The Plant Cell, Vol. 3, 371–382, April 1991 © 1991 American Society of Plant Physiologists

Characterization of *cis*-Acting Sequences Regulating Root-Specific Gene Expression in Tobacco

Yuri T. Yamamoto,[1] Christopher G. Taylor, Gregoria N. Acedo, Chi-Lien Cheng,[2] and Mark A. Conkling[3]

Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695

The expression of the tobacco root-specific gene TobRB7 was characterized. Gel blot hybridizations to RNA isolated from various tobacco tissues demonstrated that steady-state TobRB7 mRNA is not detected in expanded leaf, stem, or shoot apex tissue. To determine the spatial pattern of expression, in situ hybridization to root sections revealed that TobRB7 expression is localized to root meristem and immature central cylinder regions. The 5′ flanking region of the gene was studied with respect to its ability to direct root-specific expression. Deletions of 5′ flanking sequence were fused to the β-glucuronidase (GUS) reporter gene and transformed into tobacco. Our data demonstrated that sequences 636 base pairs from the site of transcription initiation are sufficient to direct the root-specific GUS expression in transgenic tobacco, whereas sequences 299 base pairs from the site of transcription initiation fail to direct root-specific expression. A negative regulatory element was apparent between 813 base pairs and 636 base pairs 5′ of the transcription initiation site. Histochemical localization of GUS activity in transgenic plants was consistent with in situ hybridization results: GUS activity was localized to the root meristem and central cylinder regions. GUS activity appeared 2 days post-germination in the primary root meristem. In lateral roots, GUS activity was detected from the time of initiation.

INTRODUCTION

Despite the importance of roots in plant growth and development, relatively few genes expressed specifically in roots have been isolated and the *cis*-acting sequences regulating root-specific expression have not been defined (Benfey and Chua, 1989). A root-specific hemoglobin cDNA has been isolated from the non-nodulating plant *Trema tomentosa* (Bogusz et al., 1988). A barley root-tip-specific lectin cDNA has been isolated (Lerner and Raikhel, 1989). Keller and Lamb (1989) reported isolation of a hydroxyproline-rich glycoprotein gene (HRGPnt3) expressed during lateral root initiation. Translational fusions to a β-glucuronidase (GUS) reporter gene including about 500 bp of 5′ flanking sequence exhibited GUS expression at sites of lateral root initiation, indicating that the sequences required for regulated transcription of HRGPnt3 are included in this construction.

Several tobacco cDNAs and the corresponding genomic clones of genes that are expressed in roots but not leaves have been isolated in our laboratory (Conkling et al., 1990). One of these, TobRB7, showed amino acid sequence similarity to mammalian lens fiber major intrinsic protein (Gorin et al., 1984), a soybean peribacteroid membrane nodulin (Fortin et al., 1987; Sandal and Marcker, 1988), a tonoplast protein of soybean seed storage vacuoles (Johnson et al., 1990), a membrane pore-type protein of *Escherichia coli*, the glycerol facilitator (Baker and Saier, 1990), and the *Drosophila* neurogenic gene *big brain* (Rao et al., 1990), suggesting that the TobRB7 product may be involved in membrane channeling (Yamamoto et al., 1990). Transcriptional runoff experiments and constructions in which 1.4 kb of 5′ flanking sequence-directed expression of a reporter gene in transgenic plants demonstrated that TobRB7 is regulated transcriptionally (Conkling et al., 1990). Recovered genomic clones and their correlation with genomic DNA gel hybridization experiments strongly imply that TobRB7 is a two-member gene family in tobacco (Conkling et al., 1990).

In this report, we analyze further the expression of the TobRB7 gene. In situ hybridization to root sections revealed that the gene is expressed at high levels in root meristematic and immature central cylinder regions. To test whether TobRB7 expression is meristem specific, as opposed to root specific, we measured the steady-state TobRB7 mRNA in various organs by RNA gel blot analysis. These results demonstrated that steady-state TobRB7 mRNA is present in roots but not in leaves, shoot meristems, or stems.

[1] Current address: Department of Biology, Yale University, New Haven, CT 06511.

[2] Current address: Department of Botany and Department of Biology, The University of Iowa, Iowa City, IA 52242.

[3] To whom correspondence should be addressed.

The 5′ flanking sequences of the two genes encoding the TobRB7 mRNA (designated TobRB7-5A and TobRB7-18C) were sequenced. TobRB7-5A contains three sets of repeats, designated as boxes A, B, and C. Boxes A are eight 11-bp repeats. Boxes B are two highly conserved A: T-rich sequences of about 300 bp. Boxes C are 17-bp perfect repeats. TobRB7-18C contains one B box and two C boxes. The initial 800 bp (−1 to −800) of the 5′ flanking regions of TobRB7-5A and TobRB7-18C are highly conserved. To define further the *cis*-acting sequences responsible for root-specific gene expression of TobRB7, we constructed fusion genes among various 5′ flanking regions of the TobRB7-5A gene and the bacterial reporter gene GUS (Jefferson, 1987; Jefferson et al., 1987). This deletion series was transformed into tobacco by way of *Agrobacterium*-mediated transformation, and regenerated plants were tested for the root-specific expression of the GUS enzyme. The *cis*-acting elements regulating the root-specific expression of the gene reside within its 5′ flanking region, between −636 and −299 nucleotides from the site of transcription initiation. This region (−636 to −299) corresponds to the distal half of the B box. The presence of a negative regulatory element located between −813 and −636 is also indicated. The TobRB7-5A *cis*-acting DNA sequences (−813 to −299) shown to affect qualitatively and quantitatively the expression of the GUS reporter gene include those sequences conserved in the TobRB7-18C 5′ flanking sequence (−800 to −1).

The spatial and temporal expression of TobRB7 were examined in transgenic plants. The GUS reporter gene exhibited tissue-specific GUS gene expression similar to that exhibited in in situ hybridization experiments, i.e., in the root meristem and immature central cylinder. GUS activity in transgenic plants was expressed also at the site of lateral root initiation. The tissue-specific patterns of the GUS reporter gene expression correlate well with those observed by in situ hybridizations to TobRB7 probes, implying that GUS activity profiles are relevant reporters of TobRB7 gene expression. The temporal and spatial expression of transgenic plants from the deletion series were examined. No changes in the spatial and temporal expression patterns were observed for those deletions that directed root-specific GUS expression.

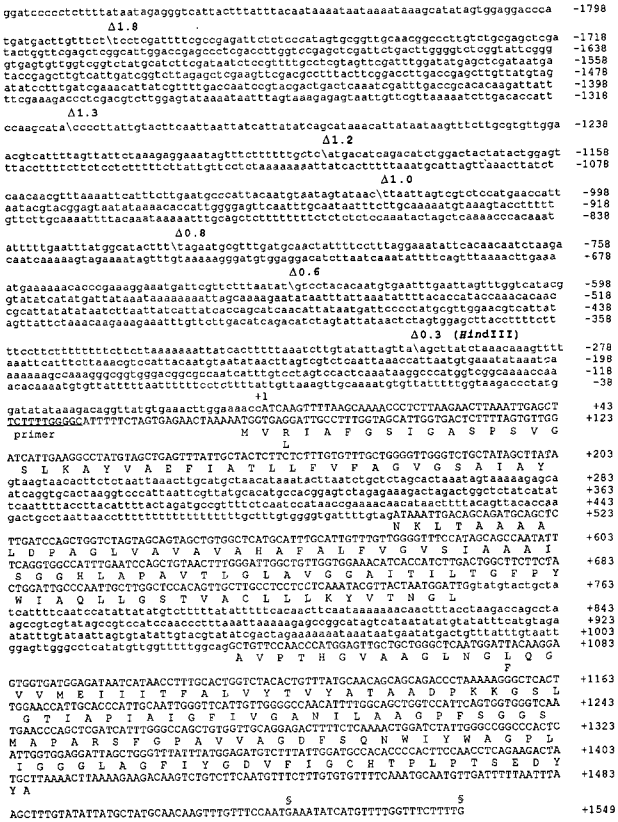


Figure 1. Nucleotide Sequence of the λ5A Clone.

