The expression of the *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*

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**SUMMARY**

We have characterized an *Arabidopsis* homeobox gene coding for a putative DNA binding protein that represents an early marker for vascular development. The full-length cDNA encodes a protein of 833 amino acids that we have designated *Athb-8*; it contains the conserved DNA binding domain that characterizes the HD-Zip family of transcription factors. RNA analysis showed that the *Athb-8* gene is expressed during the vegetative and the reproductive phases of plant growth. A higher steady-state level of the *Athb-8* mRNA was found in flowering stem and root. In situ mRNA analysis of *Arabidopsis* plants demonstrated that *Athb-8* expression is restricted to the procambial cells of embryo and developing organs. Moreover, *Athb-8*-GUS expression was found in single parenchyma cells which are differentiating into tracheary elements in wounded tobacco transgenic plants. Finally, we showed that the auxin, indole-3-acetic acid, which is involved in vascular development and differentiation, modulates the expression of the gene. Taken together, these results suggest that *Athb-8* might be a regulator of vascular development in *Arabidopsis thaliana*.

Key words: *Arabidopsis*, auxin, HD-Zip motif, procambium, vascular development, homeobox gene

**INTRODUCTION**

In flowering plants, the body organization is generated by two distinct processes. During embryogenesis, the polar axis of the plant is established, domains that set up the organization of the plant body are defined, and the primary tissue and organ systems are delineated. The primary body organization of the mature embryo may be described as a superimposition of two patterns, one axial and one radial. The apical-basal pattern is arranged along the main body axis of polarity. Shoot meristem, cotyledon(s), hypocotyl, radicle (embryonic root) and root meristem are morphologically distinct elements of the axial pattern. The radial pattern, which is perpendicular to the axis, involves the three major tissues of the primary plant body: the outer epidermis, the inner mass of ground tissue and the centrally located vascular strands (Johri, 1984; Mayer et al., 1991). During post-embryonic development, the major portion of the morphogenetic programs and patterning occurs progressively from the shoot and root meristems. Leaf primordia and the procambium, as well as the cortical ground tissue, arise from the shoot meristem (Steeves and Sussex, 1989). The root meristem is composed of few mitotically quiescent central cells surrounded by morphologically distinct initials. Further differentiation of these cells gives rise to cell files which form the epidermis and lateral root-cap, the cortex and endodermis, the stele and columella (Benfey and Schiefelbein, 1994; Doerner, 1993; Esau, 1977).

Although genetic approaches are giving significant insights into the processes involved in the formation of embryonic pattern, apical meristems and organs (reviewed by Doerner, 1993; Ma, 1994; Medford, 1992; Meyerowitz, 1994; Schiefelbein, 1994; Weigel, 1993), not much is known about developmental regulation in higher plants.

Many animal homeodomain (HD) proteins are believed to play a critical role in diverse developmental processes, including the control of pattern formation in insect and vertebrate embryos, and the specification of cell fates in many tissues (reviewed by Affolter et al., 1990). In maize, gain-of-function mutations of the *KNOTTED1* homeobox (HB) gene result in an alteration of leaf morphology (Hake, 1992). In *Arabidopsis*, mutations in the HB gene *GLABRA2* result in abnormal trichome expansion (Rerie et al., 1994). Therefore it is tempting to speculate that HD proteins in plants will be involved in differentiation and/or developmental control as they are in animals.

In an attempt to identify putative developmental regulatory genes in *Arabidopsis*, the isolation and the characterization of homeobox genes was recently undertaken (Ruberti et al., 1991). The *Arabidopsis* Athb-1 and -2 homeodomains exhibit a strong homology with the helix-3 region and the highly conserved residues of most of the HD sequences. The *Arabidopsis* homeobox genes also contain a second element that potentially codes for a leucine zipper motif (Zip), located immediately 3′ to the homeobox; therefore these gene products...
have been designated as HD-Zip proteins (Ruberti et al., 1991). Other members of the Arabidopsis HD-Zip class of proteins have been characterized (Carabelli et al., 1993; Mattsson et al., 1992; Schena and Davis, 1992), and the isolation of new members of this class of proteins has been recently reported (Schena and Davis, 1994; Sessa et al., 1994; Soderman et al., 1994). On the basis of sequence homology, we tentatively grouped the HD-Zip proteins characterized so far into four families designated HD-ZIP I-IV (Carabelli et al., 1993; Sessa et al., 1994).

We have found that HD-Zip proteins interact with DNA recognition elements in a fundamentally different fashion from the classic homeodomain proteins, thereby constituting a distinct class of regulatory proteins (Sessa et al., 1993). The apparent uniqueness of HD-Zip proteins to higher plants and the observation that light quality strongly affects the expression of the Athb-2 and -4 genes suggest that these factors might control developmental pathways that are peculiar to plants (Carabelli et al., 1993).

