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# Hypocotyl expression and light downregulation of the soybean tubulin gene, *tubB*1

**Hiroshi Tonoike, In-Seob Han[†], Ian Jongewaard[‡], Michele Doyle, Mark Guiltinan[§] and Donald E. Fosket***
*Department of Developmental and Cell Biology, University of California, Irvine, CA 92717, USA*

## Summary

**The *tubB*1 β-tubulin gene of *Glycine max* (previously named *s*β1) is highly expressed only in rapidly elongating regions of etiolated seedling hypocotyls and this expression is strongly downregulated when the seedlings are exposed to light. Primer extension demonstrated that the gene was transcribed in these tissues and contained two sites of transcriptional initiation. To determine the mechanism regulating *tubB*1 expression, a chimeric reporter gene was constructed by fusing 5' upstream regions of *tubB*1 to a promoterless β-glucuronidase (GUS) gene and these constructs were introduced into protoplasts by electroporation. Strong transient expression of the reporter gene was obtained after electroporation of chimeric constructs containing 1 kb of *tubB*1 5' upstream sequence into tobacco protoplasts. Deletion of the distal most 300 bp from the 5' sequence of *tubB*1 enhanced expression, suggesting the possibility of a negative transcriptional regulator in this region. Additional deletions of the 5' sequence reduced expression substantially. Constructs containing a *tubB*1 3' terminus were expressed at much lower levels than those containing a nopaline synthase (NOS) 3' terminus. The *tubB*1–GUS chimeric gene also was introduced into tobacco by *Agrobacterium*-mediated Ti plasmid transformation and the organ-specific expression pattern of the chimeric gene was determined in seedlings of the transgenic plants. Hypocotyls exhibited strong GUS activity when the seedlings were germinated in darkness, but lacked the GUS enzyme when the seedlings were germinated in the light. This result demonstrates that the *cis*-acting elements within the first 2 kb of the translational start site of the *tubB*1 gene are sufficient for correct expression of the gene in etiolated, elongating hypocotyl tissues, and for the downregulation of this expression by light.**

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*For correspondence (fax +1 714 856 4709).
[†]Present address: Department of Biology, University of Ulsan, Ulsan 680-749, Korea.
[‡]Present address: Committee on Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA.
[§]Present address: Department of Horticulture and Biotechnology Institute, The Pennsylvania State University, University Park, PA 16802-5807, USA.

## Introduction

Microtubules participate in numerous aspects of plant growth and development. They are structural components of the mitotic and meiotic spindle and of the phragmoplast in dividing cells. In elongating and differentiating cells, a cortical microtubule array underlies the plasma membrane and orients the deposition of cellulose microfibrils, which in turn determines the direction of cell expansion (Green *et al.*, 1987). Microtubules are formed by the regulated self-assembly of the heterodimeric protein tubulin, which is composed of one subunit each of an α- and a β-tubulin protein. Both α- and β-tubulins are encoded by relatively large multigene families in higher plants. The *Arabidopsis* genome has been shown to contain six α- and nine β-tubulin genes (Kopczak *et al.*, 1992; Snustad *et al.*, 1992), while the maize genome appears to have at least eight α-tubulin genes (Villemur *et al.*, 1992). Two members of the soybean β-tubulin multigene family have been cloned and sequenced (Guiltinan *et al.*, 1987), and there is evidence for nine additional expressed members in this multigene family (Fosket *et al.*, 1993). The significance of this tubulin gene redundancy is not known, and it is possible that many of the different α- and β-tubulin isotypes encoded by these genes are functionally interchangeable. Certainly all known α- and β-tubulin isotypes participate in the formation of microtubules, although not necessarily with the same assembly kinetics (Banerjee *et al.*, 1990; Detrich *et al.*, 1992). However, it has been demonstrated that some tubulin isotypes confer unique functions on the microtubules that contain them and other tubulin isotypes cannot substitute for these function-specific isotypes (Hoyle and Raff, 1990). The *mec*7 gene of *Caenorhabditis elegans* encodes a β-tubulin that is essential for the formation of special touch receptors containing 15 protofilament microtubules (Savage *et al.*, 1989). Also, the *Drosophila* βIII tubulin is essential for certain morphogenetic events during the embryonic development of this organism and its function cannot be replaced by other *Drosophila* β-tubulins (Kimble *et al.*, 1990).