Sequencing reactions were performed by dideoxynucleotide chain termination with Sequenase (U.S. Biochemicals). Exons are in capital letters. Introns and nontranscribed flanking regions are in lower-case letters. Predicted amino acids are shown below the corresponding codons. Two amino acid changes in the cDNA TobRB7-18C are indicated below the corresponding amino acids of the TobRB7-5A gene product. The deletion ends are shown. The base of polyA addition of the two TobRB7-5A cDNAs is indicated (§). The site of transcription initiation is indicated (+1) and the nucleotides are numbered accordingly. The primer used for primer extension is complementary to the region indicated and is underlined.

RESULTS

Clones of TobRB7

We have isolated two different, nearly full-length cDNAs and three genomic clones, λ5A, λ8D, and λ18C, corresponding to the original TobRB7 cDNA. Figure 1 shows that the predicted amino acid sequences of these two cDNAs (250 amino acids) are identical with the exception of two amino acid substitutions. Figure 2 illustrates restriction mapping data of the genomic clones and shows that λ5A and λ8D are overlapping and that the λ18C clone represents another gene. As shown in Figure 1, one nearly full-length cDNA (TobRB7-5A) and the λ5A clone show sequence identity within predicted exons. Likewise, the original TobRB7 and another full-length cDNA (TobRB7-18C) correspond to the λ18C clone (data not shown). All bands hybridizing strongly to the TobRB7 probe on tobacco genomic DNA gel blots correspond to restriction fragments predicted of these genomic clones (Conkling et al., 1990), suggesting that these genomic clones represent all members of the TobRB7 gene family in tobacco. The

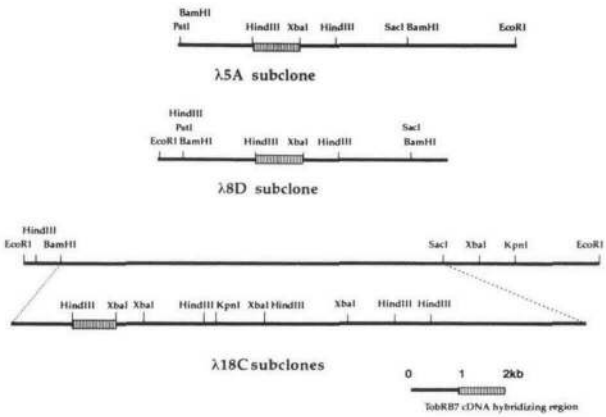


Figure 2. Restriction Maps of Genomic Clones Hybridizing to the TobRB7 cDNA.

Restriction fragments hybridizing to TobRB7 from genomic clones λ5A, λ8D, and λ18C were subcloned into pBlueScript (Stratagene, Inc.) and restriction mapped.

λ5A genomic clone was used for further deletion analyses. The nucleotide sequence of the λ5A clone from the BamHI site to the polyA addition sites of the TobRB7-5A cDNAs is shown in Figure 1.

Site of Transcription Initiation

The site of transcription initiation was determined by two complementary methods: T4 DNA polymerase extension (Hu and Davidson, 1986) and primer extension experiments (Metraux et al., 1989). This combination allowed us to determine unambiguously the site of transcription initiation.

Figure 3A shows the T4 DNA polymerase extension experiment and establishes the distance between the HindIII site (Figure 1) and the putative transcription initiation site as approximately 300 bp.

Primer extension (Metraux et al., 1989) was performed using the primer indicated in Figure 1. As shown in Figure 3B, a 38-base extension product with root RNA(A^+) and another faint band just above the major band were observed (lane 5). The faint band may be due to the steric hindrance of capping structure. No extension was detected with leaf or calf liver RNA (lanes 6 and 7, respectively).

The site indicated by the T4 DNA polymerase extension experiment (approximately 300 bp from the HindIII site, Figure 3A) agreed with the base defined by the primer extension (38 bases from the primer and 299 bp from the HindIII site). Moreover, the TATA box-like sequence (TATATAAA) found 35 bp 5′ to the indicated site further supports its identification as the site of transcription initiation.

Structure of the λ5A Genomic Clone and Comparisons with the 5′ Flanking Region of λ18C

Figure 4 summarizes the structure of the λ5A genomic clone. Three repeating units (A, B, and C) are present in the 5′ flanking region of the TobRB7-5A gene. Figure 5 illustrates the DNA sequences of these repeats. The boxes

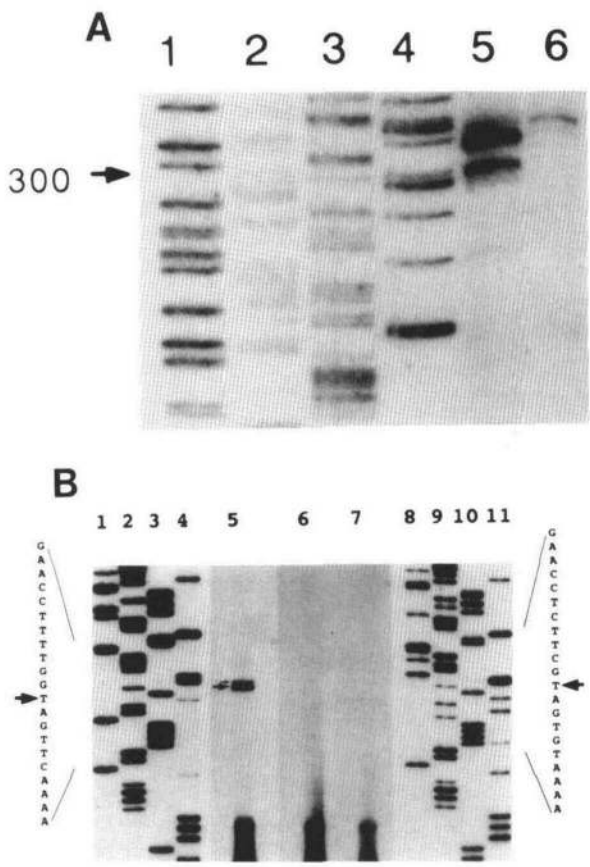


Figure 3. Identification of the Site of Transcription Initiation.

(A) T4 DNA polymerase extension. A single-strand plasmid containing the HindIII-XbaI genomic fragment (see Figures 1 and 4) was annealed with root RNA(A^+). End-labeled m13 primer was extended with T4 DNA polymerase. The length of the extension product indicates the distance between the primer and the site of transcription initiation. The extension product was electrophoresed on a 6% polyacrylamide sequencing gel with a sequencing reaction of the λ5A clone using the same primer. Lanes 1 to 4, sequence of the λ5A (GATC); lane 5, extension product with root RNA(A^+); lane 6, extension product with no RNA.

