cortical and endodermal cells was increased in ATHB-2 hypocotyls compared to controls (Fig. 2B,E). Conversely, the length of the hypocotyl cortical and endodermal cells was reduced in αATHB-2 transgenic seedlings (Fig. 2B,H). From these data we concluded that the changes in the hypocotyl length depend on a higher and lower degree of cell elongation in seedlings expressing elevated and reduced levels of ATHB-2, respectively.

Altered ATHB-2 expression affects lateral root formation

When grown on soil, ATHB-2 plants showed a reduced root apparatus compared to that of controls (data not shown). In *Arabidopsis* seedlings, the root system consists of a primary root and several lateral roots. The root apical meristem contains three tiers of initials that give rise to four sets of cell files: the stele (consisting of a central cylinder surrounded by a single layer of pericycle cells), the cortex and endodermis, the epidermis and lateral root-cap, and the columella. Lateral root formation is initiated by periclinal and anticlinal divisions in mature pericycle cells of the primary root. Upon formation of a lateral root primordium by pericycle derivatives, a meristem is formed from a subset of these cells (Dolan et al., 1993; Laskowski et al., 1995; Malamy and Benfey, 1997). To examine the effects of altered ATHB-2 expression on lateral root formation, plants were grown on agar plates set in the vertical position. We found that the number of lateral roots was significantly reduced in ATHB-2 plants compared to that of

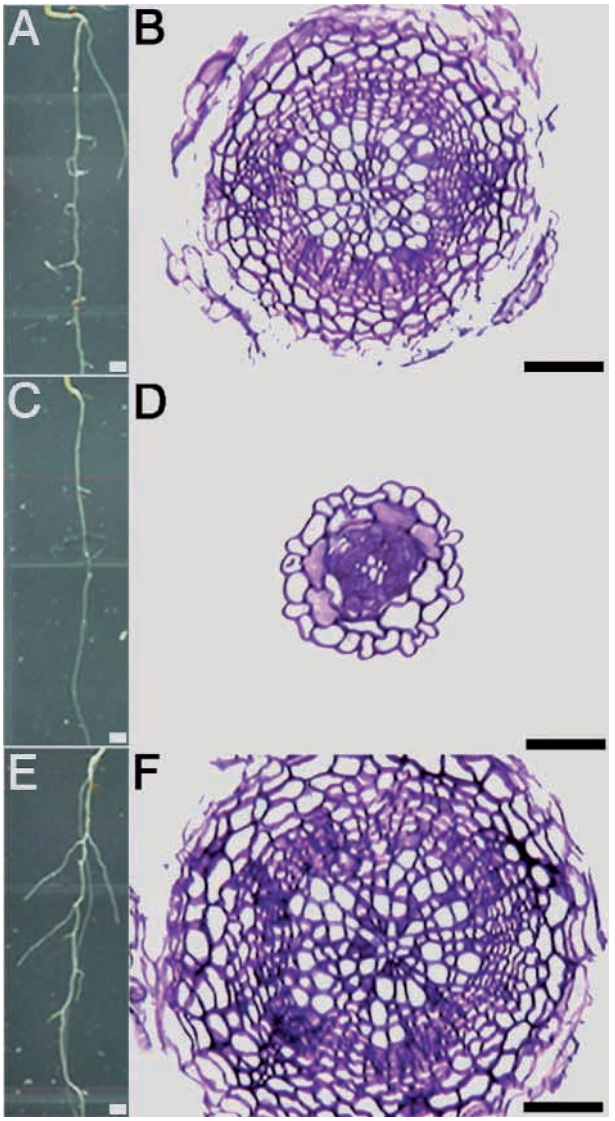

Fig. 3. Cellular organization in the roots of ATHB-2 and αATHB-2 plants. (A,B) Wild type. (C,D) ATHB-2. (E, F) αATHB-2. (A,C,E) Roots of seedlings grown for 9 days on vertically positioned agar plates in a light:dark cycle of 16:8 hours. (B,D,F) Transverse sections of plants grown for 20 days on vertically positioned agar plates in a light:dark cycle of 16:8 hours. Sections were taken 0.5 cm below the hypocotyl-root boundary. Scale bar: (A,C,E) 1 mm, (B,D,F) 50 μm.

wild-type controls (Fig. 3A,C; Table 1). Conversely, αATHB-2 seedlings exhibited a higher number of lateral roots than control plants (Fig. 3A,E; Table 1). Moreover, lateral root formation was significantly reduced in DEX-treated ATHB-2-G seedlings compared to controls (data not shown). Compared with that of the wild type, the main root of the ATHB-2 seedlings at the same age was shorter whereas that of the αATHB-2 seedlings was slightly longer (Table 1).