Here, we present the characterization of the Athb-8 gene, which encodes a novel type of HD-Zip protein. In situ mRNA analysis of Arabidopsis plants demonstrated that the Athb-8 gene is transcribed in procambia of embryo and developing organs, and during the regeneration of vascular strands. The peculiar spatial and temporal expression found suggests that the Athb-8 gene product might play a role in the regulation of vascular development in Arabidopsis thaliana.

MATERIALS AND METHODS

Plant material

A. thaliana Columbia ecotype plants were grown as previously described by Carabelli et al. (1993). Roots were obtained from plants grown for 4 weeks in liquid medium. Young seedlings were obtained from vernalized seeds germinated on solid medium and grown for 5 days in the light (GS). Soil grown plants were taken to analyze later stages. Whole plants were collected at 2 and 3 weeks and the whole aerial portion at 4 weeks. Green silique was harvested from 5 to 6 week old plants.

A. thaliana Wassilevskija (WS) ecotype plants grown as described above were used for Agrobacterium mediated transformation. Selection of transgenic seeds was done on 0.5× Murashige and Skoog salt mixture + Gamborg’s B5 vitamins in the presence of 50 μg/ml kanamycin.

Tobacco plants (Nicotiana tabacum var. SR1) were germinated on solid 1× Murashige and Skoog salt mixture + Gamborg’s B5 vitamins in the presence of 50 μg/ml kanamycin.

Screening of cDNA and genomic libraries

A λEMBL-3 library containing partially digested MboI fragments of Arabidopsis Columbia DNA (Clontech) was screened using the HB-2 degenerate oligonucleotide (5′-TGGTTYCARAAAYMGNNM-3′) that corresponds to a conserved six amino acid sequence from the helix-3 region of Athb-1 and -2 (Sessa et al., 1994). Filters were prehybridized, hybridized and washed as previously described (Ruberti et al., 1991) except that the washing temperature was 47°C. An A. thaliana Columbia cDNA library (Clontech) was screened using a 32P-labelled genomic fragment spanning the homeodomain coding region. Three independent, overlapping Athb-8 hybridizing λ cDNA clones (l21, l71, l73) were subcloned into pBluescript KSII (Stratagene) using standard techniques.

PCR cloning of the 5′ transcribed region of the Athb-8 gene

Two oligonucleotides, 73un20 and 73un21, were designed from the genomic sequence and used as upstream primers in combination with the rev73B downstream primer. Synthesis of cDNA from 250 ng of leaf poly(A)+ RNA with random hexamers was performed using the GeneAmp RNA PCR kit (Perkin Elmer-Cetus Corp.). No amplification product was obtained using the 73un21 primer. The 932 bp PCR product obtained with the 73un20 and rev73B primers was restricted with HindIII and the resulting 663 bp fragment was subcloned (p573 HindIII) into pBluescript KSII (Stratagene). The 5′ Athb-8 fragment was obtained by amplification of a genomic subclone with 73un20 and 73rev12 primers. PCR amplifications were performed according to standard protocols (Innis and Gelfand, 1990).

The sequences of the oligonucleotide primers were as follows: 73un20: 5′-AGCACACCCACCCTATAAC-3′ (from nucleotides 88 to 107 in Fig. 2A) 73un21: 5′-ACAGCCGACACATGTC-3′ (from 1 to 19) rev73B: 5′-GGGTTCAGCTCGCC-3′ (from 1736 to 1722) 73rev12: 5′-CCCTCTTTTCTCCTCAG-3′ (from 334 to 318).

DNA sequencing

Sequencing of the double stranded DNA was carried out with Sequenase 2 (USB) according to manufacturer’s instructions. Oligonucleotides were synthesized on a Beckman DNA-SM synthesizer and purified according to manufacturer’s instructions. The sequence of the four cDNAs was determined on both strands.

DNA and RNA gel blot analyses

Genomic DNA isolation, DNA restriction analysis, gel electrophoresis and Southern blot onto Hybond-N membrane (Amersham) were done as previously described (Carabelli et al., 1993). A fragment corresponding to the 3′ end of the Athb-8 cDNA (a 272 bp EcoRI DNA fragment of the 3′ Athb-8 clone, Fig. 1A) was used as probe. 32P random primed labelling of the probe and hybridization conditions were performed according to standard protocols.

