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Structure and Expression of the *Arabidopsis thaliana* Homeobox Gene *Athb-12*

Yong-Hun Lee,* Hye-Sook Oh,† Choong-Ill Cheon,† In-Taek Hwang,*[,1] Yun-Jee Kim,*[,1] and Jong-Yoon Chun*[,1,2]

**Kumho Life and Environmental Science Laboratory, 572 Sangam-Dong, Kwangsan-Gu, Kwangju, 500-712, Korea; and †Department of Biological Science, Sookmyung Women's University, Chungpa-Dong, Yongsan-Gu, Seoul, 140-742, Korea*

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We have isolated the *Arabidopsis thaliana* homeobox gene *Athb-12,* determined its structure and activation domain, demonstrated that its promoter is inducible in response to abscisic acid (ABA) treatment, and characterized the cellular distribution of its transcripts. The single intron of the gene interrupted the leucine-zipper domain region. The 5′ regulatory region of *Athb-12* can drive β-glucuronidase (GUS) expression in tobacco transgenic plants. *Athb-12* gene expression was further examined using *in situ* hybridization to determine the cellular distribution of *Athb-12* transcripts during ABA induction. A complex pattern of *Athb-12* expression was observed, often associated with regions of developing vascular tissues. Analysis of chimeras constructed from Athb-12 and the DNA-binding domain of the *Saccharomyces cerevisiae* transcription factor GAL4 revealed that the activation domain of Athb-12 lies in the C-terminal region (amino acids 180 to 235). Taken together, our data suggest that Athb-12 is a transcriptional activator important in regulating certain developmental processes as well as in the plant's response to water stress involving ABA-mediated gene expression.

Key Words: **abscisic acid; homeobox; transcriptional activation domain; water stress.**

The plant response to water stress leads to alteration of the cellular metabolism and is correlated with significant changes in gene expression (reviewed in 1–3). Many genes that respond to water stress are also induced by the exogenous application of abscisic acid (ABA) (reviewed in 1–3). It appears that water stress triggers the production of ABA, which in turn induces the transcription of various genes. However, several genes that are induced by water stress are not responsive to exogenous ABA treatment (4–7). This suggests the existence of both ABA-dependent and ABA-independent signal transcription pathways mediating the response to water stress. The structural and functional promoter analysis of ABA-inducible genes has identified several *cis*-acting ABA-responsive elements (ABRE) that mediate the ABA response of these genes (reviewed in 8).

Although a large number of water stress-inducible genes have been characterized, homeobox genes involved in water stress are poorly understood. Homeobox genes are characterized by the homeobox, a conserved 180-bp nucleotide sequence encoding a 60-amino acid DNA-binding homeodomain composed of three α-helices. The DNA-binding property of homeobox proteins suggests that these proteins may regulate the transcription of downstream target genes (9). Three *Arabidopsis* homeobox genes *Athb-6* (10), *Athb-7* (11), and *Athb-12* (12) have been reported to be induced by water stress. They are members of the homeodomain-leucine zipper (HD-Zip) family which has been found only in plants. To date, at least twenty-one distinct members of the HD-Zip family have been isolated from *Arabidopsis,* but their functions are mostly unknown. Some of the HD-Zip proteins have been characterized as transcriptional activators (13) or repressors (14) of downstream genes.

Our laboratory previously reported that the *Athb-12* message was induced by exogenous ABA treatment as well as by water stress (12). The ABA-mediated regulation mechanism of *Athb-12* involved in water stress is unknown because information about the genomic structure of the *Athb-12* gene, especially the promoter regulatory region, has not been available. Analysis of the molecular regulation of *Athb-12* may advance our understanding of how plants respond to environmental stresses such as water stress. To understand these regulatory mechanisms requires knowledge of the

[1] Present address: Department of Biological Science, Ewha Women's University, 11-1 Daehyung-dong, Seodaemun-gu, Seoul, 120-750, Korea.

[2] To whom correspondence should be addressed. Fax: 82-2-566-9831. E-mail: chun@mm.ewha.ac.kr.

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genomic structure, including the regulatory region of this gene.

MATERIALS AND METHODS

Isolation of Athb-12 genomic clones and sequencing. A Lambda ZAPII genomic library (Stratagene) containing partially digested DNA fragments of *Arabidopsis thaliana* Colombia was screened using the *Athb-12* cDNA probe as previously described (15). Approximately 1×10^6 clones were plated at a density of 5×10^4 plaques per plate. A nylon membrane (Hybond-N, Amersham) was lifted and screened with the labeled probe. The probe was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using the Random Prime DNA Labeling Kit (Boehringer Mannheim). The filters were hybridized with the radioactively probe and washed to high stringency (15). Filters were exposed to X-ray film in presence of intensifying screens at −70°C. Two positive clones were identified and recovered as phagemids by *in vivo* excision (Lambda ZAPII Kit, Stratagene) as per the manufacturer's instructions. Approximately 1.1 kb of the coding region and 2.1 kb upstream were sequenced with ABI Prism 373 automatic sequencer (Perkin-Elmer, Applied Biosystems).