Altered ATHB-2 expression affects the secondary growth of the vascular system of the root and hypocotyl

The primary root of *Arabidopsis* grows thicker by successive cell proliferation, termed secondary thickening (Dolan et al.,

Table 1. Length of the main root and number of lateral roots in wild-type and ATHB-2 transgenic lines

	Length (cm ± s.e.m.)[a]	no. ± s.e.m.[b]
wild type (Ws)	5.37±0.08	17.83±1.22
ATHB-2 (Ws)	4.12±0.07**	2.11±1.03**
αATHB-2 (Ws)	5.75±0.06**	23.44±1.27*

Seedlings were grown on agar plates in a light:dark cycle of 16:8 hours for 9 days.
[a]100 samples were measured for each line.
[b]10 samples were measured for each line.
*P<0.05; **P<0.01.

1993). The new cells produced during this process are derived from two populations of cells in the stele of the primary root. Parenchymal cells located between the primary xylem and phloem give rise to a vascular cambium composed of cells that, as thickening progresses, form an oval ring with the phloem on their outside and the xylem on their inside. To the outside of the phloem is a layer of thickened fibre cells which are derived from the pericycle of the unthickened root (Dolan et al., 1993). Transverse sections of roots in the early stages of secondary thickening revealed an increase in secondary cell proliferation in the main root of the αATHB-2 plant compared with that of the wild type at the same age (Fig. 3B,D). Conversely, the ATHB-2 plant showed reduced secondary thickening of the main root, with a root diameter of approx. 45-60% of that in the wild type (Fig. 3B,F). The three outer layers (epidermis, cortex and endodermis) were still present in both wild-type and transgenic roots (Fig. 3B,D,F).

After completion of its extension growth, the *Arabidopsis* hypocotyl also undergoes secondary thickening (Gendreau et al., 1997). Cell divisions in the central cylinder give rise to the secondary xylem, the vascular cambium, and the secondary phloem. Compared with that of the wild type, the hypocotyl of the ATHB-2 and α-ATHB-2 seedlings showed reduced and increased secondary thickening, respectively (Fig. 2C,F,I). Moreover, secondary cell proliferation of the hypocotyl was reduced in DEX-treated ATHB-2-G seedlings compared to controls (Fig. 4C,F). No other significant difference was observed in the cellular organization of the hypocotyl of transgenic seedlings. As in the controls, surrounding the central cylinder was the endodermis, and two layers of cortex cells surrounded by the epidermis. The cell numbers within each external cell layer in the hypocotyl of ATHB-2 and αATHB-2 seedlings were essentially the same as those of the wild type (Fig. 2C,F,I).

Elevated ATHB-2 levels affect auxin response pathway(s)

The ATHB-2 plants exhibit severe defects in specific cell proliferation processes such as secondary thickening and lateral root formation. Noteworthy, indole-3-acetic acid (IAA) is a key regulator of both processes (Aloni, 1995; Lomax et al., 1995). As a first step to establish a link between ATHB-2 and auxin, we investigated the effect of IAA on lateral root formation in wild-type and transgenic seedlings. To this end, wild-type and transgenic plants were grown on IAA-supplemented medium. The roots of ATHB-2 seedlings grown in the presence of IAA developed essentially the same number of lateral roots as those of wild type indicating that auxin is able to rescue the ATHB-2 lateral root phenotype (Fig. 5). Consistently, we found that the root phenotype of ATHB-2 plants can be rescued by growing the transgenic plants at high temperature (29°C) which is known to cause an increase in the level of free IAA (Gray et al., 1998; data not shown).

Table 2. Length of the hypocotyl in wild-type and *axr1-12* seedlings grown under high and low R:FR ratios (mm ± s.e.m.)