RNA was isolated and analysed as previously described (Carabelli et al., 1993). Poly(A)+ RNA was isolated from total RNA with Dynabeads Oligo(dT)25 (Dynal AS) according to manufacturer’s instructions. For northern blot analysis 10 μg of total RNA or 2.5 μg of poly(A)+ RNA from A. thaliana were denatured, electrophoresed on 1.2% agarose-1.9% formaldehyde gels and transferred onto Hybond-C extra membranes (Amersham). The amount of Athb-8 transcripts was quantitated by scanning the X-ray films with a laser densitometer (Ultroscan XL, LKB) and normalized to the amount of total RNA by transcript quantification of the nuclear gene encoding the β subunit of the mitochondrial ATPase, a gene which is known to be constitutively expressed (Kuhlemeier et al., 1987).

In situ hybridization

Linearized plasmid templates were used to generate 35S-UTP-labelled antisense and sense RNA probes by runoff transcription. Either the p71AHindIII clone (a 190 bp cDNA fragment subcloned into pBluescript KSII) or the 272 bp 3′ Athb-8 clone (Fig. 1A) were used to provide templates for the T3 and T7 polymerases. Probes were used at a final concentration of 1-1.5×10⁸ dpm/ml. Fixation of tissue, preparation of sections, hybridization and washes were carried out as described by Drews et al. (1991) with minor modifications (hybridization temperatures were 46°C and 50°C for the p71AHindIII probes and p3′ Athb-8 probes, respectively). Hybridizations with the transcribed sense RNA were performed as a negative control. Slides were exposed for 10-30 days.

Construction of promoter-GUS fusion and plant transformation

A 1.7 kb DNA fragment of Athb-8 genomic sequence upstream of the
putative initiation codon was amplified by PCR and cloned in frame with the β-glucuronidase (GUS) coding sequence in the pBII01.1 binary vector (Clontech). The pAthb-8-GUS construct was checked by sequencing and introduced into the LBA4404 Agrobacterium tumefaciens strain by standard methods. The LBA/pAthb-8-GUS strain and a corresponding control were used for Agrobacterium mediated transformation of Arabidopsis and tobacco according to the methods of Bechtold et al. (1993) and Horsch et al. (1985), respectively.

The resultant transgenic plants were screened for the level of GUS activity by standard fluorimetric assay (Jefferson et al., 1987) and for 3:1 segregation on plates containing kanamycin. Three lines of both Arabidopsis and tobacco transgenic plants were selected and used for further characterization.

Histochemical localization of GUS activity

Histochemical staining for GUS activity was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as chromogenic substrate (Jefferson et al., 1987). Freshly cut plant tissue was incubated in GUS staining solution containing 50 mM sodium phosphate pH 7.0, 0.1% Triton X-100, K3/K4 FeCN 0.5 mM, and 1 mM X-Gluc at 37°C for 1-24 hours depending on staining intensity. Photosynthetic tissues were cleared in methanol: acetic acid (3:1) and then transferred to 70% lactic acid. Photographs were taken under a Wild MZ8 stereomicroscope (Leica).

Wounding

Razor blade wounds were made in the middle of internodes that were about 10 cm behind the shoot tip. A cut penetrating approximately 75% of the diameter of the stem was made horizontally. The incision severed the cortex and vascular tissue and extended into the pith. About 3 mm under this cut, a second horizontal cut was made from the opposite side. The region between the cuts has been called ‘bridge’. Parafilm was placed in each wound to prevent possible contact between the two sides of the cut and to ensure that material could be transported in the stem only across the bridge. After wounding, the plants lost water and were splinted to maintain rigidity. In about 1 week the wounded plants started to recover, as indicated by regain of turgidity.

Arabidopsis Athb-8 expression in procambial cells

Hormone treatment

For auxin induction studies, plants were treated essentially as described by Gee et al. (1991). Leaves from 4 week old plants were cut in pieces, about 1 cm², and washed shaking for 4 hours in 10 mM potassium phosphate buffer (pH 6), 2% sucrose to remove endogenous auxins. Samples were then incubated for 1 hour in fresh buffer alone or with increasing concentrations of indole-3-acetic acid (IAA) (from 10⁻⁹ to 10⁻⁵ M), frozen in liquid N₂ and processed for RNA extraction.

Computer analysis

Comparative sequence analysis was performed with the PILEUP program from the Genetics Computer Group (Devereux, 1991).

RESULTS

Identification of a novel member of the plant HD-Zip protein family

To identify other genes coding for HD proteins, we took advantage of the strategy previously used to isolate Athb-1 and -2 (Ruberti et al., 1991). For the new screening we designed HB-2 (see Materials and methods), a degenerate oligonucleotide shorter than HB-1. HB-2 corresponds to a conserved six amino acid (aa) sequence from the helix-3 region of Athb-1 and -2. Several clones were isolated in this screening and based on cross-hybridization studies they were shown to contain different genomic fragments (Sessa et al., 1994). A clone, designated Athb-8, was further characterized. Genomic sequences flanking the homeobox region were cloned and sequenced by standard techniques.