The amino acid sequence of the tubulins encoded by these multigene families is highly conserved and there

are large regions of the known α- and β-tubulins in which there is nearly 100% amino acid identity among tubulins from diverse organisms (Fosket and Morejohn, 1992). However, the carboxyl terminal 15–20 positions are variable in sequence in both α- and β-tubulins, although this region exhibits a conservation of charged residues. The carboxyl terminal variable region, plus an immediately adjacent conserved sequence, represents a regulatory domain in both α- and β-tubulins (Cross *et al.*, 1991; Maccioni *et al.*, 1986). The regulatory domain is on the surface of the tubulin dimer, as well as the microtubule surface, where it can interact with other molecules, including microtubule-associated proteins. As a result, the carboxyl terminal variable domain is in a position to influence the nature of the interactions that occur between microtubules and other cellular structures. Since the carboxyl terminal sequences of tubulin isotypes differ, it is reasonable to expect that each isotype will interact with a different spectrum of cellular proteins (Cowan *et al.*, 1988). This means that the isotypic composition of the microtubule is likely to be the key determinant of its function. As a result, it is important to understand how cells regulate the expression of their tubulin genes.

The structure and developmental expression of two members of the relatively large soybean β-tubulin multigene family have been analyzed (Guiltinan *et al.*, 1987; Han *et al.*, 1991). One, designated *tubB*2, is expressed in many different tissues and organs, while the other, designated *tubB*1, encodes the most diverged known angiosperm β-tubulin and exhibits a unique pattern of developmental expression. The highest levels of *tubB*1 gene transcripts are found in etiolated hypocotyls during their elongation, and these transcripts disappear after elongation is complete. Expression of the *tubB*1 gene also is regulated by light. Transcripts of the *tubB*1 gene rapidly disappear from the hypocotyls when etiolated seedlings are exposed to light, and they are virtually undetectable after 12 h of illumination. The gene is expressed weakly in some of the tissues of light-grown soybean plants, however. Most notably, the hypocotyls of light-grown seedlings contain *tubB*1 transcripts, although at markedly lower levels than the dark-grown hypocotyls. In the mature plant, *tubB*1 gene transcripts were not detected in vegetative buds or young leaves, which contain high levels of transcripts from other tubulin genes, but the *tubB*1 gene is weakly expressed in expanded leaves (Han *et al.*, 1991).

To identify the factors regulating the expression of the *tubB*1 gene, various fragments from the 5' upstream region of the *tubB*1 gene were fused to a GUS reporter gene containing either a NOS or *tubB*1 3' terminus, and the chimeric constructs were analyzed for activity after electroporation into tobacco protoplasts. Additionally, the expression pattern of a chimeric GUS reporter gene, driven by a 2 kb fragment from the 5' region of the *tubB*1 gene, was determined in transgenic tobacco plants. Here we show that a fragment from the *tubB*1 5' upstream region was able to drive strong transient expression of the chimeric gene in electroporated protoplasts. Seedlings of transgenic tobacco plants containing the chimeric gene exhibited a pattern of GUS expression similar to the pattern of *tubB*1 expression in soybean seedlings. These data indicate that the developmental regulation of the *tubB*1 gene in soybean is mediated primarily, but not necessarily exclusively, at the transcriptional level.

## Results

### *Mapping the transcriptional start site of the* tubB1 *gene*

The site of *tubB*1 transcriptional initiation was mapped by primer extension. An 18 base oligonucleotide was synthesized whose sequence corresponded to a region near the start of translation in the first exon of the *tubB*1 gene which was identified as a sequence that did not occur in the soybean *tubB*2 tubulin gene. The end-labeled oligomer was hybridized to RNA extracted from light-grown and etiolated soybean seedling and extended with reverse transcriptase. No products were obtained when RNA extracted from light-grown seedlings was used. Two products were observed in approximately equal amounts when RNA extracted from dark-grown seedlings was analyzed; one was a 248 base product and the second consisted of 159 bases (Figure 1). This demonstrates that two different transcriptional start sites are utilized when the *tubB*1 gene is transcribed in etiolated seedlings. Initiation of transcription from the first site would result in a 38 base transcribed, untranslated leader that is 73% A + T, while the second would be 127 bases long and 62% A + T (Figure 2). S1 nuclease protection experiments lead to the same conclusions (data not shown).

### *Development of a transient assay system*

The *Escherichia coli* β-glucuronidase (GUS) gene was used as a reporter for the analysis of the promoter activity of the 5' upstream region of the *tubB*1 gene (Jefferson *et al.*, 1987). The ability of the 5' upstream region of the *tubB*1 gene to drive the expression of a promoterless GUS reporter gene initially was examined in a transient expression system after the chimeric constructs were electroporated into protoplasts of cultured BY-2 tobacco cells. A fluorescent assay in which a substrate for the enzyme, 4-methylumbelliferyl glucuronide is converted to the fluorescent product, 4-methyl umbelliferone (4-MU), by GUS enzyme activity was used as a measure of GUS gene expression. A 2 kb fragment from the 5' upstream region of the *tubB*1 gene was able to drive the expression