(B) Primer extension. Lanes 1 to 4, sequencing reaction for the λ5A (CTAG); lane 5, the extension product with root RNA(A^+); lanes 6 and 7, controls with tobacco leaf RNA(A^+) and calf liver RNA, respectively; lanes 8 to 11, sequencing reaction for the λ18C (CTAG). The sequencing reactions were exposed overnight and the extension products were exposed for 2 days.

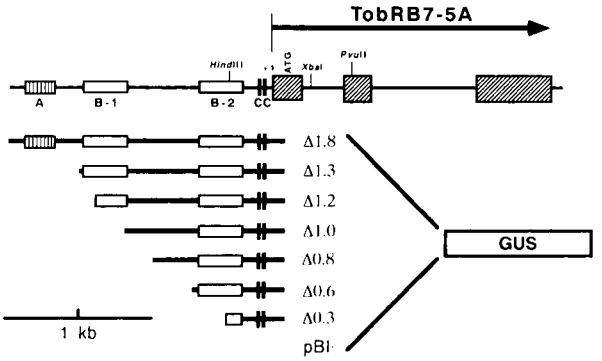


Figure 4. Structure of the λ5A Gene and the Deletions Used in This Study.

Restriction sites used in this study are indicated. The site of transcription initiation is indicated (+1). Boxes are three repeating units: boxes A (striped), eight highly conserved repeats from −1726 to −1483 (see Figure 5A); boxes B (open), about 300 bp of highly conserved repeats at regions −1290 to −973 and −501 to −198 (see Figure 5B), boxes C (solid), 17-bp perfect repeats at −114 to −98 and −68 to −52 (see Figure 5C). The initiating ATG of the translation product is indicated. The deletion series is also indicated.

A are eight highly conserved repeats at regions −1726 to −1483 (Figure 5A). Boxes B1-5A and B2-5A are two conserved A:T-rich (>70%) stretches of DNA at regions −1290 to −973 and −501 to −198 (Figure 5B). Boxes C1-5A and C2-5A are 17-bp perfect repeats (Figure 5C) found at −114 to −98 and −68 to −52.

Characterization of independent TobRB7 isolates from a primary tobacco root cDNA library identified approximately equal numbers of cDNA clones corresponding to λ5A and λ18C. This suggests that each gene is expressed at approximately equal levels. Figure 6 compares the DNA sequences of the 5′ flanking regions of λ18C and λ5A genomic clones. Sequences from −1 to approximately −800 of λ5A and λ18C are highly homologous (>90% sequence identity). No significant homology was observed for >1 kb of sequence distal of that shown in Figure 6. 5′ flanking regions of the TobRB7-18C gene include one copy of the B box (B-18C) (Figure 5B) and two copies of the C boxes (C1-18C and C2-18C) (Figure 5C). No TobRB7-18C sequences corresponding to a second B box or the A boxes were observed within 2 kb of the site of transcription initiation.

The 5′ Flanking Region Contains Root-Specific *cis*-Acting Elements

The *cis*-acting sequences responsible for root-specific expression of the TobRB7-5A gene were defined by deletion analysis. Figure 4 shows all deletions and their locations with respect to the structure of the TobRB7-5A genomic clone. The precise end point of each deletion is shown in Figure 1. Fusion to the GUS reporter gene was accomplished by introducing a BamHI restriction site at +70 (6 bp 5′ of the initiating ATG of the TobRB7-5A gene product) and ligating the appropriate deletion to the BamHI site of pBI101. Excised tissues (roots and leaves) from transgenic plants were tested for GUS activity using the quantitative fluorometric assay described by Jefferson (1987).

Figure 7 and Table 1 summarize the quantitative analysis of GUS expression in transgenic plants. For each deletion in the series, a large number of independent transformants was regenerated and assayed. Transformants exhibited significant variation in levels of expression that did not correlate with copy number. Such variation is commonly observed and is attributed to "position effects." GUS expression was root specific for constructs including at least 636 bp (Δ0.6) 5′ of the initiation of transcription. Constructs including 299 bp of 5′ flanking sequence (Δ0.3) exhibited a loss in GUS expression levels in roots that was equivalent to "promoterless" pBI101 transformants. These data are consistent for a positive, *cis*-acting root-specific

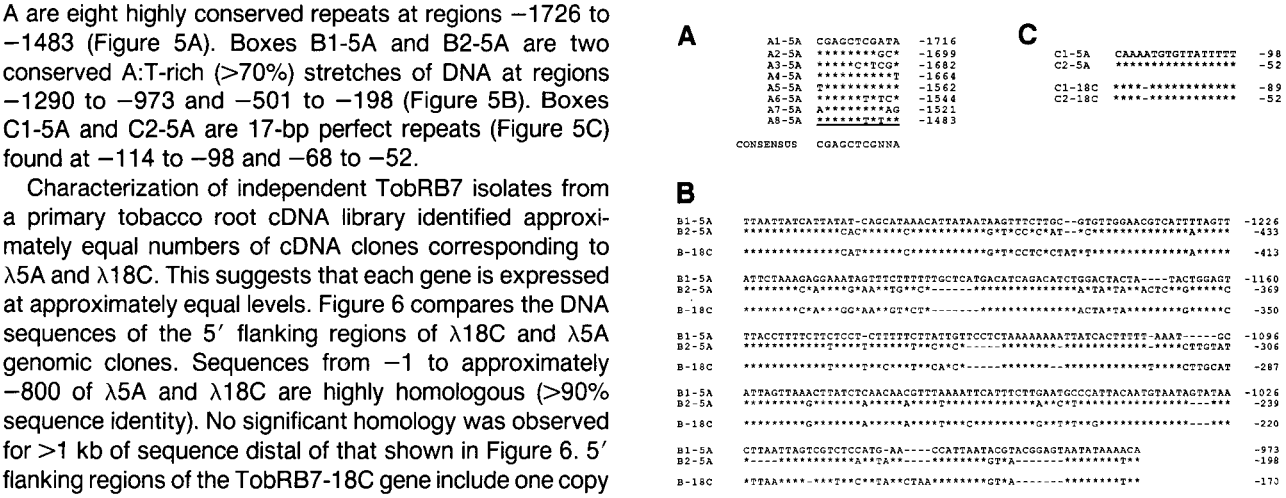


Figure 5. Nucleotide Sequences of Repeated Regions in the 5′ Flanking Regions of the TobRB7-5A and TobRB7-18C Genes.

An asterisk represents nucleotide identity and a minus sign represents gaps.

(A) Nucleotide sequence of boxes A. Eight sequences designated as boxes A are aligned. The consensus sequence is shown.

(B) Nucleotide sequence of boxes B. Boxes B1-5A and B2-5A (from the TobRB7-5A gene) and box B-18C (from the TobRB7-18C gene) are aligned.

(C) Nucleotide sequences of boxes C. Boxes C1-5A and C2-5A (from the TobRB7-5A gene) and boxes C1-18C and C2-18C (from the TobRB7-18C gene) are aligned.