To determine the entire coding sequence, the HB-2 hybridizing genomic fragment was used to screen an Arabidopsis cDNA library. Three clones were obtained and sequenced. No differences in the DNA sequence were observed between the overlapping regions of different cDNA inserts. A single long open reading frame, starting within the homeobox region, was
identified in this sequence. The same primers used for sequencing the cDNA clones were utilized for DNA sequence analysis of the corresponding genomic clone. To determine which genomic sequences located 5' to the Athb-8 cDNA are transcribed, synthesis of cDNA from poly(A)^+ RNA and PCR amplification were performed. Random hexanucleotide primers were used to prime first strand cDNA synthesis from *A. thaliana* leaf poly(A)^+ RNA. An attempt to obtain a PCR product using the forward primer 73un21 with the reverse primer rev73B was unsuccessful. However, a 932 bp product was obtained with the 73un20 and rev73B primers. DNA sequence analysis of this subcloned fragment (p5'73HindIII)

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**Fig. 2.** DNA and amino acid sequences of Athb-8. (A) The genomic DNA sequence is shown. Dotted lines represent 5' and 3' untranslated regions as deduced from cDNA sequences. Dashed lines represent introns. The deduced amino acid sequence is shown below the sequence of each exon. Two ATGs present in the 5' untranslated region are underlined. **(B)** Amino acid comparison of the Athb-8 HD-Zip domain with other Athb HD-Zip domains (Carabelli et al., 1993; Mattsson et al., 1992; Ruberti et al., 1991; Soderman et al., 1994). Single letter code designates amino acid residues. The positions of the introns in Athb-1, -2 and -8 are indicated with white arrowheads. EMBL Accession No. Z50851 ATHB8GN.
revealed that the 5′ end of the Athb-8 gene contains a 413 bp intron.

DNA sequence comparison of the deduced cDNA with the genomic clones showed that the Athb-8 gene is split by 17 introns (Fig. 1A). A single long open reading frame is identified in the cDNA sequence. There are two ATG codons in this frame toward the 5′ end of the cDNA. We tentatively assigned the first ATG codon as the initiation codon and deduced the protein sequence from this codon. DNA sequence analysis of the 308 bp-long 5′ untranslated leader sequence revealed that it contains two short open reading frame of 13 and 3 amino acids (Fig. 2A) which might be involved in translational regulation of Athb-8.

Southern blot analysis of A. thaliana DNA showed that the p3′Athb-8 cDNA (see Fig. 1A) hybridizes to single BamHI and EcoRI fragments of approximately 5 and 0.7 kb, respectively (Fig. 1B).

The predicted protein contains 833 aas with a calculated relative molecular mass of 92x10^3. The protein is characterized by a HD-Zip motif confined to the N terminus of the polypeptide. Amino acid sequence analysis revealed a particular organization of the Athb-8 HD-Zip domain. The Athb-8 HD domain contains two invariant amino acid residues (W48, F49, N51, R53) and seven out of the eight highly conserved residues (Q12, L16, Y20, L40, V47, R55, K57) of the homeodomain consensus sequence. This conservation could be observed only by looping out four amino acid residues between the putative helices 2 and 3 of the Athb-8 HD. Moreover, the exact spatial register between the homeodomain and leucine zipper found in all the HD-Zip proteins characterized so far is not conserved in the Athb-8 HD-Zip domain. The best alignment of Athb-8 with the prototype Athb-1 HD-Zip domain could be obtained by looping out four amino acid residues between the putative helix 3 of the homeodomain and the first leucine residue of the zipper (Fig. 2B). The DNA binding properties of the Athb-8 HD-Zip domain will be published elsewhere (Sessa G., Steinleider C., Baima S., Morelli G. and Ruberti I., unpublished data). From database searches, no obvious stretches of significant similarity between Athb-8 and other known proteins have been found.

**Regulation of Athb-8 during plant development**

To investigate Athb-8 gene expression in plants, RNA blot hybridization was used to study the abundance of Athb-8 transcripts in different Arabidopsis tissues. A blot containing poly(A)^+ RNA from entire plants of different age and individual organs was probed with a 5′ end fragment of the Athb-8 cDNA. One unique band of approximately 3.4 kb hybridized with the probe in all samples (Fig. 3). After normalization, we calculated that Athb-8 transcripts are slightly enriched (1.3 fold) in 2-week and 3-week old plant (Fig. 3, lane 2 and 3) with respect to green seedlings and 4-week old plants (Fig. 3, lane 1 and 4), respectively. The analysis of RNA isolated from different organs of 5-week old flowering plants revealed a quantitative difference in the Athb-8 expression pattern. Athb-8 transcripts are 5.8-fold enriched in stem (Fig. 3, lane 5) and 1.5-fold in flower (Fig. 3, lane 7) with respect to leaf (Fig. 3, lane 6). The lowest expression is found in siliques (Fig. 3, lane 9). In addition, hybridization of RNA isolated from roots of plants grown on liquid medium for 4 weeks showed that the Athb-8 gene is expressed at a higher steady state level in this organ than in the leaf (4.7-fold enriched, Fig. 3, lane 8).