	High R:FR	Low R:FR
wild type (Col)	1.11±0.03	2.29±0.08**
axr1-12 (Col)	0.96±0.04	1.06±0.05**

Seedlings were grown in high and low R:FR ratios for 7 days.
10 samples were measured for each condition.
**P<0.01.

Several recent studies have also implicated the auxin response and the auxin transport systems in hypocotyl elongation of light-grown *Arabidopsis* seedlings (Boerjan et al., 1995; Romano et al., 1995; Gray et al., 1998; Jensen et al., 1998). Low R:FR ratios cause a significant increase in hypocotyl elongation of young seedlings (Smith and Whitelam, 1997; Table 2). To investigate whether auxin was involved in mediating shade-induced hypocotyl elongation, the *axr1-12* mutant, severely impaired in auxin response (Hobbie and Estelle, 1994), was examined. When grown in low R:FR ratios, the hypocotyls of the *axr1-12* seedlings elongated significantly less than those of wild type (Table 2), indicating a role for the auxin in the shade avoidance response. To investigate whether an active transport of auxin is needed for the shade-induced hypocotyl elongation, the auxin transport inhibitor naphthylphthalamic acid (NPA) was used. NPA was able to significantly reduce hypocotyl elongation of wild-type seedlings in response to low R:FR ratios (Table 3). Consistently with a role of ATHB-2 in the shade avoidance response, NPA was also able to reduce hypocotyl elongation of ATHB-2 transgenic seedlings grown in high R:FR ratios (Table 3).

Taken together, these data strongly suggest that shade-induced hypocotyl elongation depends on actively transported auxin and ATHB-2 activity interferes with auxin response pathway(s).

Transcriptional role of ATHB-2 in the regulation of cell elongation and secondary growth of the vascular system

The analysis of ATHB-2, αATHB-2 and ATHB-2-G transgenic plants indicated that the ATHB-2 protein is involved in the regulation of fundamental processes such as cell elongation

Table 3. Length of the hypocotyl in wild-type seedlings grown under high and low R:FR ratios and in ATHB-2 seedlings grown under high R:FR ratios (mm ± s.e.m.)

	–NPA	+NPA
wild type (Ws) high R:FR	1.53±0.04	1.04±0.04**
wild type (Ws) low R:FR	2.28±0.10	1.28±0.08**
ATHB-2 (Ws) high R:FR	2.35±0.05	1.31±0.04**

Seedlings were grown in high and low R:FR ratios for 7 days in the absence and in the presence of NPA (3 μM).
10 samples were measured for each condition.
**P<0.01.

and secondary thickening. As a first step towards understanding the molecular mechanism through which the ATHB-2 protein regulates these processes, we investigated whether it acts as a transcription factor.

ATHB-2 negatively regulates reporter gene expression in a transient assay

In vitro DNA binding studies have demonstrated previously that HD-Zip proteins act as DNA binding proteins (Sessa et al., 1993, 1998b). In particular, the study of the DNA binding properties of ATHB-1 and -2, members of the HD-Zip I and II protein families, respectively (Carabelli et al., 1993), has shown that they interact with DNA as homodimers and recognize two 9 bp pseudopalindromic sequences that differ at the central position (BS-1 and BS-2, respectively; Sessa et al., 1993). Studies on effects of HD-Zip proteins on gene expression have only been reported for ATHB-1 which was capable of transiently activating a reporter gene with ATHB-1 target sites upstream of the truncated CaMV 35S -46 promoter in tobacco and *Arabidopsis* leaves (Aoyama et al., 1995; Sessa et al., 1998a), and Oshox1, a rice HD-Zip II protein, which was found to repress reporter gene activity in rice suspension cells (Meijer et al., 1997).