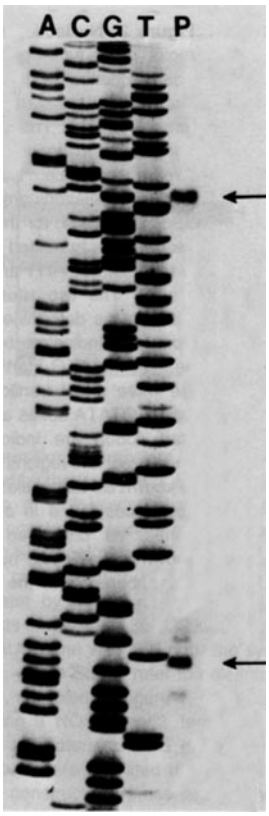


**Figure 1.** Analysis of transcriptional initiation in the *tubB*1 gene by primer extension.
The 18 mer oligonucleotide 5'-TAATGCATCCATTGAAAG-3', which primed at position 103–120 in the first exon of the *tubB*1 gene, was end-labeled with polynucleotide kinase, hybridized to total RNA extracted from 7-day-old soybean seedlings and the primer was extended with reverse transcriptase. The arrows indicate the two major primer extension products (lane P).

of the GUS reporter gene, as did a 5' deletion in which the most distal 1 kb of the sequence was removed. Constructs with 2 and 1 kb of *tubB*1 5' upstream sequence gave equivalent GUS activity in the electroporated protoplasts. Deletion of an additional 300 bp of 5' sequence actually increased the observed GUS activity in transient assays. However, chimeric constructs in which the GUS gene was driven by either a 448 bp fragment or a 118 bp fragment from the *tubB*1 5' sequence showed substantially less activity (Figure 3). Thus, transient expression data suggest that elements required for the transcription of the *tubB*1 gene lie somewhere within the first 1 kb of the 5' upstream region.

The level of transient GUS expression was affected by the 3' terminus of the chimeric construct. Substantially higher levels of GUS were obtained with constructs containing the 3' terminus from the plant-expressible bacterial NOS gene than with constructs containing the *tubB*1 3' terminus. This reduction in GUS activity was not due to a specific interaction between the *tubB*1 3' terminus and the *tubB*1 5' sequence, since a similar reduction in expression levels was observed in constructs containing the *tubB*1 3' terminus in which the GUS gene was driven by the CaMV 35S promoter (Figure 4).

### *Analysis of the activity of the* tubB1 *promoter in transgenic tobacco*

A chimeric construct containing the promoterless GUS gene fused to a 2 kb fragment of the 5' upstream region of the *tubB*1 gene was introduced into tobacco by *Agrobacterium tumefaciens*-mediated Ti transformation. The chimeric *tubB*1–GUS constructs were cloned into the T-DNA of the *Agrobacterium* Ti plasmid, along with a selectable antibiotic resistance gene under the control of a generic plant active promoter, and transgenic tobacco plants were regenerated from cultured leaf disks after they were exposed to the bacteria. A total of 169 tobacco plants were regenerated. Mature leaves from these $R_0$ plants were tested for GUS activity with the fluorimetric assay. The level of GUS expression was highly variable and 40 of the regenerates exhibited no detectable GUS activity. Since the *tubB*1 gene in soybean was expressed most strongly in the seedling hypocotyl and only weakly in mature leaves, all regenerates were carried through to the next level of analysis in which the GUS activity of $R_1$ seedlings was determined. The $R_0$ plants were self-fertilized and the seeds produced were germinated in darkness on agar plates and tested for GUS activity with the histochemical assay 7 days after sowing. All $R_0$ plants that exhibited GUS activity in their mature leaves also exhibited GUS activity in the hypocotyls of some fraction of their $R_1$ progeny. In addition, the $R_1$ progeny of 21 of the 40 $R_0$ plants with GUS-negative leaves exhibited GUS activity in the seedling hypocotyls. Although a portion of the $R_1$ progeny of most of the regenerates exhibited GUS activity, only 60 $R_0$ plants produced $R_1$ seedlings that gave the 3:1 segregation ratio for GUS-expression to nonexpression that would be expected if the regenerates had received a single copy of the chimeric gene. Qualitatively, the pattern of expression of the chimeric gene in seedlings representing the $R_1$ generation, and in six cases also the $R_2$ generation, is very similar to the pattern of expression of the endogenous *tubB*1 gene in soybean seedling. Specifically, only the hypocotyls of etiolated seedlings stained an intense blue as a result of liberation of the blue indigo dye from the X-Gluc substrate by GUS enzyme. No GUS activity was observed in the roots or in cotyledons of 7-day-old etiolated transgenic seedlings. Because the GUS protein is stable, GUS enzyme levels could not be expected to reflect a rapid decrease in the levels of transcript of the chimeric gene in response to illumination, if this were to occur. Therefore, to determine the effects of