**Cell type-specific expression pattern of the Athb-8 gene**

We have attempted to determine more precisely the cellular distribution of the Athb-8 mRNA in the stem by in situ hybridization of anti-sense RNA probes to longitudinal sections of fixed tissue. As probes we used the Athb-8 35S-labelled antisense RNA, transcribed from two subclones lacking the HD-Zip motif (respectively, p71 Δ HindIII and p3′Athb-8 probe, see Fig. 1A) whereas the corresponding 35S-sense RNAs have been used as control probes. A specific signal was found clearly associated with the vascular regions of stem and pedicel, but it was absent in the sections hybridized with the control probe (data not shown). To better localize the Athb-8 expression, transverse sections of the stem were also analyzed. After autoradiography, sections were stained with toluidine blue and micrographs were taken with a bright-field (Fig. 4A,D,E,G) or a dark-field (Fig. 4B,C,F,H) microscope. Interestingly, the signal is localized to the layer of cells located between the xylem and the phloem elements, but not around them (Fig. 4B,D). This region, formed by procambial cells, is the meristem responsible for the formation of the vasculature in the primary body of the plant. In contrast, no hybridization signal was revealed in completely differentiated vascular strands of the stem, where the procambium is no longer present (data not shown). Control experiments using sense probe demonstrated the specificity of the signal (Fig. 4C).

To determine if the Athb-8 expression is associated with vascular development in other organs, in situ analysis was also performed on longitudinal sections of developing flowers (Fig. 4E,F) and cross-sections of cauline leaves (Fig. 4G,H). The hybridization signals in these organs were found associated with regions of active vascularization. Similar results were also obtained in 2-week old plants both in the rosette stem and in the leaf (data not shown).
Several genes have been shown to be expressed in regions of the meristems, as well as in procambium. To test whether *Athb-8* expression is procambium specific, as opposed to meristem specific, we analyzed longitudinal sections of vegetative and floral apices. No hybridization signal was detected in these two meristems. Instead, it was possible to detect a low hybridization signal on a side of the vegetative meristem where the initiation of the procambium in associ-
ation with the young leaf primordium occurs (data not shown).

A similar analysis was also performed on longitudinal and transverse sections of root apices. In situ hybridization revealed Athb-8 mRNA localization in a region corresponding to the meristem and elongation zone (Fig. 5A). The specific signal in the elongation zone of the root is clearly associated with the central cell file corresponding to the procambium (Fig. 5B, C). During the analysis we occasionally observed lateral root primordia. Lateral root formation begins by periclinal and anticlinal divisions in pericycle cells of the primary root (Dolan et al., 1993). Upon formation of a dome-shaped structure (primordium) by pericycle derivatives, a new root meristem is derived from a subset of these cells. A significant level of Athb-8 expression is visible (just above the periphery of the vascular cylinder) in the center of the primordium (data not shown).

Finally, we have performed in situ hybridization experiments to determine if the Athb-8 gene is transcribed during embryogenesis. Analysis of longitudinal sections of siliques revealed a low hybridization signal in the central region of the embryo at the linear cotyledon stage (Fig. 5D). This group of narrow elongated cells is the procambium, which extend into the cotyledons in this stage embryo.

To characterize further the cell-specific expression of the Athb-8 gene, we analyzed the expression of the Athb-8-GUS chimeric gene in Arabidopsis. For this purpose, we fused the 1.7-kb DNA fragment lying directly upstream from the first ATG of the Athb-8 coding region in frame with the GUS reporter gene (Jefferson et al. 1987). The entire leader sequence of the Athb-8 mRNA, which contains two short open reading frames and an intron, was included in the construct. In such a construct, GUS should undergo the same transcriptional and translational regulation as Athb-8. Transgenic Arabidopsis seedlings harboring the chimeric Athb-8-GUS gene were analyzed by histochemical staining. In cotyledons GUS staining is visible along vascular strands (Fig. 6A), which are easily identified by the autofluorescence of tracheid cells (Fig. 6B). The GUS staining is also visible in regions of cotyledons and leaflets where the vasculature will be formed (indicated by arrows, Fig. 6A). In the primary root, a specific GUS staining is found in the meristem and in the elongation zone, and it is associated with central files of cells corresponding to the procambium and the columella (Fig. 6C).
**Athb-8 is expressed during the early stages of revascularization**

One of the most widely used approaches to study determinants of vascular differentiation is the analysis of revascularization after severing vascular bundles. We therefore decided to investigate Athb-8 expression under these particular experimental conditions.