To test whether ATHB-2 can regulate transcription, the entire ATHB-2 coding sequence or the HD-Zip-2 coding sequence fused to the sequence encoding the transactivation domain of the herpes simplex virus transactivation domain VP16 (amino acids 413-490; Dalrymple et al., 1985) transcribed from the −343 CaMV 35S promoter (ATHB-2 and HD-Zip-2-V, respectively) were used as the transacting genes in particle bombardment experiments with *Arabidopsis* leaves. As a negative control, the ATHB-2 coding sequence was replaced with the HD-Zip-2 coding sequence (HD-Zip-2). ATHB-1 was used as a positive control. Plasmids carrying the luciferase (LUC) reporter gene (de Wet et al., 1987) with the ATHB-1 binding sequence (5′-CAATTATTG-3′, BS-1; Sessa et al., 1993) or the ATHB-2 binding sequence (5′-CAATGATTG-3′, BS-2; Sessa et al., 1993, 1994) or its mutant sequence (5′-CAATGGATTG-3′, mBS-2; Sessa et al., 1994) placed 5′ of the −46 CaMV 35S promoter, were used as reporter plasmids. Plasmids carrying the transacting genes also contained a β-glucuronidase (GUS) gene (Jefferson et al., 1987) transcribed from the −343 CaMV 35S promoter, and its activity was used as an internal control. Each transacting plasmid was co-bombarded with a reporter plasmid into *Arabidopsis* leaves. Twenty hours after bombardment, the leaves were assayed for LUC and GUS activities. LUC activity was normalized with respect to GUS activity, and the normalized value was considered to represent the strength of the transacting function of each transcription factor. The results are shown in Fig. 6A. HD-Zip-2-V, but not ATHB-2, displayed strong transcriptional activation from the promoter with the ATHB-2 binding site, whereas no transactivation was detected with the mutant binding sequence. HD-Zip-2 was inactive with either the wild-type or mutant binding sequences. Similar results were obtained in particle bombardment experiments performed in *Arabidopsis* hypocotyls (data not shown).

To investigate further the role of ATHB-2, we took advantage of our previous discovery that ATHB-1 has the ability to recognize, although less efficiently, the ATHB-2 binding site in vitro (Sessa et al., 1997). Reciprocally, ATHB-2 recognises the ATHB-1 binding site with a reduced affinity relative to the ATHB-2 binding sequence (Sessa et al., 1997). Thus, we investigated whether ATHB-2 has the ability to reduce the transactivation mediated by ATHB-1 in a binding site-specific manner. To this end, ATHB-1 and ATHB-2 in a 1:3 ratio were co-bombarded with a reporter bearing either the BS-1 site or the BS-2 site. As a control, ATHB-1 was also co-bombarded with HD-Zip-2-V. The results are shown in Fig. 6B. Co-bombardment of ATHB-1 and -2 results in a reduced transactivation of the reporter gene containing the ATHB-1 binding site (approx. 4-fold) and more significantly of that bearing the ATHB-2 binding site (approx. 8-fold) compared to that observed when ATHB-1 by itself was bombarded with the reporter containing the BS-1 and BS-2 sequence, respectively. As expected, ATHB-1 together with HD-Zip-2-V was more effective in the transactivation of both BS-1 and BS-2 reporter genes. To prove that the observed reduction in ATHB-1-mediated transactivation was attributable to the binding of ATHB-2 to BS-1 or BS-2, we constructed a derivative of ATHB-2 with a single amino acid substitution at position 51 of the HD (mATHB-2) which prevents DNA binding in vitro (Sessa et al., 1997). Co-bombardment of ATHB-1 and mATHB-2 results in the same transactivation as that of ATHB-1 alone (Fig. 6B).

Taken together, these results demonstrated that ATHB-2 functions as a negative regulator of gene expression in a transient assay.

Expression of HD-Zip-2-V-G in transgenic plants reduces cell elongation and increases secondary thickening in the hypocotyl

To investigate whether ATHB-2 does indeed act as a transcriptional repressor in the cell elongation process, we generated transgenic plants expressing a derivative of ATHB-2 with the same DNA binding specificity but opposite regulatory properties, inducible by glucocorticoids. To this end, the HD-Zip-2-V coding sequence fused to a DNA fragment encoding the glucocorticoid receptor domain (Miesfeld et al., 1986) transcribed from the CaMV 35S promoter (HD-Zip-2-V-G) was introduced into *Arabidopsis* using the vacuum infiltration method (Bechtold et al., 1993). 20 independent primary transgenic plants were regenerated in the presence of kanamycin. The induction experiment was done with T3 generations of 10 lines. In the presence of dexamethasone all of the HD-Zip-2-V-G lines had a shorter hypocotyl than that of control plants (see Fig. 7A,D showing a representative line grown in the absence and in the presence of DEX, respectively). Moreover, a more detailed analysis of a representative transgenic line (in which the expression level of the transgene was 25-fold that of the *ATHB-2* gene) revealed that the longitudinal length of hypocotyl epidermal cells was significantly reduced in the presence of dexamethasone (Fig. 7B,E). Finally, cross sections of DEX-treated seedlings showed an increase in the secondary thickening of the hypocotyl compared to controls (Fig. 7C,F). Taken together, these results strongly suggest that ATHB-2 may indeed act as a repressor in vivo.