Primer extension. Primer extension was carried out using an oligonucleotide (5′-GGTTTTCACCAGATCTTGTAAGTTTCTTGA-3′, PE1) complementary to positions +58 to +87 of the *Athb-12* coding sequence as described previously with minor modification (16, 17). Briefly, the oligonucleotide was end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Pharmacia Biotech) and T4 polynucleotide. In 20 μl of hybridization buffer (150 mM KCl, 10 mM Tris–Cl, pH 8.3, 1 mM EDTA), the radiolabeled primer was mixed with 15 μg of total RNA isolated either from *Arabidopsis* plants treated with ABA (the final concentration in the medium was 10 μM) or from yeast. The mixtures were heated at 65°C for 90 min and slowly cooled to room temperature. The reaction conditions were adjusted to 75 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each dNTP, and 100 units of Superscript II reverse transcriptase (Life Technologies, Inc.) in a final volume of 50 μl. The reactions were incubated at 42°C for 60 min and terminated by the addition of 1 μl of 0.5 M EDTA. The extended products were recovered by ethanol precipitation after RNase A digestion and phenol extraction, and analyzed on a 6% sequencing gel. The identical primer was used in parallel sequencing reactions to determine the exact position of the transcription start site.

Construction of a chimeric Athb-12-GUS gene and plant transformation. Approximately 2.1 kb of the 5′ upstream region of *Athb-12* (12) was fused to the β-glucuronidase gene (GUS) in the plant transformation vector pBI101 (Clontech, CA). The 2.1-kb fragment was amplified by PCR using the 5′ oligonucleotide 5′-CTCAAGCTT-TCTTAATGGTGCCAAATG-3′ (introducing a *Hin*dIII site) and the 3′ oligonucleotide 5′-CTCTCTAGAGGTTTTCACCAGATCTTGTAA-GTT-3′ (introducing a *Xba*I site) and subcloned in the *Hin*dIII and *Xba*I sites of pBI101 to produce the plasmid p2.1. This *Athb-12*::GUS fusion construct (p2.1) was mobilized into *Agrobacterium tumefaciens* GV3101 by means of direct DNA transformation (18). Transgenic tobacco plants were obtained by the standard leaf disc method of transformation (19) and grown in a controlled environment of 25°C, 80% ambient humidity, with day and night periods of 18 and 6 h, respectively. Leaf discs of *Nicotiana tabacum* cv. Havana were infected with GV3101 *Agrobacterium tumefaciens* strain containing *Athb12*::GUS fusion construct. Plant transformants were selected on MS agar medium supplemented with 500 μg/ml cefotoxime and 300 μg/ml kanamycin. Regenerated plants were transferred onto the hormone free MS medium with 100 μg/ml of kanamycin. T2 transgenic plants were used for characterization.

Histochemical localization of GUS activity. Seeds used for GUS analysis were surface-sterilized, sown on sterile filter paper placed on top of MS medium containing 30% sucrose and 400 μg/ml kanamycin and allowed to germinate at 22°C with a 16 h light/8 h dark cycle. After 15 days, the filter paper and seedlings were transferred to MS plates containing sucrose and kanamycin with or without 100 μM ABA, and cultured for 5 days to minimize any potential stresses occurring during ABA treatment. Seedlings and hand-cut sections of the transgenic tobacco plants were then stained for GUS activity. Histochemical localization of GUS activity *in situ* was performed according to the method of Jefferson *et al.* (20). Samples were fixed in 0.3% formaldehyde, 50 mM $NaPO_4$ pH 7.0, 10 mM MES, 0.3 M mannitol, washed with 50 mM $NaPO_4$ pH 7.0, and immersed in a GUS reaction buffer (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 100 mM $NaPO_4$ pH 7.0, 0.1% Triton X-100, 0.1 mM KFeCN, 1 mM EDTA). The samples were incubated in the dark at 37°C for 12 to 24 h. For better visualization of the stained samples, chlorophyll was extracted from the seedlings with 100% ethanol or acetone and seedlings subsequently stored in 50% ethanol. Photographs were taken under a stereomicroscope (Leica, Germany).