As a convenient model system to address this question, we have chosen transgenic tobacco plants harboring the Athb-8-GUS chimeric gene. In fact, tobacco is easy to manipulate in wounding experiments, and Athb-8 expression can be easily monitored by histochemical detection of GUS activity.

To ascertain if the Athb-8 promoter sequence chosen was also active in transgenic tobacco plants, we analyzed GUS expression by histochemical staining (Fig. 7). GUS staining is visible in the vascular system of seedlings (Fig. 7A), in the elongation zone of the primary root (Fig. 7B) and in lateral root primordia (Fig. 7C). Moreover, GUS activity is detectable in embryos starting at the heart stage (Fig. 7D). Therefore the expression pattern of the chimeric gene in tobacco is coincident with that of the Athb-8 gene in Arabidopsis.

To determine whether the Athb-8 promoter is active during revascularization, we analyzed young internodes wounded by a double cut. The two partially overlapping transverse cuts (about 3 mm apart) severed all the vascular bundles but maintained a narrow bridge of pith cells connecting the apical and basal portion of the stem. After wounding the apical portion withered and stopped growing but it regained a healthy appearance in approximately 7 days, suggesting that functional connections had been re-established. We followed the expression of Athb-8-GUS gene in wounded stems by histochemical staining at different time points after cutting. Unwounded stems do not show any specific staining (Fig. 8A,B). Conversely, stems stained 1 hour after the wound appeared diffusely blue coloured indicating that the Athb-8 promoter is wound inducible (Fig. 8C). A rapid induction of Athb-8 upon wounding was also observed in leaves in both tobacco and Arabidopsis transgenic plants (data not shown). Eight hours after wounding GUS activity was lower and the pattern of blue staining was changed. GUS activity was mainly restricted to the portion of the stem above the cut region (Fig. 8D). The staining of a straight and narrow portion of the bridge shows that the expression is maintained preferentially on the side of the nearest upper leaf trace (Fig. 8D). Sometimes we found that the GUS activity is retained in the basal portion of the stem around the uncut vascular bundles (Fig. 8D). However, in stems in which the upper cut severed all the vascular bundles of the nearest leaf no staining was visible in the bridge (data not shown). About 7 days after wounding the expression of GUS activity in recovered plants was mainly present in the bridge and in a narrow region immediately above the margin of the upper cut (Fig. 8E).

To understand better the localization of Athb-8-GUS expression we analyzed in more detail the wounded region of

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**Fig. 7. Athb-8-GUS gene expression in transgenic tobacco plants.** Histochemical localization of GUS activity in (A) seedling, (B) primary root, (C) lateral root, (D) heart stage embryo. (B,C) Dark-field micrographs. The scale bar represents 1 mm in A and 100 μm in B,C and D.
recovered stems. The analysis of transverse sections of the stem showed that new vessels with typical lignified secondary walls had been formed in the pith from parenchyma cells which had undergone cell division and redifferentiation (Fig. 8F,G). Many small, intensely blue stained cells resembling a wound cambium are often present near the new rows of tracheary elements (Fig. 8G). Although new vessel elements differentiate preferentially in close proximity to the old vascular system, we focused our attention on more clearly distinguishable vascular elements that are formed in the pith as isolated cells, small clusters or strands. Sections of stems at earlier phases of recovery showed that many single or grouped parenchyma cells positively stain for GUS activity (Fig. 8H). These cells are most likely vascular cell precursors at very early stages of differentiation because we occasionally observed cells which had both a pale blue colour and the typical pattern of secondary wall deposition (Fig. 8I).

From these experiments we can therefore conclude that Athb-8 gene is expressed at early stages of vascular cells determination during revascularization.