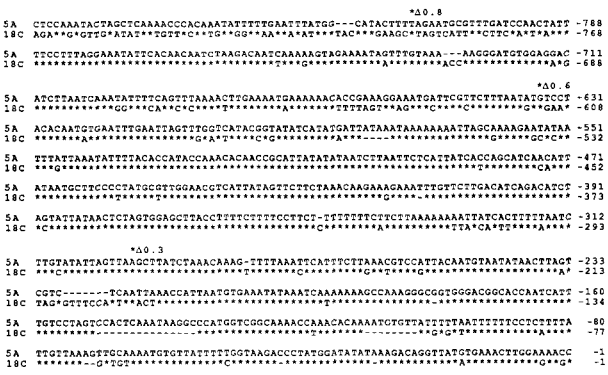


Figure 6. Comparison of the Nucleotide Sequences of the 5′ Flanking Regions of TobRB7-5A and TobRB7-18C.

5′ flanking sequences starting at −1 were aligned. An asterisk represents nucleotide identity and a minus sign represents gaps.

regulatory sequence located between 636 bp and 299 bp 5′ of the initiation of transcription.

Quantitative analysis also uncovered an apparent negative, *cis*-acting regulatory sequence located between 813 bp and 636 bp 5′ of the initiation of transcription. Transformants of the Δ0.6 deletion exhibited higher average GUS activity levels in both roots and leaves (Figure 7). Because this might result from a few transformants exhibiting exceptionally high activity, we examined the median GUS activity levels for the transformants. The median GUS activity level in roots of the Δ0.6 transformants was 1156 pmol of methylumbelliferone (MU)/mg of protein/min, whereas those for the Δ1.8, Δ1.3, Δ1.2, Δ1.0, and Δ0.8 ranged from 327 to 728 pmol of MU/mg of protein/min. These data further support the presence of a negative element located between 813 bp and 636 bp 5′ of the initiation of transcription. However, the median root:leaf ratio of the Δ0.6 transformants was essentially identical to that of the other deletions exhibiting root-specific GUS expression. Because the negative element appears to affect similarly GUS activity levels both in roots and in leaves, it appears that this element is not tissue specific.

Spatial and Temporal Localization of TobRB7 Gene Expression

The organ-specific accumulation of TobRB7 mRNA was examined by RNA gel hybridization. As shown in Figure 8, RNA isolated from expanded young and old leaves, stems, and the shoot apex did not hybridize to the TobRB7 probe, whereas root RNA showed strong hybridization.

As shown in Figure 9, in situ hybridization (Meyerowitz, 1987; Smith et al., 1987) to tobacco root thin sections using an antisense TobRB7 RNA probe localized strong hybridization to the root meristematic and immature central cylinder regions. Sense TobRB7 RNA probes showed no hybridization (data not shown).

To investigate further the temporal and spatial tissue-specific distribution of TobRB7 gene expression, representative transformants of the promoter deletion series were allowed to self and set seeds. Surface-sterilized seeds of transgenic plants were germinated, and at various times after germination the developing seedlings were stained for GUS activity. Wild-type seeds resulting from segregation, seeds from the Δ0.3 deletion, and seeds from the promoterless pBI101 showed no staining (data not shown).

The developmental time course is shown in Figure 10 and did not vary significantly for any of the deletions

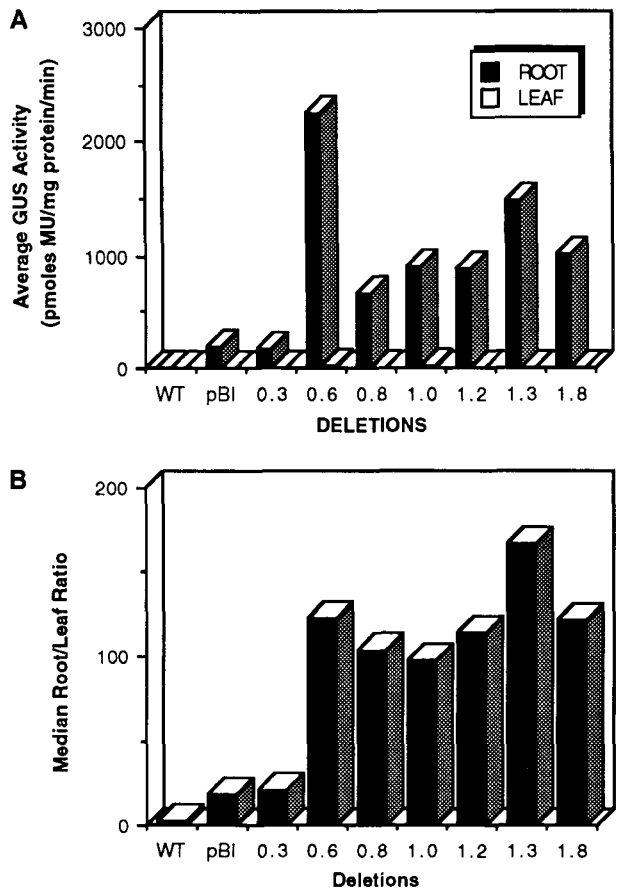


Figure 7. GUS Activities in Roots and Leaves of Transgenic Plants.

(A) Average GUS activities from roots (solid bars) and leaves (open bars) for each deletion construction were plotted.
(B) Median root: leaf GUS activity ratios for each transgenic plant were calculated and the median for each deletion construction was plotted.

Table 1. GUS Activities in Roots and Leaves of Transgenic Plants

	No. of Plants	Average GUS activity (Range of Activities)		Median Ratio (Roots:Leaves)
		Roots	Leaves	
Wild type	8	4 (1–11)	0.7 (0.17–2.26)	2.8
pBI-0.0	21	187 (4–614)	6.9 (0.18–95.7)	19.0
pBI-Δ0.3	21	160 (1–586)	5.2 (0.8–28.4)	21.1
pBI-Δ0.6	22	2242 (4–11,540)	24.7 (0.05–217.5)	122.3
pBI-Δ0.8	17	652 (2–3394)	4.8 (0.03–23.5)	103.2
pBI-Δ1.0	9	804 (3–2068)	55.7 (1.72–373.4)	97.1
pBI-Δ1.2	23	881 (2–4688)	4.3 (0.14–22.4)	113.5
pBI-Δ1.3	24	1475 (5–14,110)	3.0 (0.14–8.9)	166.4
pBI-Δ1.8	18	1007 (1–4274)	6.5 (0.3–20.0)	121.3

Summary of GUS activities for the deletion series. pBI-0.0 is the "promoterless" plasmid pBI101.1. GUS activities in roots and leaves of transgenic plants were quantified fluorometrically. The number of independent transformants analyzed for each construction is shown.

analyzed. Root-specific GUS activity was observed as early as 2 days postgermination. GUS activity continued to be observed in the meristem and early central cylinder regions early in development in a pattern similar to that observed in in situ hybridization analysis (Figure 9). However, later in development (day 6 and mature roots), GUS activity extended to more mature central cylinder tissue than was observed for the TobRB7 mRNA by in situ hybridization and did not appear in the meristem. We believe this result reflects the stability of the GUS gene product relative to TobRB7 mRNA. In situ hybridizations to roots of mature transgenic plants using antisense RNA GUS probes (data not shown) revealed GUS mRNA localization similar to that observed with the TobRB7 probe (Figure 9). In addition, GUS activity was observed early at the site of lateral root initiation (Figure 10). As the lateral root matured, GUS activity was observed in the immature central cylinder regions of the lateral root.