DISCUSSION

The *Arabidopsis ATHB-2* gene was shown earlier to be rapidly

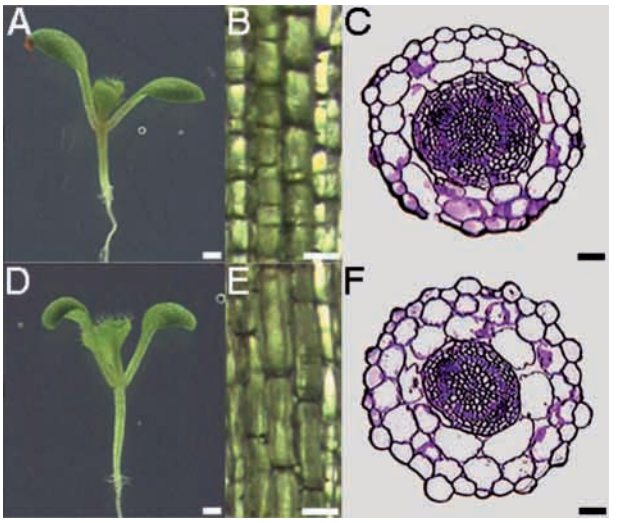

Fig. 4. Phenotypes of ATHB-2-G transgenic seedlings. (A-F) ATHB-2-G seedlings grown for 7 days on agar medium alone (A-C) or on agar medium with 10 μM DEX (D-F). (A,D) Side view of the seedlings. Note the longer hypocotyl in the DEX-treated seedlings. (B,E) Magnified views of A and D, respectively, showing hypocotyl epidermal cells. (C,F) Transverse sections showing the cellular organization of the hypocotyl. Scale bar, (A,D) 500 μm, (B,C,E,F) 50 μm.

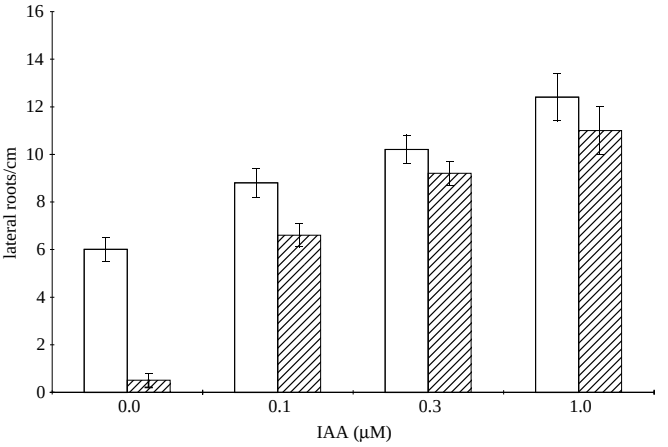

Fig. 5. IAA is able to rescue the ATHB-2 lateral root phenotype. Wild-type (white bars) and ATHB-2 (hatched bars) seedlings were grown on agar plates in a light:dark cycle of 16:8 hours for 5 days and then transferred to IAA-supplemented medium. Following 4 days of growth, lateral roots were counted on the portion of primary root which had grown in the absence of IAA. 10 samples were measured for each treatment.

and strongly induced by changes in the R:FR ratio that induces the shade avoidance response in many plants (Carabelli et al., 1993, 1996; Steindler, 1997). The experiments described here provide evidence that the *ATHB-2* gene encodes a transcription factor involved in the regulation of cell elongation, a fundamental developmental process of the plant cell, as well as in specific cell proliferation processes such as lateral root formation and secondary growth of the vascular system. Taken together, the data indicate that ATHB-2 plays a major role in phytochrome-regulated growth responses such as shade avoidance and neighbour detection that produce dramatic changes in the development of a plant.