Fig. 8. Athb-8-GUS gene expression during wound recovery. Localization of GUS activity in control (A,B) and wounded stems 1 hour (C), 8 hours (D) and 1 week (E-I) after cutting. (A,C,D,E) Outside view. All the stems are oriented with the apical end upward. An arrow indicates the position of the leaf trace in D. (B) Hand-cut transverse section of the control stem. (F-I) Hand-cut transverse sections through the bridge region. A partially differentiated tracheary element showing a pale blue colour is indicated by an arrowhead in I. P, parenchyma; TE, tracheary elements; V, vascular ring; WC, wound cambium. The scale bar represents 500 μm in A, C, D, E and 100 μm in F-I.
Auxin induces Athb-8 mRNA accumulation

It is very well known that auxins are hormones that regulate various aspects of plant growth and development, such as cell elongation, cell division, cell differentiation and morphogenesis (review by Estelle, 1992). The formation of the vascular apparatus involves several of these aspects and there is convincing evidence that an auxin flux is responsible for the orderly pattern of vascular differentiation from leaves to root (Sachs, 1969; 1981). Moreover, the stimulus for vascular redifferentiation after wounding is thought to be the auxin released from the severed bundle itself (Steeves and Sussex, 1989).

Therefore, we analyzed the effect of auxin on the expression of the Athb-8 gene. In a preliminary experiment leaf tissues were incubated in the presence of 50 μM indol-3-acetic acid (IAA) for 30 minutes and 1 hour and the correspondent RNAs were analyzed by northern blot (see Materials and Methods). The result obtained revealed a higher steady state level of Athb-8 transcripts in the auxin-treated tissues with respect to the control samples, with a higher accumulation after 1 hour (data not shown).

To study dose-dependent accumulation of Athb-8 mRNA, leaf tissues were incubated for 1 hour with \(10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10 \) M IAA and the correspondent RNAs were analyzed. A 2.6- and a 4-fold increase in the steady state level of Athb-8 mRNA occurs in leaves treated with \(10^{-5}\) and \(10^{-4}\) M IAA, respectively (Fig. 9). Similar results were also obtained with the synthetic auxins naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid, while no induction was observed with 2-(p-chlorophenoxy)-naphthalenelacetic acid and 2,4-dichlorophenoxyacetic acid, respectively (data not shown).

**DISCUSSION**

In this study, we describe the characterization of an Arabidopsis gene, Athb-8, encoding a homeodomain-leucine zipper protein of 833 amino acids which belongs to the HD-ZIP III family previously described (Sessa et al., 1994).

Homeobox genes in plants, by analogy to the functional roles of the products of animal HB genes, are thought to code for transcriptional regulators which mediate important developmental processes. This hypothesis has been supported by the analysis of the maize KNOTTED-1 (KN1) gene, the first isolated and well characterized plant HB gene (Hake, 1992).

So far, no mutationally defined loci can be correlated with genes encoding HD-Zip factors although a less conserved leucine-zipper domain is present in Athb-10 (Sessa et al., 1994) which corresponds to GLABRA2, a homeodomain protein involved in trichome development (Rerie et al., 1994). Interestingly, outside the HD-Zip region we found that Athb-8 shares some homologies with GLABRA2. The complete alignment of Athb-8 with GLABRA2 will be published elsewhere (Sessa G., Steindler C., Baima S., Morelli G. and Ruberti I., unpublished data). In addition, the expression analysis of Athb-1 (Aoyama et al., 1993), Athb-2/HAT4 and Athb-4 (Carabelli et al., 1993; Schena et al., 1993) in Arabidopsis and in transgenic plants suggested that HD-Zip proteins may control important aspects of plant development.

Here, we show that Athb-8 expression is associated with the development of the vascular system. Vascular development involves first the formation from the apical meristems of specific cells (procambium) which are committed at some point to differentiate into xylem and phloem. Subsequently, it involves the cytodifferentiation of these procambial cells into more specialized vascular cells (Aloni, 1987; Esau, 1977; Shininger, 1979). In the young, terminal regions of the stem and in differentiating leaves the procambium consists of discrete cellular strands composed of elongated cells. In the roots, the procambium is a core of tissue from which the vascular cylinder originates. So far, procambial cells have been described only cytologically and there are no specific biochemical characteristics attributable to this cell type. Moreover, it is not possible to define the stage at which the cells are committed to xylem or phloem differentiation unless they have clearly differentiated. Various efforts have been made to identify specific markers of vascular development. Recently, many cDNA clones for genes expressed preferentially in cells that redifferentiate into tracheary elements have been isolated from Zinnia elegans (Demura and Fukuda, 1994). A genetic approach has been taken by different groups to isolate developmental mutants. Several of them have mutations that interfere with the normal pattern of vascular development (Bowman et al., 1994), but only the Arabidopsis wooden leg mutant shows a specific defect in the vascular tissue (Scheres et al., 1995).