DISCUSSION

This report presents a comprehensive analysis of a plant gene exhibiting root-specific expression. Earlier, we reported the isolation of four transcriptionally regulated, root-specific genes from tobacco (Conkling et al., 1990). Here, we characterize one of these genes, TobRB7, with respect to its structure, the *cis*-acting sequences responsible for regulated expression, and its tissue-specific patterns of expression. In situ hybridization analyses demonstrated that the genes encoding the TobRB7 mRNA are expressed in the root meristem and immature central cylinder (Figure 9). Steady-state mRNA could not be detected in shoot meristem, leaf, or stem tissue (Figure 8), demonstrating that expression is root specific and not meristem specific.

The reporter gene (GUS), when driven by the 5′ flanking sequence of the TobRB7-5A gene, exhibited tissue-specific expression patterns similar, but not identical, to those observed by in situ hybridization experiments using TobRB7 probes (Figures 9 and 10). In situ hybridization experiments of transgenic plants using a GUS hybridization probe exhibited hybridization patterns similar to those

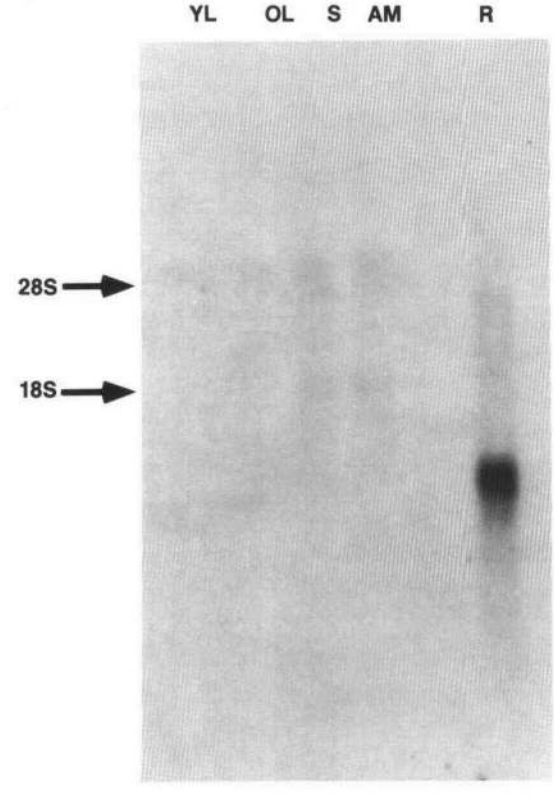


Figure 8. RNA Gel Blot Analysis.

Total tobacco RNA extracted from young leaves (YL), old leaves (OL), stems (S), shoot apical meristems (AM), and roots (R) was subjected to electrophoresis, transferred to a nitrocellulose filter, and hybridized to a probe of the TobRB7 cDNA. All lanes except R contained 10 μg of total RNA; R contained 2 μg of total RNA. The positions of the 28S and 18S rRNAs are shown.

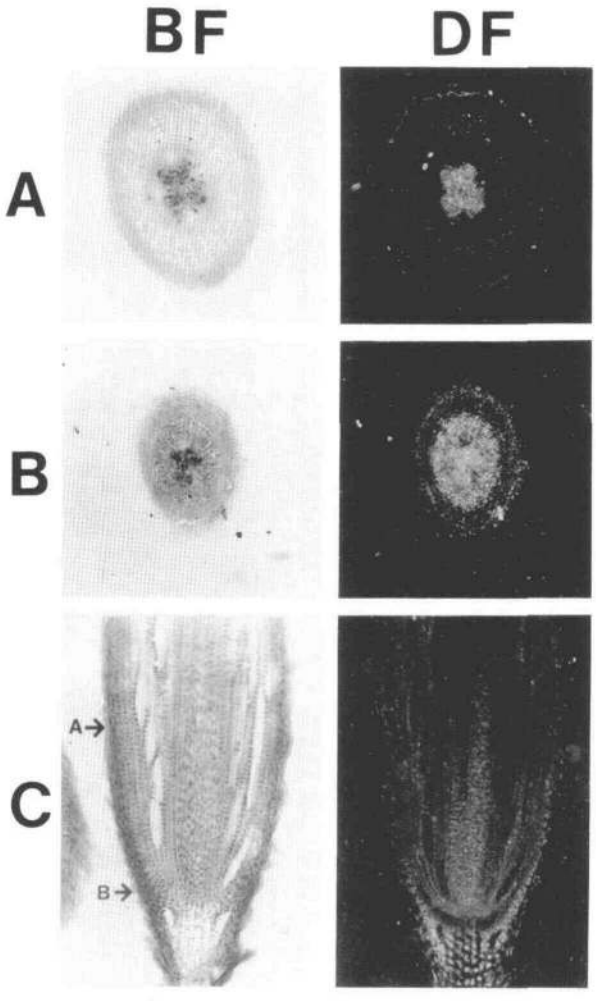


Figure 9. In Situ Localization of TobRB7 mRNA in Paraffin Sections of Tobacco Roots.

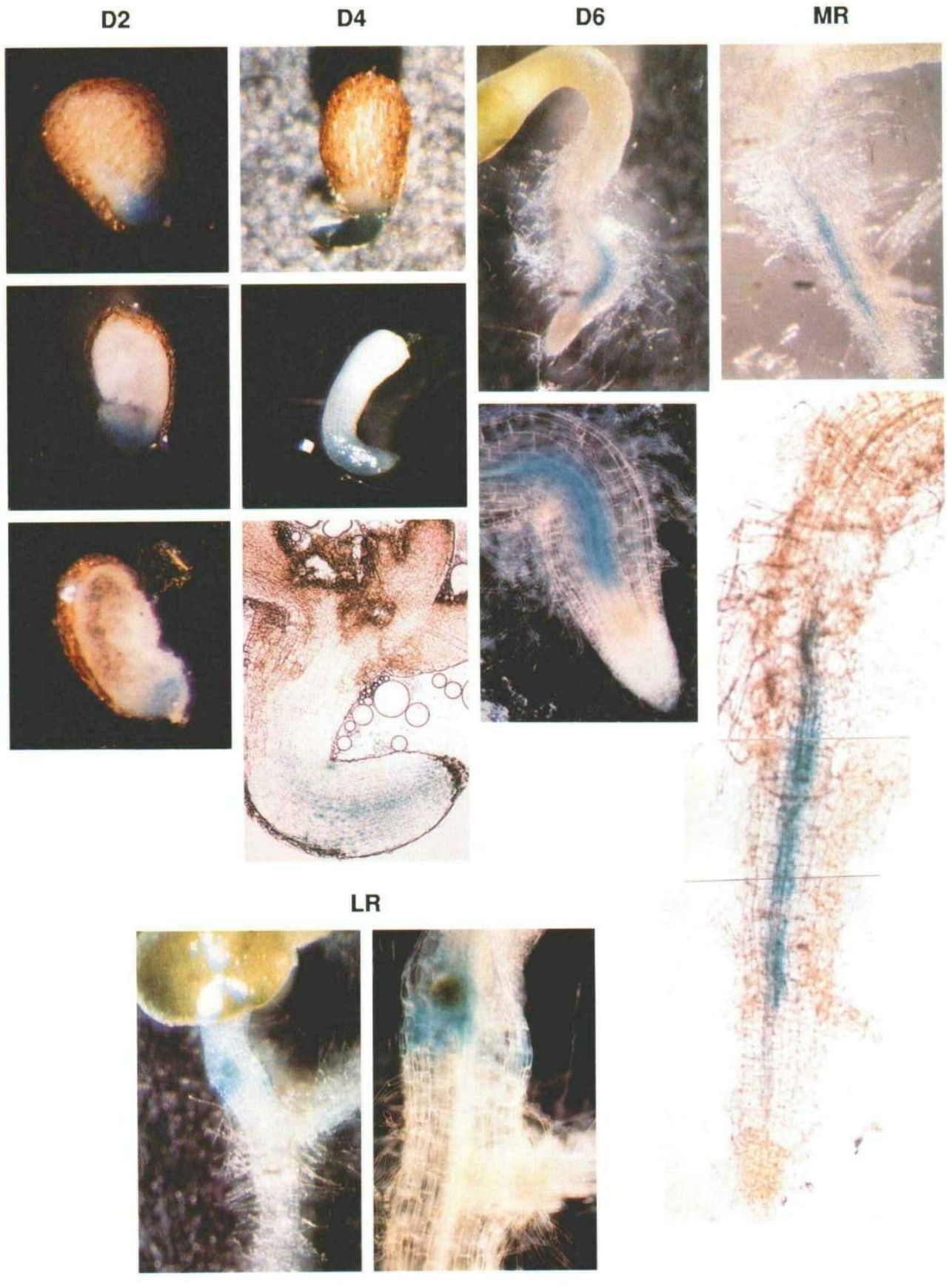
Conditions were basically as described (Meyerowitz, 1987; Smith et al., 1987). After exposure, the slides were developed and visualized under bright-field (BF) and dark-field (DF) microscopy.
(A) Transection of root through elongation region.
(B) Transection of root through meristematic region.
(C) Longitudinal section of root. A→ and B→ indicate approximate positions of transections A and B, respectively.