ATHB-2 enhances cell elongation

We have shown that alterations in ATHB-2 levels can dramatically alter cell shape, specifically affecting the direction of cell expansion. In cotyledons and hypocotyl**,** elevated levels of ATHB-2 promotes cell elongation along the main axis of the plant; conversely, reduced levels of ATHB-2 cause increased expansion of the cells in a direction orthogonally to the main axis of the plant.

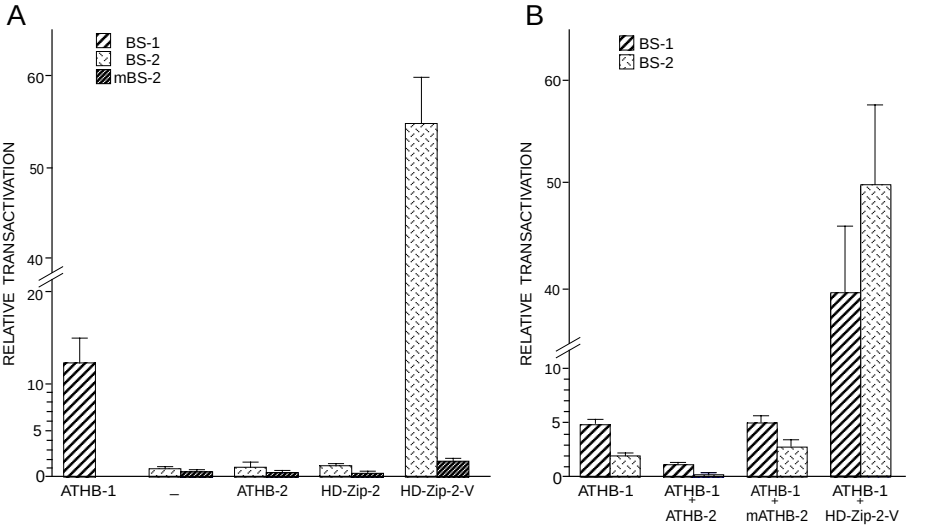

Fig. 6. ATHB-2 acts as a negative regulator of gene expression in a transient assay. (A) HD-Zip-2-V, but not ATHB-2, transactivates reporter gene expression. A plasmid carrying either ATHB-1, ATHB-2, HD-Zip-2 or HD-Zip-2-V and a reporter plasmid carrying a LUC gene, containing at its promoter either six copies of the ATHB-1 binding sequence (5′-CAATTATTG-3′; BS-1), six copies of the ATHB-2 binding sequence (5′-CAATGATTG-3′; BS-2) or its mutant sequence (5′-CAATGGTTG-3′; mBS-2) were co-bombarded into *Arabidopsis* leaves. The plasmids of the transacting genes also carried a GUS gene driven by the CaMV 35S promoter, which was used as an internal control. Twenty hours after bombardment, the leaves were assayed for LUC and GUS activities. LUC activity was normalized with respect to GUS activity for each bombardment experiment. The average values of three independent experiments were calculated for each combination of transacting and reporter genes. (–) represents non-bombarded leaves. Bars indicate standard deviations. (B) ATHB-2 reduces the transactivation mediated by ATHB-1. ATHB-1 or ATHB-1 in combination with either ATHB-2, mATHB-2 or HD-Zip-2-V and a LUC gene containing at its promoter either six copies of the BS-1 sequence or six copies of the BS-2 sequence were co-bombarded into *Arabidopsis* leaves. Assays for LUC activity and normalization were as described in A. The average values of three independent experiments were calculated for each combination of transacting and reporter genes. Bars indicate standard deviations.

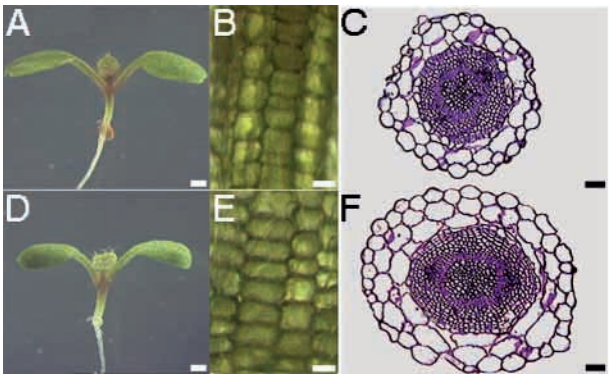

Fig. 7. Phenotypes of HD-Zip-2-V-G transgenic seedlings. (A,D) Side view of HD-Zip-2-V-G seedlings grown for 7 days on agar medium alone (A) or with 0.005 μM DEX (D). (B,E) Hypocotyl epidermal cells of HD-Zip-2-V-G seedlings grown for 14 days on agar medium with (E) or without (B) 0.001 μM DEX (C,F) Transverse sections showing the cellular organization of the hypocotyl of seedlings in B and E, respectively. Scale bar, (A,D) 500 μm, (B,C,E,F) 50 μm.