In situ hybridization. Six-week-old *Arabidopsis* plants were placed in water with and without ABA (the final concentration of ABA in the medium was 10 μM). After 2–4 h, each sample was separated into roots and stems and cut into 1 cm long pieces. These roots and stems were fixed, dehydrated and embedded in Paraplast X-TRA (Oxford, USA). Thin sections (10 μm) were prepared from paraffin-embedded stems and roots, and used for *Athb-12* mRNA localization as described previously (21). The pBluescript SK vector containing the *Athb-12* cDNA (about 400 bp) was used to make sense and antisense digoxigenin (DIG)-labeled *Athb-12* RNA probes using the DIG RNA-labeling Kit from Boehringer Mannheim. These were then fragmented to give probes of about 150 nucleotides before hybridization (22). DIG-labeled *Athb-12* RNA probes were hybridized with *Arabidopsis* stem and root sections. The hybridization signals were detected by incubating with an anti-DIG-antibody alkaline phosphatase conjugate (DIG Nucleic Acid Detection Kit, Boehringer Mannheim, Germany). Sections were counter-stained with 0.1% Safranin-Fast green to show anatomical structure.

Plasmid construction. Various segments of the *Athb-12* gene were fused in-frame 3′ to the sequence of the DNA-binding domain (DBD) of GAL4 (residues 1–143) which was under the control of the yeast ADH1 promoter. These segments were generated by PCR using *Pfu* DNA polymerase (Stratagene) and 5′- and 3′-primers containing *Eco*RI and *Bam*HI sites, respectively. The PCR products were digested with *Eco*RI and *Bam*HI and ligated into the corresponding sites of pGBT9 (Clontech, CA). The fusion constructs were verified by sequence analysis.

Yeast cell culture and transformation. Saccharomyces cerevisiae HF7C (23) was grown in YPD [1% yeast extract, 2% peptone, 2% D-glucose, 1.8% agar (only in plate)] or synthetic minimal medium (SD) [0.67% yeast nitrogen base, 2% D-glucose, (10×) amino acids dropout solution deficient in tryptophan or histidine, 1.8% agar (only in plates)]. Yeast was transformed with appropriate plasmids by the lithium acetate method (24), and the transformants were selected on SD medium. Transcriptional activation function of the pGBT9::Athb12 constructs was investigated by β-galactosidase assay. Filter and liquid β-galactosidase assays were performed as described in the Clontech protocol.

RESULTS

Isolation and Characterization of the Arabidopsis thaliana Homeobox Gene Athb-12

We screened approximately 1×10^6 recombinants from an *Arabidopsis thaliana* genomic library using a ^{32}P-labeled *Athb-12* cDNA probe. Two positive clones were isolated, each containing ~4 and 5 kb of the *Arabidopsis thaliana* genomic DNA. Restriction map-

ping and Southern blot analysis revealed that both clones contained the entire *Athb-12* cDNA sequence. The genomic DNA insert was characterized by Southern blot hybridization, PCR and DNA sequencing. The *Athb-12* gene spans ~1.1 kb and contains a single intron. The intron occurs in the leucine-zipper domain region. The entire DNA sequence of the *Athb-12* gene is shown in Fig. 1. Intron-exon boundaries were determined from the sequence discrepancies between genomic DNA and cDNA. The presence of consensus splice donor-acceptor sequences was confirmed (25). During the course of this work, the sequence of a 142-kb *Arabidopsis* genomic DNA from a BAC clone, which includes the *Athb-12* gene, was released in NCBI database (GenBank Accession No. AL138642).

The 5′-flanking region of the *Athb-12* gene was sequenced (Fig. 1) and its transcription start site determined by primer extension using the PE1 primer, which is located just next to the first codon, ATG (+88). With total RNA from whole *Arabidopsis* plants used as templates, an extended product which terminated 87 nucleotides upstream of the translation start site was observed (Fig. 2). No signal was observed in the negative control where yeast RNA was used as a template (data not shown). Sequence analysis of the 5′-flanking region identified a putative TATA box sequence and the presence of previously characterized regulatory element sequences such as the ACGT sequence and the drought responsive element (DRE) sequence (26). The ACGT sequence is the core element of A, C, G, boxes (27) and of ABRE sequences (28). The TATA box element (TATATAA) is located at positions −36 to −30, possibly directing transcription from the indicated position +1. The ACGT sequence was found at positions −186, −364, −592, −738, −863, and −872 and the DRE element (ACCGAC) was at position −858. Recently, MYC- and MYB-related transcription factors isolated from *Arabidopsis* have been reported to function as transcriptional activators in the promoter of the drought- and ABA-inducible gene *rd22* (29). The *Athb-12* promoter also has two putative binding sites (CACATG) for MYC-related bHLH DNA binding proteins at position −607 and −985, and three putative recognition sites (TGGTTAG) for MYB-related proteins at position −395, −967, and −1666.