observed with TobRB7 probes. Thus, RNA of the GUS reporter gene (when transcribed from 5′ flanking sequences of the TobRB7-5A gene) accumulated in tissue-specific patterns similar to those of TobRB7 RNA. At this time, we cannot be certain whether the GUS activity observed in more mature regions of the vascular cylinder reflects the stability of the GUS gene product or whether the TobRB7-5A promoter is expressed at lower levels in other regions of the root that are not detected in our in situ hybridization conditions but maintain sufficient GUS activity for histochemical staining. The similarities of the expression patterns of TobRB7 and the GUS reporter gene demonstrate that the 5′ flanking sequence directs expression of the reporter gene in biologically relevant fashions.

In situ localization of TobRB7 mRNA and localization of GUS activity to the root meristem and early central cylinder imply that the TobRB7 gene product may be required early in root development. This notion is supported further by histochemical analysis of developing transgenic seedlings. Root-specific GUS activity driven by the TobRB7-5A 5′ flanking sequence was observed in seedlings as early as 2 days postgermination. In lateral roots, expression began at initiation and remained in the meristem and developing central cylinder. This expression at lateral root initiation sites is distinct from that reported by Keller and Lamb (1989) for the HRGPnt3 gene. The HRGPnt3 gene appears to be expressed transiently only at the site of lateral root initiation, whereas TobRB7 is expressed throughout development of the lateral root.

TobRB7 is encoded in tobacco by an apparent two-member gene family (TobRB7-5A and TobRB7-18C). Two strongly hybridizing fragments were detected in DNA gel hybridization of tobacco genomic DNA digested with a variety of restriction endonucleases using TobRB7 as a probe (Conkling et al., 1990). All restriction fragments detected on the genomic DNA gel blots corresponded to restriction fragments of the isolated genomic clones, implying that all of the TobRB7 genes had been isolated. Both genes were expressed at approximately equal levels and encoded mRNAs whose predicted translation products are almost identical (two amino acid substitutions in 250 amino acids). Because tobacco is an amphidiploid species, these two genes may be two homeologous genes. We are examining this hypothesis by characterizing TobRB7 homologs from the tobacco progenitor species *Nicotiana sylvestris* and *N. otophora* or *N. tomentosiformis*.

Both the TobRB7-5A and TobRB7-18C genes have three exons and two introns. The exon/intron junctions are conserved for these genes. Three sets of repeats, designated as boxes A, B, and C, are apparent within the 5′ flanking region of the TobRB7-5A gene (Figures 4 and 5). Boxes A are eight 11-bp repeats located between −1726 and −1483. The sequences are conserved highly (Figure 5A) and their consensus includes a perfect palindrome of 8 bases (CGAGCTCG). Boxes B are two highly conserved sequences of about 300 bp found at −1290 to −973 and −501 to −198 (Figure 5B). The B boxes are rich in A:T (72% for the distal box, 73% for the proximal box). Boxes C are 17-bp perfect repeats at −68 to −52 and −114 to −98 (Figure 5C). The TobRB7-18C gene contains one copy of a B-like box (Figure 5B) and two C-like boxes (Figure 5C). 5′ flanking sequences of TobRB7-5A and TobRB7-18C are highly homologous for the proximal 800 nucleotide residues (−1 to −790), but then diverge (Figure 6).



Deletion analysis identified the *cis*-acting elements responsible for the root-specific expression of the TobRB7-5A gene. Deletion of the A boxes and the B1-5A box did not result in significant qualitative or quantitative changes in the root-specific expression of the GUS reporter gene. Deletions including 636 bp (Δ0.6) of 5′ flanking sequence were sufficient to direct root-specific expression of the GUS reporter gene. Deletions containing only 299 bp of 5′ flanking sequence (Δ0.3) exhibited only basal levels of GUS expression in both leaves and roots. This region (−636 to −299), therefore, contains positive *cis*-acting sequence(s) required for root-specific expression. An apparent negative regulatory region was uncovered between −813 and −636 of the TobRB7-5A gene. Because root-specific gene expression was abolished in Δ0.3 transformants, 5′ nested deletions could not dissect further the TobRB7-5A 5′ flanking sequence. Based on this deletion analysis, the *cis*-acting sequences regulating root-specific expression of the TobRB7-5A gene reside within 800 bp 5′ of the initiation of transcription (a negative regulatory element located between −813 and −636 and a positive, root-specific regulatory element located between −636 and −299). It is significant that sequences to approximately −800 are conserved in the 5′ flanking region of the TobRB7-18C gene (Figure 6), a closely related root-specific gene that is expressed at approximately equal levels to the TobRB7-5A gene.

We reported previously that the root-specific expression of TobRB7 is, at least in part, regulated transcriptionally (Conkling et al., 1990). In these earlier experiments, the TobRB7-5A gene was fused to the GUS reporter gene at the PvuII site in the second exon (see Figure 4) and included about 1.4 kb of 5′ flanking sequence. Thus, this construction contained the first TobRB7-5A intron. Transient expression analyses of monocot genes indicate that inclusion of the first intron often results in higher levels of reporter gene expression (Callis et al., 1988). GUS activities in roots of five TobRB7/GUS transgenic plants carrying the first intron were quantified (Conkling et al., 1990). GUS activity levels (average, 220; range, 100 to 530 pmol of MU/mg of protein/min) were compared with activity levels reported for constructions containing similar 5′ flanking sequences and lacking the first intron (Table 1). Thus, it appears that inclusion of the TobRB7-5A first intron does not result in higher expression levels in stable transformants and, if anything, may lower expression levels.