Some evidence indicates that polarity-specific expansion processes play important roles in regulating the growth of plant organs. For example, the *ANGUSTIFOLIA* and *ROTUNDIFOLIA3* genes control leaf morphology by regulating cell expansion in the leaf-width and leaf-length direction, respectively (Tsuge et al., 1996). The characterization of the *sabre* mutation, which results in abnormal cortex cell expansion in the root, has suggested that the extent or direction of cell expansion could be determined by the equilibrium between pairs of counteracting activities (Aeschbacher et al., 1995). Our characterization of the ATHB-2 protein revealed that it acts as a transcriptional repressor in transient expression assays. Remarkably, we have found that expression in transgenic plants of a derivative of ATHB-2 with the same DNA binding specificity but opposite regulatory properties (HD-Zip-2-V) results in a shift in the orientation of cell expansion toward radial expansion in the hypocotyl. Extrapolating from the model suggested for the action of SABRE, we propose that ATHB-2 and a member of the same protein family (HD-Zip II; Morelli et al., 1998) with regulatory properties analogous to those of HD-Zip-2-V (ATHB-X) produce opposite effects on cell expansion. We also postulate that the two activities are in a dynamic equilibrium. Under this hypothesis raising ATHB-2 levels (as in the ATHB-2 and ATHB-2-G plants) causes the equilibrium to be shifted toward longitudinal expansion. Reducing ATHB-2 below wild-type levels (as in αATHB-2 plants) or raising ATHB-X levels (as simulated in DEX-treated HD-Zip-2-V-G plants) shifts the equilibrium toward radial expansion. A prediction of this hypothesis is that reducing the levels of ATHB-X should cause increased elongation in the hypocotyl.

If this hypothesis is correct, it suggests that changes in the R:FR ratio cause an increase in extension growth (shade avoidance response) by inducing *ATHB-2* gene expression and therefore changing the equilibrium between ATHB-2 and ATHB-X.

ATHB-2 inhibits secondary vascular cell proliferation and lateral root formation

There is considerable evidence indicating that IAA is a key regulator of vascular development and cambial growth (Aloni, 1995). Developing buds and young shoots are major sources of auxin, which is transported in a basipetal polar fashion (Lomax et al., 1995). In the'canalization hypothesis' Sachs proposed that the diffusion of this hormone from an auxin source induces the formation of a polar auxin transport system along a narrow file of procambial cells; the polar transport of auxin should result in the formation of vascular strands (Sachs, 1981). Indeed, several experiments support the hypothesis that the auxin polar transport and its regulation induces formation of primary as well as secondary vascular tissues (Aloni, 1995 and references therein; Gälweiler et al., 1998; Uggla et al., 1998; Mattsson et al., 1999).

Also, several studies have shown that lateral root initiation is dependent on IAA transported from the shoot into the root. For example, removal of the auxin-synthesizing tissues (Wightman and Thimann, 1980) or treatment of seedlings with specific inhibitors of polar auxin transport such as N-1-naphthylphthalamic acid abolishes lateral root formation (Muday and Haworth, 1994; Reed and Muday, 1996). Moreover, it has been recently shown that mutations in *TIR3* resulting in a reduction in auxin polar transport dramatically affect lateral root production (Ruegger et al., 1997).

We have shown that changes in ATHB-2 levels can alter the above mentioned cell proliferation processes which are regulated by auxin response pathways. In particular, elevated levels of ATHB-2 dramatically reduce the number of lateral roots and inhibit secondary thickening in the hypocotyl and in the primary root. Furthermore, plants with reduced levels of ATHB-2 partially phenocopy plants exposed to high levels of auxin, enhancing lateral root formation and secondary growth of the vascular system. In view of the finding that IAA is able to rescue the ATHB-2 lateral root phenotype, and because of the reciprocal phenotypes obtained in ATHB-2 and αATHB-2 plants, it seems likely that ATHB-2 inhibits secondary vascular cell proliferation and lateral root formation by affecting either the response to or the distribution of auxin.