Analysis of the Athb-12 Promoter in Transgenic Tobacco Plants

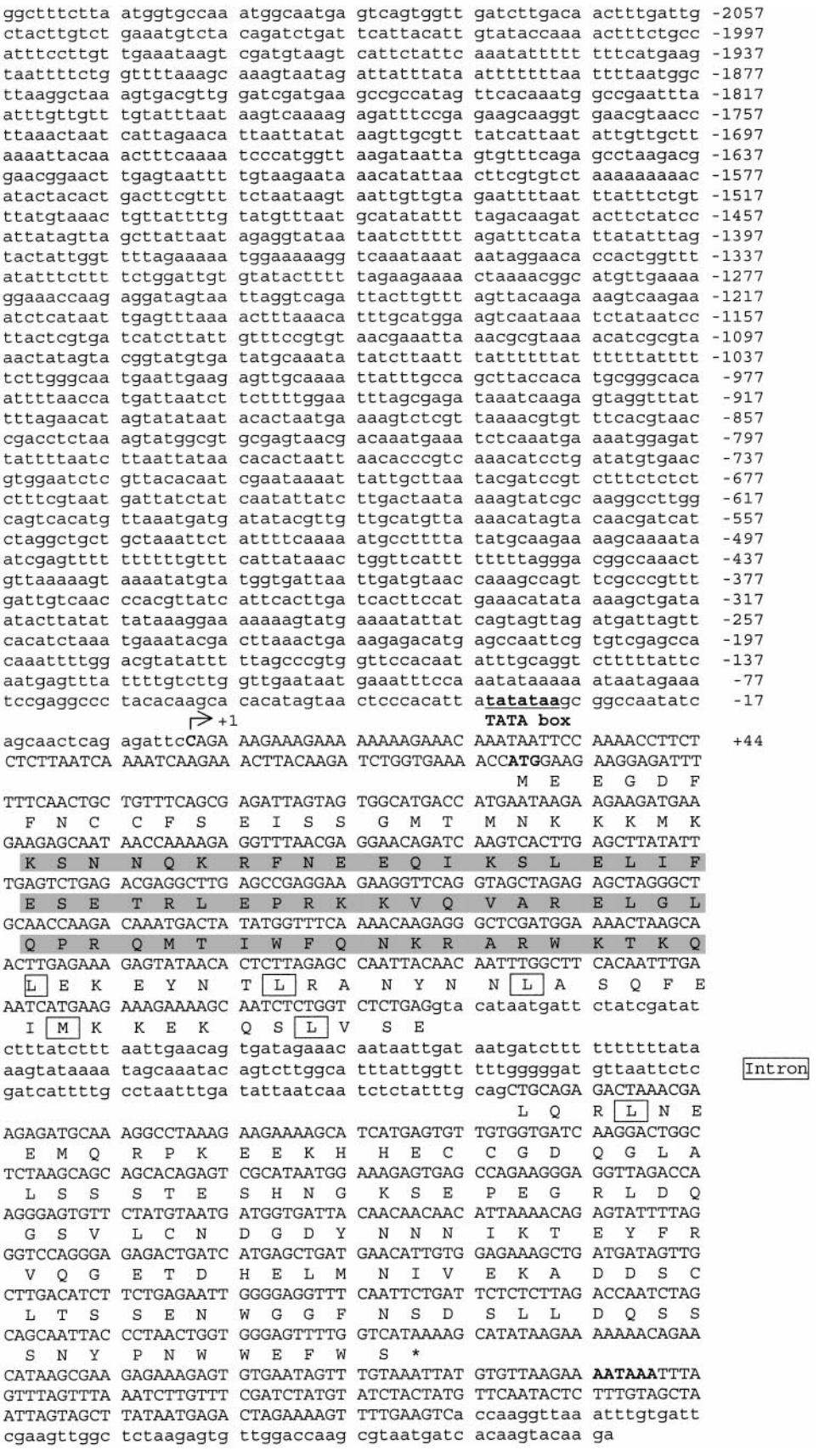
We have previously shown that *Athb-12* transcripts were induced by exogenous ABA treatment (12). It indicates that the *Athb-12* gene has an ABA-inducible promoter. Thus, to determine whether the 5′-flanking DNA associated with the *Athb-12* gene was capable of inducing the gene expression by exogenous ABA treatment, the 5′-flanking region of *Athb-12* was fused to the GUS reporter gene and the expression of this chimeric fusion gene (*Athb-12::*GUS) examined in transgenic plants. This construct includes the sequence extending 2.1-kb upstream from the first codon, ATG (+88). We carried out this promoter analysis in tobacco plants because an *Athb-12* homologue was observed in tobacco plants by genomic Southern blot analysis (data not shown). Histochemical staining of the transgenic plants carrying the *Athb-12::*GUS gene construct is shown in Fig. 3. Weak expression of the *Athb-12::*GUS fusion gene was observed in the shoot meristem region of plants raised under normal growth conditions. In contrast, the ABA-treatment of transgenic tobacco plants containing the *Athb-12::*GUS fusion gene strongly induced GUS expression in stem, petiole, leaves around shoot meristem, and elongation region of the root. These results clearly indicate that the 2.1-kb sequence of the 5′-flanking region of *Athb-12* contains all *cis*-elements responsible for ABA regulation of *Athb-12* gene. In particular, GUS staining was more strongly detected in young leaf, at the junction of root and stem, and in lateral root. These expression patterns of *Athb-12::*GUS fusion plants are distinct from that of pBI121 plants, in which GUS staining was ubiquitous with no regard for leaf age or different structures of the stem or root. Furthermore, we examined by hand-cut sections the expression pattern of the *Athb-12::*GUS fusion gene in stem, leaf and root (Fig. 4). GUS expression was detected in the vascular bundles and epidermis of stem and root, and in the vein of leaf.