Promoter sequences of many eukaryotic genes are frequently combinatorial; often, multiple *cis*-acting elements act upon a gene to regulate gene transcription (Benfey et al., 1989) (reviewed in Maniatis et al., 1987; Jones et al., 1988). We believe the positive *cis*-acting sequences regulating root-specific expression reside within the 5′ half of the repeated DNA region we have termed the B box. The B box is repeated in the TobRB7-5A 5′ flanking sequence and is present within the TobRB7-18C flanking sequence. Deletions of the distal B box (B1-5A) did not result in statistically significant changes in GUS activity levels. However, Δ1.8 and Δ1.3 transgenic plants, which contain an intact B-1 box, exhibited slightly higher GUS activities than did Δ1.2, Δ1.0, and Δ0.8 transgenic plants, which contain sequential deletions of the B-1 box. Thus, duplication of the B box may lead to slightly increased levels of TobRB7-5A gene transcription. We are testing the ability of the B-1 box to direct root-specific gene expression independent of the B-2 box.

The spatial and temporal expression of the GUS reporter gene directed by the TobRB7-5A promoter deletion series were examined. All deletions that directed root-specific GUS expression exhibited the same temporal and spatial patterns of GUS expression (Figure 10). This implies either that the TobRB7-5A promoter is not combinatorial in its tissue-specific regulation or that the deletion series was not sufficiently fine to dissect multiple *cis*-acting elements. However, an element whose presence in the deletion series decreased expression levels in roots was present in the TobRB7-5A promoter. Our analysis established at least two regulatory elements (a positive element between bases −636 and −299 and a negative element between bases −813 and −636) that act in concert to regulate TobRB7-5A gene expression.

METHODS

Strains

Nicotiana tabacum cv Wisconsin 38 was used for cloning and gene characterization. *N. tabacum* SR1 was the recipient of *Agrobacterium*-mediated transformation. *Escherichia coli* DK1 competent cells were prepared according to Maniatis et al. (1982). *A. tumefaciens* LBA4404 (Hoekema et al., 1983) was the host for plant transformation using the binary vector system of Jefferson et al. (1987). Isolation of the tobacco root-specific cDNA clone TobRB7 and the corresponding genomic clones was reported previously (Conkling et al., 1990).

Figure 10. Histochemical Localization of GUS Activity in Developing Transgenic Plants.

Sequential stages 2 days (D2), 4 days (D4), and 6 days (D6) postgermination of germinating seedlings carrying the GUS reporter gene under control of the TobRB7 promoter are shown. MR illustrates a mature root of a transgenic plant stained for GUS activity. LR shows sites of lateral root initiation.

RNA Preparation and Analysis

Tissue for RNA preparations was harvested and immediately frozen in liquid nitrogen. For root RNA, hydroponically grown roots were harvested approximately 1 cm from the root tip. The top two expanded leaves were harvested for young leaf RNA preparations. The eighth to tenth leaves from the top were harvested for old leaf RNA preparations. Shoot apices of about 3 mm were dissected for apical meristem RNA preparations. Total RNA was prepared either using a modification of Chirgwin et al. (1979) or a modification of the RNA mini-preparation of Wadsworth et al. (1988). Poly(A^+) RNA was selected by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972).

Total RNA was subjected to electrophoresis on a 1% agarose-6% formaldehyde gel (Goldberg, 1980) and transferred directly in 20 × SSC to a nitrocellulose filter. The filter was hybridized to a random-primed (Feinberg and Vogelstein, 1983) probe (5×10^5 cpm/mL hybridization solution) of the TobRB7 cDNA. Filters were exposed for 16 hr with an intensifying screen.

Construction of cDNA Libraries

cDNA libraries were constructed using a modification of the RNase H/DNA polymerase I method of Gubler and Hoffman (1983). A cDNA library of mRNA isolated from tobacco root tissue constructed in pUX-DBE (a gift from J.-Y. Sheen) using BstXI adaptors was used to isolate full-length counterparts of TobRB7.

Hybridization Conditions and DNA Sequencing

All nucleic acid hybridizations were performed at 65°C in aqueous conditions: 5 × SSC (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% each Ficoll, BSA, polyvinylpyrolidone), 25 mM sodium phosphate (pH 7.4), 5 mM EDTA, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA. Filters were washed two times (15 min each) at room temperature in 1 × SSC, 0.1% SDS. Then they were washed two or three times (15 min each) at 42°C in 0.2 × SSC, 0.1% SDS.

DNA sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) using Sequenase (U.S. Biochemicals). Unidirectional deletion series were constructed as described (Henikoff, 1984) except that S1 nuclease was substituted for mung bean nuclease. In all cases, both DNA strands were sequenced.

T4 DNA Polymerase Extension

The extension reaction was performed according to Hu and Davidson (1986) except that blunt-ending buffer [67 mM potassium acetate, 33 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ 0.5 mM dithiothreitol] was used. Two micrograms of tobacco root RNA(A^+) (Figure 3A, lane 5) was heated at 65°C for 10 min. As a control, diethyl pyrocarbonate-treated dH_2O was substituted for the RNA (Figure 3A, lane 6). Approximately 0.5 μg of single-strand template (HindIII-XbaI fragment inserted in M13mp18) in 80% formamide (Fluka AG, Switzerland) hybridization buffer [40 mM Na-Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA] was added and incubated at 80°C for an additional 10 min. The sample was cooled to 37°C and incubated for 16 hr. The annealed mixture was ethanol precipitated once in 2.5 M ammonium acetate and once in 0.3 M sodium acetate. The dried pellet was resuspended in 1 × blunt-ending buffer and 2 ng of γ-^{32}P-labeled m13 universal primer was added. To anneal, the mixture was heated at 65°C for 10 min and then at 37°C for 60 min. One unit of T4 DNA polymerase (Boehringer Mannheim Biochemicals) was added and the mixture was incubated at 37°C in the presence of 3.3 mM deoxynucleotide triphosphates for 60 min. The reaction was dried and electrophoresed on a 6% sequencing gel with a sequencing reaction using the same template and primer as marker (Figure 3A, lanes 1 to 4, GATC).

Primer Extension

Primer extension experiments were carried out according to Metraux et al. (1989). One microgram of tobacco root (Figure 3B, lane 5) or leaf RNA(A^+) (lane 6) or 1 mg of calf liver total RNA (lane 7) in 1 × reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3 at 42°C), 10 mM $MgCl_2$, 70 mM KCl) was incubated at 75°C for 10 min. The sample was cooled to 65°C and 60 fmol of γ-^{32}P-labeled primer (see Figure 1) was added. The sample was cooled slowly (2 hr) to 37°C. The annealed mixture was incubated at 42°C with 30 units of avian myeloblastosis virus reverse transcriptase (Promega Biotec) in the presence of 0.6 mM deoxynucleotide triphosphates, 4 mM dithiothreitol, 100 μg/mL BSA for 30 min. Ten units of RNase inhibitor (Boehringer Mannheim Biochemicals) was included. After the reaction, extension products were ethanol precipitated in 0.3 M sodium acetate and electrophoresed on a 10% sequencing gel with sequencing reactions of the λ5A (Figure 3B, lanes 1 to 4) and the λ18C clones (lanes 8 to 11) using the same primer (CTAG). The sequencing reaction was exposed overnight and the extension products were exposed for 2 days.

Construction of Deletions

The deletion series was constructed using the polymerase chain reaction (PCR) to place a BamHI restriction site adjacent to the site of translation initiation. The templates were various deletions of the TobRB7-5A 5′ flanking sequence generated by ExoIII/SI treatments (Henikoff, 1984). An oligonucleotide complementary to the 5′ untranslated region of the TobRB7-5A gene and containing a BamHI restriction site at the +70 site was synthesized and used as a PCR primer. An oligonucleotide complementary to the cloning vector beyond the polylinker cloning site served as the opposing primer. Amplified products were digested with BamHI (newly introduced at +70) and XbaI (from the cloning vector) and cloned as a cassette into pBI101.1 (Jefferson, 1987) that had been digested with BamHI and XbaI. All fusions were sequenced at a junction point between each deletion and the GUS gene from a primer complementary to the 5′ end of the GUS gene.