We have also found that expression in transgenic plants of HD-Zip-2-V results in an increase of secondary vascular growth in the hypocotyl, a phenotype reciprocal to that obtained with elevated levels of ATHB-2. This suggests that, as for the orientation of cell expansion, the rate of secondary cell proliferation might be determined by the equilibrium between pairs of counteracting activities. Recently, we have shown that ATHB-8, encoded by an auxin-regulated gene specifically expressed in provascular cells (Baima et al., 1995), enhances secondary vascular growth both in *Arabidopsis* and tobacco transgenic plants (S. Baima, A. M., M. Possenti, P. Pane, I. R., M. M. Altamura and G. M., unpublished results). The *ATHB-8* gene encodes an HD-Zip transcription factor of the HD-ZIP III family, and in vitro DNA binding studies revealed that HD-Zip III proteins recognize a 11 bp pseudopalindromic sequence that is efficiently recognized by ATHB-2 (Sessa et al., 1998b; G. S., unpublished results). Thus, elevated levels of ATHB-2 might inhibit secondary cell proliferation by competing with ATHB-8 for binding to downstream genes involved in secondary growth.

A model for ATHB-2 function in shade avoidance responses

In most plants, primary growth is essentially equivalent to grow in length. The stems and roots of many plants also increase in diameter, in regions that have stopped elongating, by the activity of lateral meristems. To maximize the capability of an organ to grow in length a mechanism tightly coupling vascular development with elongation growth should exist and be very effective. From the analysis of transgenic plants with altered ATHB-2 level, we deduce that the increase in extension growth of a seedling in low R:FR ratios is the consequence of two events: a change in the orientation of cell expansion toward elongation in cells that do not divide, as the epidermal and cortical cells in the hypocotyl, and the inhibition of cambial cell proliferation and differentiation that contribute to radial growth. Likewise, secondary growth of the vascular system is significantly reduced in wild-type seedlings grown under low R:FR ratios compared to those grown under high R:FR ratio (C. S. and A. M., unpublished data). Mutations in *HY5*, a gene encoding a b-Zip protein required in the light-dependent inhibition of hypocotyl elongation during the de-etiolation process, also cause an increase in longitudinal expansion of hypocotyl epidermal cells and inhibition of secondary cell proliferation (Oyama et al., 1997). Again, this fact implies that, at least in the hypocotyl, these two processes are tightly coupled. It has been suggested that the HY5 protein may be involved in the signaling pathway mediated by auxin (Oyama et al., 1997). It is well known that many developmental processes depend on regulated auxin transport. For example, a gradient of auxin concentration from tip to base is believed to be responsible for differential elongation rates in different regions of the shoot (Sanchez-Bravo et al., 1992) and for differential rate of xylem cell differentiation (Aloni and Zimmermann, 1983). In addition, a higher lateral transport of auxin which enhances an auxin-dependent elongation process, has been recently demonstrated in the *Arabidopsis* hypocotyl (Boerjan et al., 1995; Romano et al., 1995; Gray et al., 1998; Jensen et al., 1998). Moreover, in the case of tropic responses, lateral redistribution of auxin gives rise to differential growth rates, resulting in curvature of the growing organ (Lehman et al., 1996). In analogy, we propose that extension growth phenomena induced by neighbour detection and shade are the result of a laterally symmetric redistribution of auxin regulated by ATHB-2. Among other possibilities, this might be achieved straightforwardly by increasing the amount of auxin actively transported in non-vascular cells, for example in epidermal and cortical cells of the hypocotyl, to the detriment of that transported through the developing vascular system. This hypothesis is supported by the finding that the polar auxin-transport inhibitor NPA was able to significantly reduce hypocotyl elongation of wild-type seedlings in response to low R:FR ratios and of ATHB-2 seedlings under normal light conditions. A net reduction of auxin transported through the vascular system might result in (i) a letup in vascular differentiation; (ii) a decrease in auxin concentration reaching the root, resulting in a strong reduction of lateral root formation and, eventually, in a slower growth of the main root. This hypothesis is currently under investigation.

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