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GCATTAAATC ATTTTTATTA TTGAACTTGC GCATTAAAAC ACACGACAAC -921

AAAATGTTAA TTCTTCAAAT TATGTTATCG CTTATGCTAA AAAAAATGAA -871
                                          N.p.CAB identity
AAATAATGAT ATCGCTTAAG ATTCTCATTC ATAATTTCTC TTTTGAAAAA -821

ATAAGGGCAA ACAATCTTTC CAGCAGTTTC AGATATTCCA CGAATAAATG -771

CCAGTTAATT GGGCAATGTT GCGGTTTTTT AATTAGTTTT TTAAGGAAGA -721

CCAGGGATTC CCACACCATT ACCTAACTCT TGCTTTAGTG GCATGAAAAT -671

AGTTGTCTTG AGAAGGATAC ACTTTCAACA TGCTCAATAG GGACTTATAT -621
                        >Sd2
AACTATGGGC ATACATATTT TTTATCAATC ATTATAATCT GACTAATCTC -571
                AT-rich
TATACGATAT GCAGTCAAAG AGTTATTAAT TTTTATCATA AATTAAAAAC -521

TCAATTAATG TCACATAAAA ATTGGTCAAA ATTATAAACA ATCTGGGACT -471

AACTAATAAT TTTTGGAGAC CATTAAACTT AGTTTAACTT TATCTAATAT -421

ACTTATCCAC TACTCTGTCA CAAACCTAGA ACGTTAACAC CGTGTATAGT -371
      >Sd3
AAAGGAGGTG AAAAATAGTT GTCAAATCAA TCATTGTGGA GAGAAGCATG -321
        Box II
GTGAGCAAAC GGGGAAATGG ACAATTGGAG ATAGTATTGA CAGATGGGAT -271
                              I Box
ATCAAGTGGT AGGAAGAACT GATAATTCAG ATACATAAGG CCACAAGGCC -221
                    Box II
ATGAGAACAA TCATGCAACT TCTTGTCACT TTCCACGTCC AGCTCATTAC -171

ACTACTTGGA GTAAGACATA GCCCCTTCCC ATCAAACCCA CATGAACCTA -121

TTAATTGAAC TTCTATTCAT TTTTATTTTC CACTTGACAC AAAGAAAGGC -71
TATA Box                        +1'       >Sd4
AAGTACCATT TTTCTCAACC TTATCACCCT TTTGCATAAA AGGGCCACCC -21
                    LRE                 TATA BOX
CTTCTTCTTC TTCCCTTCTC AACTTGTACT ACAATTTCTG GATTGAATTA +30
                      +1
TCTTCAAAAT G
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**Figure 2.** Nucleotide sequence of the 5' region of the *tubB*1 gene.
The sequence of the first 1011 nt upstream from the start codon of the *tubB*1 gene is shown. This represents the Sd1 deletion from the original 2 kb of 5' upstream sequence. The positions of the additional deletions are indicated as >Sd2, >Sd3 and >Sd4; for the Sd2 deletion, the sequence is truncated at –648, while Sd3 is truncated at –411 and Sd4 is truncated at –80. The two sites of transcriptional initiation, as determined by primer extension, are indicated by +1 for the site nearest the ATG start codon and +1' for the more distal transcriptional start site. Putative TATA boxes and the translational start codon are indicated in bold and underlined, and regions exhibiting homology to known *cis*-acting elements or conserved sequences found in other light-regulated genes, as discussed in the text, are shown in bold. The nucleotide sequence is numbered from the first transcriptional start site.

light on the expression of the chimeric gene, the GUS histochemical staining patterns of etiolated and light-grown seedlings were compared. In contrast to the strong GUS expression observed in hypocotyls of etiolated seedlings, no GUS activity was found in hypocotyls of light-grown seedlings, while the cotyledons and leaves did exhibit GUS staining (Figure 5). GUS activity within the cotyledons of light-grown seedlings was most evident in the vascular tissues. The expression of the chimeric *tubB*1–GUS construct in older transgenic tobacco plants was confined largely to two structures of mature leaves, trichomes and vascular tissue, and there was some expression in the internal phloem of older stems (data not shown).