Cellular Localization of Athb-12 mRNA Expression in Arabidopsis Stem and Root

We next examined *Athb-12* expression in *Arabidopsis* plants by *in situ* hybridization to determine the cellular distribution of its expression (Fig. 5). Sense and antisense *Athb-12* riboprobes labeled with DIG were hybridized to *Arabidopsis* stem and root sections. *Athb-12* transcripts were found clearly associated with the vascular region of stem and root (Fig. 5). The hybridization signals were localized to the vascular region and epidermis of stem and the vascular region, cortex, and epidermis of root. No hybridization signal was observed with the sense probe in the stem and root sections (Fig. 5). In experiments where plants were treated with ABA, the intensity of staining increased but the cellular pattern of staining did not differ from that of non-ABA-treated plants.

Identification of the Athb-12 Activation Domain

As homeobox proteins are well known for their role as transcription factors, we next examined the transcriptional activity of the Athb-12 protein. The intact yeast GAL4 protein is a strong transcriptional activator of responsive promoters in yeast, whereas the GAL4 DBD alone is not. Thus fusion of the GAL4 DBD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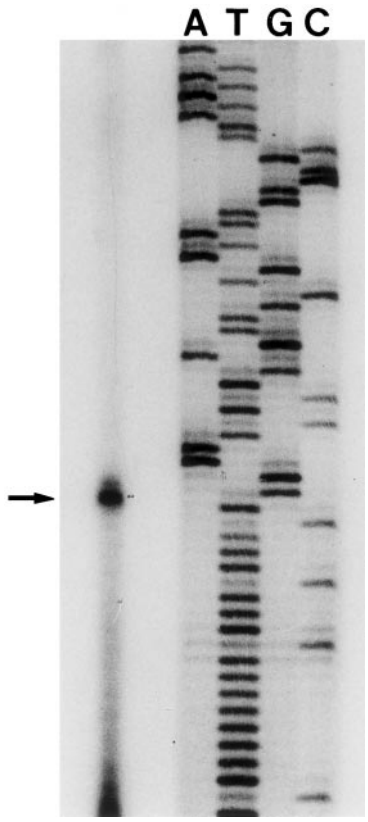

FIG. 2. Determination of the transcription initiation site of the *Athb-12* gene by primer extension analysis. Total RNA prepared from *Arabidopsis* plants treated with ABA was hybridized with the PE1 primer (see Materials and Methods). Extension products were analyzed by electrophoresis on a denaturing polyacrylamide gel alongside a sequencing ladder of *Athb-12* genomic DNA prepared using the same primer. The extension product is indicated by an arrow.