Tobacco Transformation, Regeneration, and GUS Assays

Direct transformation of *Agrobacterium* was conducted as described by An et al. (1988). Leaf disc transformation and plant

regeneration were performed as described by An et al. (1986). The excised leaf and root tissues were assayed for the GUS expression according to Jefferson (1987). GUS activities were quantified for independent transgenic plants using the fluorogenic assay. Proteins were extracted and incubated in the presence of 1 mM 4-methylumbelliferyl β-D-glucuronide at 37°C. Samples were taken at 5 min, 30 min, and 1.5 hr and the enzyme reaction was stopped in 0.2 M Na_2CO_3. The fluorometer was calibrated with 10 nM and 100 nM MU. Protein concentration was determined according to Bradford (1976).

For histochemical staining, regenerated plants were allowed to self and surface-sterilized seeds were germinated on Murashige and Skoog (1962) (MS) medium containing 300 μg/mL kanamycin. Seeds for controls were germinated on MS medium lacking kanamycin. Plants, seeds, or excised root and leaf tissue were incubated at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 25 mM sodium phosphate buffer (pH 7.0), 1% DMSO.

In Situ Hybridizations

Conditions were basically as described (Meyerowitz, 1987; Smith et al., 1987). Briefly, tobacco roots were fixed in phosphate-buffered glutaraldehyde, embedded in Paraplast Plus (Monoject, Inc., St. Louis, MO), and 8-μm sections cut. ^{35}S-labeled RNA probes of the antisense strand of TobRB7 were hydrolyzed by alkaline treatment to yield a 100-base to 250-base mass average length. Approximately 5×10^6 cpm labeled RNA/mL of hybridization solution was used. Hybridizations were in 50% formamide for 16 hr at 42°C. After exposure, the slides were developed and visualized under bright-field and dark-field microscopy.

ACKNOWLEDGMENTS

We thank Dr. Jen Sheen for the gift of pUX-DBE. We also thank the staff of the Southeastern Plant Environmental Laboratories for their assistance in the propagation of transgenic plants. These studies were supported by Grant DMB-8811077 from the National Science Foundation. Y.T.Y. was supported in part by a grant from the North Carolina Tobacco Foundation, Inc. C.G.T. was supported in part as a U.S. Department of Agriculture Food and Agricultural Sciences National Needs Graduate Fellow (88-38420-3825).

Received January 24, 1991; accepted February 27, 1991.

REFERENCES

An, G., Watson, B.D., and Chiang, C.C. (1986). Transformation of tobacco, tomato, potato, and *Arabidopsis thaliana* using a binary vector system. Plant Physiol. **81,** 301–305.

An, G., Ebert, P.R., Mitra, A., and Ha, S.B. (1988). Binary vectors. In Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. A3/1–19.

Aviv, H., and Leder P. (1972). Purification of biologically active globin mRNA by chromatography on oligothymidylic acid. Proc. Natl. Acad. Sci. USA **69,** 1409–1412.

Baker, M.E., and Saier, M.H. (1990). A common ancestor for bovine lens fiber major intrinsic protein, soybean nodulin-26, and *E. coli* glycerol facilitator. Cell **60,** 185–186.

Benfey, P.N., and Chua, N.-H. (1989). Regulated genes in transgenic plants. Science **244,** 174–181.

Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8,** 2195–2202.

Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J., and Peacock, W.J. (1988). Functioning haemoglobin genes in non-nodulating plants. Nature **331,** 178–180.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Callis, J., Fromm, M., and Walbot, V. (1988). Introns increase gene expression in cultured maize cells. Genes Dev. **1,** 1183–1200.

Chirgwin J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18,** 5294–5299.

Conkling, M.A., Cheng, C.-L., Yamamoto, Y.T., and Goodman, H.M. (1990). Isolation of transcriptionally regulated root-specific genes from tobacco. Plant Physiol. **93,** 1203–1211.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Fortin, M.G., Morrison, N.A., and Verma, D.P.S. (1987). Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment. Nucl. Acids Res **15,** 813–824.

Goldberg, D.A. (1980). Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA **77,** 5794–5798.

Gorin, M.B., Yancy, S.B., Cline, J., Revel, J.-P., and Horwitz, J. (1984). The major intrinsic protein (MIP) of the bovine lens fiber membrane: Characterization and structure based on cDNA cloning. Cell **39,** 49–59.

Gubler, U., and Hoffman, B.J. (1983). A simple and efficient method for generating cDNA libraries. Gene **25,** 263–269.

Henikoff, S. (1984). Unidirectional ExoIII deletions for DNA sequencing. Gene **28,** 351–359.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983). A binary plant vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303,** 179–180.

Hu, M.C.-T., and Davidson, N. (1986). Mapping transcription start points on cloned genomic DNA with T4 DNA polymerase: A precise and convenient technique. Gene **42,** 21–29.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J. **6,** 3901–3907.

Johnson, K.D., Höfte, H., and Chrispeels, M.J. (1990). An intrinsic tonoplast protein of protein storage vacuoles in seeds is structurally related to a bacterial solute transporter (GlpF). Plant Cell **2,** 525–532.

Jones, N.C., Rigby, W.J., and Ziff, E.B. (1988). *Trans*-acting protein factors and the regulation of eukaryotic transcription: Lessons from studies on DNA tumor viruses. Genes Dev. **2,** 267–281.

Keller, B., and Lamb, C.J. (1989). Specific expression of a novel cell wall hydroxyproline-rich glycoprotein in lateral root initiation. Genes Dev. **3,** 1639–1646.

Lerner, D.R., and Raikhel, N.V. (1989). Cloning and characterization of root-specific barley lectin. Plant Physiol **91,** 124–129.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Maniatis, T., Goodbourn, S., and Fischer, J.A. (1987) Regulation of inducible and tissue-specific gene expression. Science **236,** 1237–1244.

Metraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA **86,** 896–900.

Meyerowitz, E. (1987). In situ hybridization to RNA in plant tissue. Plant Mol. Biol. Rep. **5,** 242–250.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Rao, Y., Jan, L.Y., and Jan, Y.N. (1990). Similarity of the product of the *Drosophila* neurogenic gene *big brain* to transmembrane channel proteins. Nature **345,** 163–167.

Sandal, N.N., and Marcker, K.A. (1988). Soybean nodulin 26 is homologous to the major intrinsic protein of the bovine lens fiber membrane. Nucl. Acids Res. **16,** 9347.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Smith, A., Hinchee, M., and Horsch, R. (1987). Cell and tissue specific expression by in situ hybridization in floral tissues. Plant Mol. Biol. Rep. **5,** 237–241.

Wadsworth, G.J., Redinbaugh, M.G., and Scandalios, J.G. (1988). A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal. Biochem. **172,** 279–283.

Yamamoto, Y.T., Cheng, C.-L., and Conkling, M.A. (1990). Root-specific genes from tobacco and *Arabidopsis* homologous to an evolutionarily conserved gene family of membrane channel proteins. Nucl. Acids Res. **18,** 7449.