Qualitatively, a consistent pattern of GUS expression was found in etiolated transgenic seedling; when GUS activity was observed, it was confined to the hypocotyl. The pattern of tissue- and organ-specific GUS expression was more variable in light-grown plants, however. Approximately 11% of the transgenic plants lacked GUS expression in mature leaves, although the gene was expressed in etiolated seedling hypocotyls. Quantitatively, the levels of expression of the chimeric *tubB*1–GUS gene are highly variable among different $R_0$ transformants and among the progeny of the individual transformants. Southern analysis of genomic DNA from 10 $R_0$ plants demonstrated that regenerates exhibiting a 3:1 segregation ratio of GUS expression to nonexpression among their $R_1$ progeny had received a single copy of the *tubB*1–GUS gene. In contrast, $R_0$ plants in which all of the $R_1$ progeny expressed GUS (i.e. the $R_1$ seedlings exhibited a 1:0 ratio of GUS expression) received multiple copies of the chimeric gene (data not shown). There was considerably more variability in the observed levels of GUS expression among plants with a high chimeric gene copy number and in their progeny than among those with a single copy of the gene. GUS activity ranged from zero to 212 times background, with an average of 104 ± 16 ($n$ = 20) in the mature leaves of $R_0$ plants whose progeny exhibited a 1:0 GUS expression ratio. Conversely, $R_0$ plants whose progeny gave a 3:1 GUS segregation ratio ranged from zero to a 36-fold

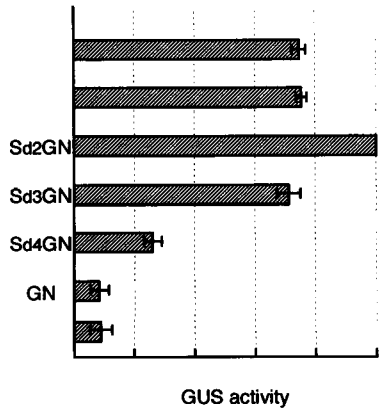


**Figure 3.** The expression in transient assays of chimeric GUS gene constructs is affected by the length of 5' *tubB*1 sequence.
Equivalent amounts of pBluescript plasmid DNA containing chimeric *tubB*1–GUS gene constructs were electroporated into BY-2 tobacco protoplasts with two electrical pulses, 1 sec apart, at a field strength of 800 V $cm^{-1}$ and a pulse duration of 150 μsec and GUS activity was determined 70 h later by means of a fluorescence assay (Jefferson, 1987). The constructs tested were SGN, 2 kb of *tubB*1 5' sequence fused to a promoterless GUS gene with a Ti nopaline synthase gene (NOS) 3' terminus; Sd1–4GN, where Sd1–4 refer to deletions of the 2 kb *tubB*1 5' upstream sequence, as noted in Figure 1, each fused to a promoterless GUS gene containing a NOS gene 3' terminus; and GN, a construct containing only the promoterless GUS gene with a NOS 3' terminus. These electroporations were repeated three times and the average expression for each construct is presented, along with the standard deviation of the mean.

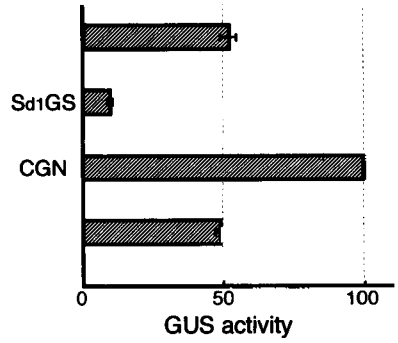


**Figure 4.** The effect of the 3' terminus on the expression of chimeric GUS gene constructs in transient assays.
Plasmid DNA containing different chimeric GUS gene constructs was electroporated into BY-2 tobacco protoplasts as described in the legend for Figure 3. GUS activity was determined 70 h after electroporation. The constructs were designated as shown in Figure 3, except that the chimeric genes contained either the 3' terminus of a NOS gene (N) or the 3' terminus of the *tubB*1 gene (S), and C indicates the CaMV 35S promoter. The electroporations were repeated three times and the average expression of each construct is presented, along with the standard deviation of the mean.

stimulation of GUS activity in mature leaves, with an average of 18 ± 20 ($n$ = 54). Seedlings from $R_0$ plants whose progeny gave a 3:1 ratio of GUS expression, exhibited approximately a twofold variation in the levels of GUS expression among those seedlings in which expression was observed (Figure 6a and b). In contrast, the $R_1$ progeny of an $R_0$ plant that contained multiple copies of the GUS gene exhibited expression levels that varied by a factor of approximately 10 among the expressing $R_1$ progeny (Figure 6c).