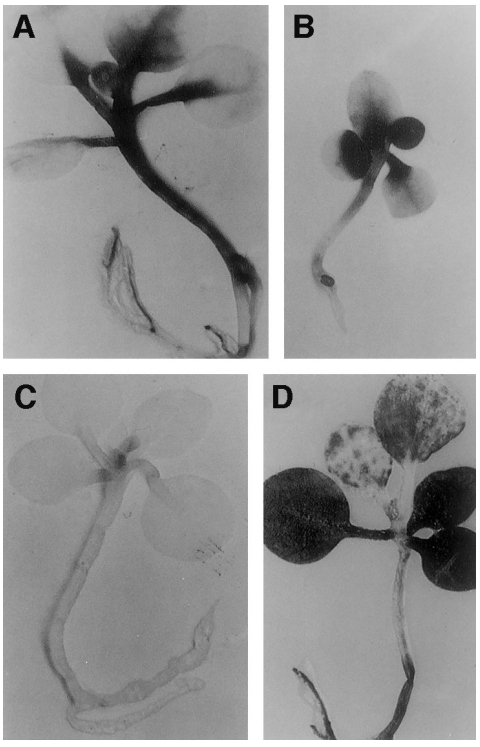

FIG. 3. Histochemical localization of GUS activity in tobacco plants transformed with *Athb-12* promoter-GUS fusion construct. The transgenic plants were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide after treatment either with (A and B) or without ABA (C). As a positive control, pBI121-transgenic plants were treated in the same way as A, B, and C. The GUS activity in the pBI121 plants was the same after both treatments (D).

to heterologous polypeptides provides an assay system for transcriptional activation domains distinct from those involved in DNA binding (30). It has been shown that many transcription factors from plants and animals have transcriptional activation domains that are functional in yeast (31–34). Therefore we used this yeast one-hybrid assay system to determine the transcriptional activation domain of Athb-12. Various portions of *Athb-12* cDNA were fused to the DBD of the yeast transcription factor GAL4 (Fig. 6A). The effect of these fusion proteins on transcription of a *lacZ* reporter gene linked to multiple upstream copies of the GAL4 DNA-binding site was determined (Fig. 6B). Full-length Athb-12 (amino acids 1 to 235) fused to the GAL4 DBD leads to high-level transcription of the *lacZ* reporter gene. Deletion of the C-terminal 112 amino acids of Athb-12 (Athb-12 1–124) eliminates transcriptional activation, despite this construct containing the intact homeodomain and leucine-zipper domain of Athb-12. Interestingly, when only the C-terminal region (Athb-12 123–235) of Athb-12 was fused with GAL4 DBD, the transcription level of *lacZ* was increased 1.5 fold compared to that of the full-length Athb-12. Transformed yeast containing various fusion constructs assayed by the filter lift assay gave results consistent with the above liquid culture assays (Fig. 6C). These results indicate that the transcriptional activation domain of Athb-12 is located within amino acids 123–235 of the protein.

To better define and localize the Athb-12 activation domain, we generated more DBD fusion constructs by a series of deletions in the C-terminal region of Athb-12. Deletion of amino acids 209–235 (Athb-12 123–208), 180–235 (Athb-12 123–179), and 151–235

FIG. 1. Complete nucleotide sequences of the *Athb-12* gene and its 5′-flanking promoter region. The nucleotide sequence of exons is in uppercase and that of the intron is in lowercase. The deduced amino acid sequence is shown. The transcription start site is indicated with a bent arrow (+1) and the putative TATA box underlined. The homeodomain is shaded and the leucine and methionine in the proposed leucine-zipper motif are boxed. The asterisk represents the termination codon. The putative polyadenylation signal is shown in boldface type.

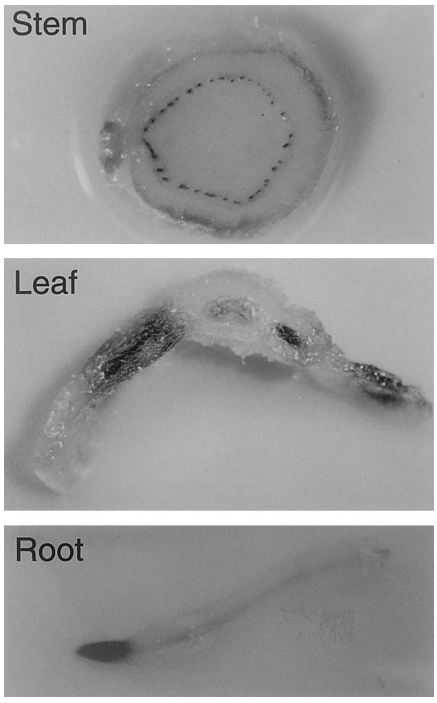

FIG. 4. *Athb-12*::GUS gene expression in each different tissues of transgenic tobacco plants. Stem, leaf, and root were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide overnight. Samples were hand-sectioned and observed under a microscope. GUS activity was restricted to specific regions.

(Athb-12 123–150) in the C-terminal region eliminated β-galactosidase activity, whereas deletion of the N-terminal portions containing the homeodomain and leucine-zipper from such DBD fusions (Athb-12 151–235 and Athb-12 180–235) increased the transcriptional activity of the fusion protein (Fig. 6B). Corroborating results were obtained with the filter lift assay (Fig. 6C). Thus the activation domain resides within amino acids 180–235 of the Athb-12 protein.