We have examined the expression of Athb-8 in several plant organs. RNA gel analysis and in situ hybridization studies of Athb-8 expression in Arabidopsis led to the conclusion that the gene is mainly restricted to the procambium. These data have been confirmed also by histochemical localization of Athb-8-GUS expression in Arabidopsis and tobacco transgenic plants. By comparing the expression of the gene in the vegetative meristems of the plant with that in the floral meristems and in the embryos, we can draw the conclusion that the Athb-8 expression is not linked to the mitotic activity per se, but it is rather restricted to the procambial cells. Moreover, in all the tissues analyzed, the expression of Athb-8 in the vascular system is confined to procambial cells and it is never found in terminally differentiated vascular cells.

Histological studies have revealed that progenitors of procambium can generally be discerned during the transition from the globular to the heart stage of embryogenesis (review by West and Harada, 1993). We were able to detect a low but sig-
nificant level of Athb-8 expression in the torpedo stage of the Arabidopsis embryo. This means that Athb-8 is always expressed in provascular cells even when they are not differentiating into more mature vascular cells. Therefore, Athb-8 might be considered a specific molecular marker of early vascular development. Consistent with this hypothesis is the observation that the Athb-8-GUS chimeric gene is induced early during re-vascularization of wounded stems. When a vascular bundle is severed, nearby parenchyma cells resume cell division and redifferentiate into both xylem and phloem (Steeves and Sussex, 1989). Therefore, differentiation of tracheary elements after wounding has been widely used as an experimental model system to study vascular development without the superimposition of spatial and temporal factors such as in the apical meristem.

We cut internodes of Athb-8-GUS transgenic tobacco plants so that only a narrow transverse bridge connected the apical and basal portion of the stem. In this way, new tracheary elements were differentiaitied in the middle of the pith to form an horizontal vascular connection between the old, interrupted vascular strands. We were able to detect GUS staining in single parenchyma cells of the bridge which were not distinguishable from the neighbour cells for any other morphological aspect. As we occasionally observed a lighter staining in single tracheary cells at early stages of differentiation, we concluded that the single stained parenchyma cells in the pith are the precursor of these vascular elements.

It is well known that auxin is the major signal involved in the control of several aspects of plant vascular differentiation (Aloni, 1987). One of the main peculiarities of auxin is that, of all the known plant hormones, it is the one that exhibits polar transport (Kaldewey, 1984). At early stages of plant development, an auxin polar transport system may be involved in the establishment of bilateral symmetry in embryos (Liu et al., 1993). In postembryonic development, young growing leaves or developing buds induce the formation of vascular tissue below them. In a series of elegant experiments performed in pea seedlings, Sachs provided evidence supporting the hypothesis that an auxin flux determines the orderly pattern of vascular differentiation from leaves to root (Sachs, 1969, 1981). In the ‘canalization hypothesis’ he proposed that the diffusion of auxin 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 finally results in the formation of vascular strands (Sachs, 1981, 1991). This hypothesis has been widely supported by experimental data on vascular redifferentiation in wounded stems (Gersani, 1985; Sachs, 1981; Sussex et al., 1972). In our experimental system, detection of GUS activity in stems at different times after wounding revealed interesting features of the regulation of Athb-8 expression. Athb-8 is strongly and rapidly (1 hour) induced at wound sites. Few hours later, the gene expression becomes regulated in a polar fashion, being more expressed in the apical than in the basal portion of the cut region. Moreover, the position of the leaf trace nearest the upper cut strongly influenced the time of activation of Athb-8 expression in the bridge region. All these observations implicated auxin in the regulation of Athb-8 expression. In fact, it has been shown that several auxin-regulated genes are also wound inducible (An et al., 1990; Eberner et al., 1993). It has been suggested that flavonoids released after wounding may act as natural inhibitors of auxin transport (Jacobs and Rubery, 1988), resulting in locally high auxin concentration. This fact may explain the dual regulation of the auxin-regulated genes and therefore the wound inducible expression of the Athb-8-GUS gene. Moreover, the local increase of auxin descending along the leaf traces and coming out from severed vascular bundles (Gersani, 1985; Sussex et al., 1972) can well explain the polar and asymmetrical GUS staining of wounded Athb-8-GUS plants. A more direct confirmation that auxin might have a role in regulating Athb-8 expression in wounded plants comes from the results obtained incubating Arabidopsis tissues in the presence of IAA. In fact, a four-fold accumulation of the Athb-8 mRNA has been observed in wounded leaves 1 hour after the beginning of hormone treatment (Fig. 9). Similar results were also obtained in cut flowering stem (S. B., unpublished data).

In conclusion, the expression pattern and the auxin regulation of the gene encoding Athb-8 suggest a role for this factor in provascular specification. A more detailed study aimed at the functional assessment of Athb-8 in transgenic plants as well as the analysis of mutants with specifically altered vascular development will help to define the role of Athb-8 in the determination and differentiation of provascular cells and the regulatory cascades in which this gene participates.

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