## Discussion

Previously we demonstrated that the *tubB*1 β-tubulin gene of soybean exhibits a complex pattern of developmental expression. The gene is expressed strongly only in elongating regions of seedling hypocotyls and then only when the seedlings are germinated in darkness. Furthermore, *tubB*1 transcripts rapidly disappear from the hypocotyls when etiolated seedlings are exposed to light (Han *et al.*, 1991). Although the gene is not expressed strongly in any of the tissues and organs of the adult plant tested, low levels of *tubB*1 gene transcripts are detected in mature leaves of light-grown soybean plants. The results presented here show that many aspects of this developmental expression pattern are controlled at the

**Figure 5.** Histochemical demonstration of GUS activity in hypocotyls of etiolated seedlings of transgenic tobacco containing a *tubB*1 promoter–GUS chimeric gene.
Seeds of a transgenic tobacco plant containing a chimeric *tubB*1–GUS gene were germinated and grown for 4 days either in darkness or under a 12 h photoperiod, and then stained for GUS activity using the chromogenic substrate X-Gluc. The blue color indicates GUS activity.

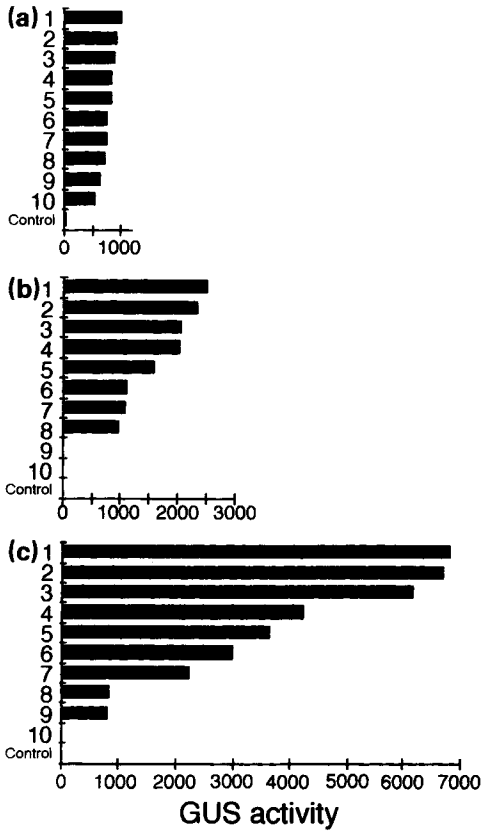


**Figure 6.** Variations in the GUS expression levels among progeny of different transformed tobacco plants.
Seeds obtained by selfing the $R_0$ transgenic plants were germinated on kanamycin-containing selection medium. Those that were able to grow and produce true leaves (kanamycin-resistant) were selected for transfer to soil. One month later the mature leaves of the same age from 10 progeny of each transformant were analyzed for GUS activity using the fluorescent assay. The graph plots GUS activity in the 10 randomly selected progeny from three of these transgenic plants as a function of plant number. (a) Transformant no. 8; (b) Transformant no. 1; (c) Transformant no. 4.

transcriptional level by *cis*-acting elements within the *tubB*1 gene itself. We demonstrate that a 1 kb 5' fragment of the *tubB*1 gene is able to drive the transient expression of a GUS reporter gene when the *tubB*1–GUS chimeric gene construct is electroporated into tobacco protoplasts. The level of transient GUS expression under the control of *tubB*1 putative promoter is nearly as great as that observed when the GUS gene is driven by CaMV 35S promoter when both contain the NOS 3' terminus. Furthermore, hypocotyls of tobacco plants that had been transformed with the chimeric *tubB*1–GUS construct exhibit strong GUS activity when the seedlings are dark-grown, but not if they are germinated in the light. Thus, the putative promoter region of the *tubB*1 gene alone is able to target the expression of the gene to the correct organ and mediate its regulation by light in this tissue.

The expression of many other plant genes is regulated by light. These include a number of nuclear genes encoding chloroplast proteins, such as chlorophyll *a*/*b* binding protein (*cab*) genes, and genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*S) (Dean *et al.*, 1989; Tobin and Silverthorne, 1985). *Cis*-acting elements have been identified in the 5' upstream regions of these genes that are responsible for the regulation of their expression (Gilmartin *et al.*, 1990; Kuhlemeier *et al.*, 1987). Furthermore, these regulatory elements will confer both light-regulation and tissue-specific expression on a reporter gene when they are fused to the truncated CaMV 35S promoter that drives the reporter (Lam and Chua, 1990). Some of these *cis*-acting elements have been shown to be binding sites for transcription factors necessary for the expression of these genes when plants are exposed to light (Gilmartin *et al.*, 1992; Green *et al.*, 1988; Perisic and Lam, 1992). The 5' upstream sequence of the *tubB*1 gene contains some sequences with homology to other conserved regulatory elements. Most striking is the region from –866 to –882 that contains a 17 bp sequence with 100% identity to a portion of a *Nicotiana plumbaginifolia cab* gene that was shown to contain a negative regulatory element (Castresana *et al.*, 1988). The *tubB*1 gene also contains an AT-rich sequence from –596 to –607 that is nearly identical to the consensus sequence for the AT-1 transcription factor binding site that is present in several *cab* and *rbc*S genes (Datta and Cashmore, 1989), and sequences at –45 to –52, –245 to –251, –283 to –293, and –353 to –363 are similar to the box II and I box regulatory elements found repeated in many light-regulated genes (Giuliano *et al.*, 1988; Kuhlemeier *et al.*, 1988). We are evaluating the significance of these regions for the regulation of *tubB*1 gene transcription.