DISCUSSION

We previously isolated a cDNA clone, designated *Athb-12*, that encodes a new member of the HD-Zip family. In the present study we have characterized the organization, genomic structure, and promoter function of the *Athb-12* gene. We have also identified a transcriptional activation domain located in the C-terminal region. The *Athb-12* gene spans approximately 1.1 kb and contains two exons. The leucine zipper region is interrupted by the one intron at position 96/97 of the leucine-zipper motif. The position of this intron is interesting as there are reports that common intron positions can be a useful marker to identify relationships among different homeobox genes (reviewed in 35). The recently identified *Arabidopsis Athb-6* gene (10), which is one of the water stress-inducible HD-Zip family members, has two introns with the second intron at the identical position 96/97 in the leucine-zipper motif.

Previous Northern blot analysis revealed that *Athb-12* mRNA was detected in stem, leaf, flower and root as well as in seedling (12). However, *in situ* hybridization and promoter-GUS analyses showed a complex pattern of *Athb-12* expression. *Athb-12* transcripts are more strongly expressed in stem and petiole, and in the junction of stem and root. In root, its expression was high in lateral roots rather than in primary roots. Interestingly, *Athb-12* expression was strongly detected in young leaf, whereas in mature leaf its expression was only weakly detected, mainly in leaf vein. Some of the *Athb-12* expression patterns are similar to those of the other *Arabidopsis* homeobox genes such as *Athb-8* (36) and *prha* (37) which have been shown to be expressed in the vascular regions. Thus, Athb-12 may also have a role in vascular development as well as in plants' response to water stress. The expression pattern of *Athb-12* mRNA is distinct from that of *Athb-6*, in that *Athb-6* expression was restricted to regions of cell division and/or differentiation in cotyledons, leaves, roots, and capels. Thus, Athb-6 has been proposed to have a function related to cell division and/or differentiation in developing organs. The different structures and expression patterns of *Athb-12* and *Athb-6* genes indicate that both genes are regulated in different manners.

Since inspection of the amino acid sequence of Athb-12 revealed that the C-terminal region is as acidic as those of many transactivators, including yeast GAL4 (38, reviewed in 39), we investigated whether Athb-12 could act as a transcriptional activator. Athb-12 fused to a heterologous DBD exhibited strong activating properties. The deletion of the C-terminal acidic domain abolished Athb-12 ability to function as a transcription activator, whereas deletion of the N-terminal region including the homeodomain and

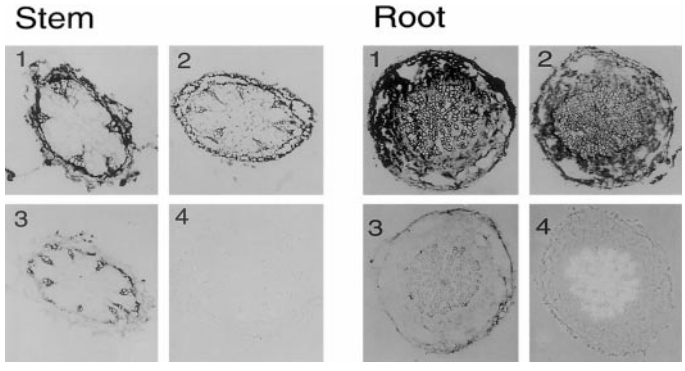

FIG. 5. *In situ* hybridization analysis of *Athb-12* expression in stems and roots of *Arabidopsis thaliana*. Samples were hybridized with *Athb-12* DIG-labeled antisense (1 and 2) or sense (4) riboprobe after treatment either with (1) without ABA (2). Each tissue was stained with safranin and fastgreen (3).