The *tubB*1 gene differs from most light-regulated genes in that its expression in the hypocotyl is negatively regulated by light. A few other genes, including NADPH-protochlorophyllide oxidoreductase (Tomizawa *et al.*, 1989), asparagine synthase (Gilmartin *et al.*, 1990; Tsai and Coruzzi, 1990), the gene encoding phytochrome (Bruce and Quail, 1990; Bruce *et al.*, 1989) and genes represented by several cDNAs (Okubara and Tobin, 1991) are also negatively regulated by light and this regulation is mediated by the phytochrome system. Additionally, some dark-expressed genes in etiolated pea seedling, identified as cDNAs, are negatively regulated by blue light (Marrs and Kaufman, 1991; Warpeha *et al.*, 1989). The *cis*-acting elements responsible for the negative regulation of the transcription of these genes have not been fully characterized, but GT-1 binding sites have been identified in the phytochrome gene (Dehesh *et al.*, 1990; Kay *et al.*, 1989). Light also brings about the rapid loss of type-1 phytochrome and β-tubulin mRNA from etiolated tissues (Colbert *et al.*, 1990; Cotton *et al.*, 1990).

The light-induced downregulation of tubulin gene transcripts may be a general property of tubulin gene expression in etiolated stem tissue since illumination with either red or white light has been shown to result in the rapid loss of β-tubulin mRNA from etiolated *Avena,* barley and soybean seedlings (Bustos *et al.*, 1989; Colbert *et al.*, 1990). However, the mechanism responsible for the light-induced destabilization of β-tubulin transcripts has not been determined. It may be independent of the effects of light on the transcription of the *tubB*1 gene.

### *Transcriptional start*

Two different *tubB*1 transcriptional initiation sites were identified by primer extension. The sequences surrounding these transcriptional start sites are very similar to each other and to the consensus transcription start sites for plant genes, CTCATCA (Joshi, 1987). Similarly, the length of the transcribed, untranslated sequence in each case is consistent with other plant genes, as is the fact that the sequences of both leaders would be over 60% A + T (Joshi, 1987). Thus it is plausible that there are two different signals for transcriptional initiation within the *tubB*1 gene. There is a putative TATA box 29 bases upstream from the first transcriptional start site, while there is a second putative TATA box 25 bases upstream from the second site (Figure 1). However, the sequences of both of the two putative TATA boxes are significantly diverged from that of the consensus plant TATA box (Joshi, 1987), and these elements may not represent TATA boxes at all, although they could still represent binding sites for TFIID. The transcription factor complex TFIID contains a TATA box binding polypeptide and plays a key role in transcription because it nucleates the formation of the initiation complex (Pugh and Tjian, 1992). TFIID is also required for transcription from promoters lacking TATA boxes (Pugh and Tjian, 1991; Zhou *et al.*, 1992).

## Experimental procedures

### *Culture of BY-2 cells and protoplast isolation*

The BY-2 tobacco cell line was the generous gift of Dr Matsumoto, Japan Tobacco, Yokohama, Japan. The cells were cultured as previously described (Okada *et al.*, 1986). Protoplasts were isolated 3–4 days after the cells were subcultured to fresh medium by resuspending 4 ml of packed cells in 40 ml of a filter-sterilized solution containing 1% cellulase Onozuka RS (Yakult Honsha Co., Ltd, Tokyo), 0.5% macerozyme Onozuka R10 (Yakult Honsha Co.), 0.1% Pectolyase Y23 (Seishin Pharmaceutical Co., Ltd, Tokyo), 0.5% bovine serum albumin, 0.01% 2-mercaptoethanol, 50 mM $CaCl_2$, 10 mM sodium acetate, pH 5.8, and 0.25 M mannitol. Protoplasts were released after 2 h of gentle shaking in the wall-digestion solution, and isolated by filtration through a 60 μm mesh filter to remove cells with undigested walls.