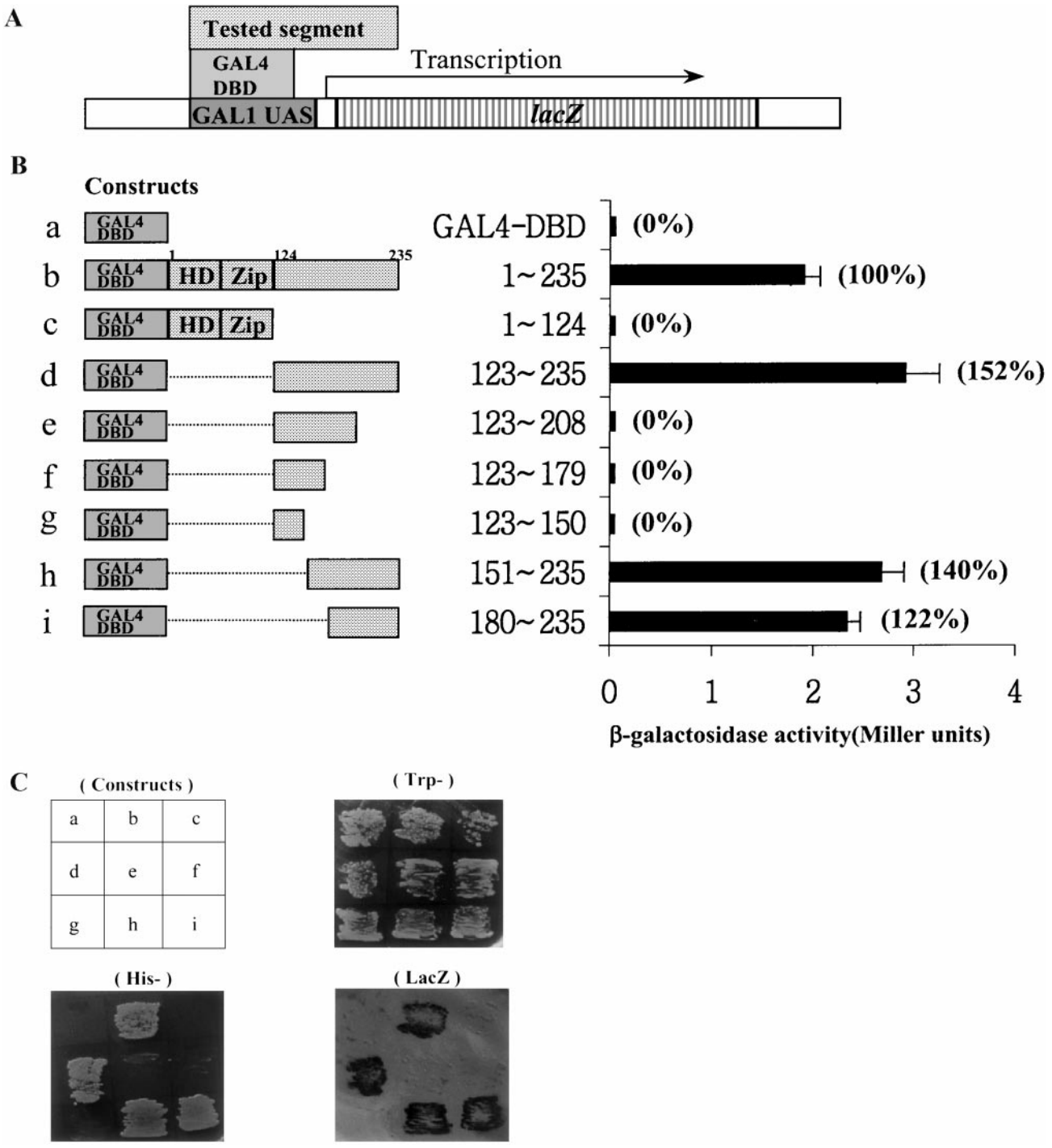

FIG. 6. Mapping of transcriptional activation domains in Athb-12 by fusion to the GAL4 DBD in a yeast genetic system. (A) Schematic diagram of a yeast one-hybrid assay system to determine the transcriptional activation domain of Athb-12. (B) Various segments of Athb-12 were expressed as fusion proteins with the GAL4 DBD in strains bearing the GAL1 upstream activation sequence (UAS)-*lacZ* reporter gene as shown in the schematic diagram. GAL4 DBD alone was used as a negative control. β-galactosidase activities were measured and expressed relative to the value obtained for the full-length Athb-12-DBD fusion. The results are the means $\pm$ SE of 3 to 5 independent transformations. (C) The constructs were also assayed colorimetrically for β-galactosidase activity by the filter lift assay. All yeast strains containing these fusion constructs grew in medium deficient in tryptophan, whereas only yeast strains containing constructs b, d, h, and i grew in medium deficient in histidine. This indicates that the constructs b, d, h, and i contained the transcriptional activation domain of Athb-12.

leucine-zipper motif did not affect the transcriptional activity. Taken together with our findings that *Athb-12* expression is induced by ABA-mediated water stress and has a complex pattern during development, this suggests that Athb-12 is a transcriptional activator important in regulating certain developmental processes as well as in plants' response to water stress involving ABA-mediated gene expression. The studies described here provide a starting point for the examination of the regulation of the *Athb-12* gene in more detail. Further work is required to define factors and elements necessary for *Athb-12* activation in response to water stress.

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