### *Electroporation*

Protoplasts were collected by centrifugation at 50 ***g*** for 5 min. The protoplast pellet was washed once in the same solution without enzymes, then washed by seven additional resuspensions with fresh electroporation buffer solution (1 mM MES, pH 5.8, 2.5 mM $CaCl_2$, 0.225 M sorbitol and 0.225 M mannitol) after pelleting the protoplasts by centrifugation at 50 ***g*** for 5 min. The density of the washed, protoplast suspension was determined by counting and the density was adjusted with electroporation buffer. Electroporation was carried out at 4°C with a Shimadzu SSH-1 electroporator at a DNA concentration of 25 μg $ml^{-1}$ and a protoplast density of $2 \times 10^6$ protoplasts $ml^{-1}$. The protoplasts were kept at 4°C for 15 min after electroporation and then warmed to room temperature, after which the protoplasts were mixed first with an equal volume of electroporation buffer and then with an equivalent volume of 2× culture medium containing sorbitol plus mannitol and transferred to a 24-well microtiter plate which was sealed with Parafilm and incubated in the dark at 22°C. Electroporation conditions were optimized using a chimeric construct containing the GUS gene driven by 1 kb of 5' upstream sequence from the *tubB*1 gene. Maximum GUS activity was observed when the construct was electroporated into BY-2 protoplasts with a field strength of 800 V $cm^{-1}$ for 150 μsec. The GUS enzyme activity of the electroporated protoplasts increased with time after electroporation, reaching a maximum after 67 h and remaining constant for many hours thereafter. As a result, GUS expression levels were routinely determined 70 h after electroporation.

### *GUS enzyme assays*

Fluorimetric assays for GUS activity in tissue or cell homogenates were conducted by measuring the fluorescence of 4-methylumbelliferone formed as a result of GUS cleavage of 4-methylumbelliferyl-D-glucuronide, as described by Jefferson (1987). Samples were homogenized in a 50 mM phosphate buffer containing 10 mM EDTA, 0.1% Triton X-100, 0.1% lauroylsarcosine and 10 mM 2-mercaptoethanol and clarified by centrifugation. Aliquots of the supernatant were mixed with an equal volume of extraction buffer containing 0.7 mg $ml^{-1}$ 4-methylumbelliferone and the reaction was allowed to proceed for 1–20 h before it was terminated by the addition of 0.2 M $Na_2CO_3$. Fluorescence was determined with a Hoefer DNA fluorometer, Model TKO 100. Total protein of the homogenates was determined by the Bio-Rad protein assay using the manufacturer's suggested procedure. Histochemical assays for GUS were conducted by a modification of the procedure of McCabe *et al.* (1988). Whole seedlings or organ fragments were vacuum infiltrated with a solution containing 0.5 mg $ml^{-1}$ X-Gluc in 100 mM phosphate buffer (pH 7.5). After incubation overnight in the staining solution, the seedlings were treated with 70% ethanol and photographed.

### *Plasmid constructions*

Standard recombinant DNA methods were used (Sambrook *et al.*, 1989). A λ clone designated MG-2 was shown by restriction analysis and sequencing to contain an 8.1 kb insert of soybean genomic DNA bearing a β-tubulin gene originally designated sβ1 (Guiltinan *et al.*, 1987). The name of the gene was changed to *tubB*1 in this paper to conform to accepted nomenclature for plant genes. A 2.4 kb *XbaI-XhoI* fragment of MG-2 containing the 5' terminus of the *tubB*1 gene and the proximal 2 kb of up-

stream sequence was subcloned into a pUC19 vector. Since there were no convenient restriction sites near the ATG translational start codon, to which the GUS fusions could be made, the clone was opened with *PstI* and a timed sequence of digestions was made with *Bal*31 nuclease. These were cloned into pUC19 and screened to identify inserts with a probability of containing fragments from which the β-tubulin coding sequence was eliminated. Prospective clones were sequenced to determine the precise extent of the deletion and one clone, designated pSB1BP3, which terminated 4 bp 5' from the ATG translational start codon, was selected for fusion to the reporter gene. The insert in pSB1BP3, which contained 2 kb of 5' upstream sequence from the *tubB*1 gene, was inserted into the binary *Agrobacterium* Ti vector pBI101, containing a promoterless *Escherichia coli* GUS gene (Jefferson *et al.*, 1986), to create the plasmid pSB1GUS. The structure of the chimeric *tubB*1–GUS gene was verified by restriction analysis and sequencing.

Plasmids for electroporation and transient expression were derived from pSB1GUS. The entire *tubB*1–GUS chimeric gene was cut from the pSB1GUS plasmid and ligated to pUC19. A series of five 5' deletions from the initial 2 kb of *tubB*1 5' sequence was generated by digestion with exonuclease III. These were then ligated into pGEM (Promega) and pBluescript (Stratagene) vectors and the extent of each of the deletions was determined by sequencing. The extent of each of the deletions is shown in Figure 2.

### *Plant transformation*

The chimeric *tubB*1–GUS gene in the pBI101-derived plasmid pSB1GUS was transferred into *Agrobacterium tumefaciens* LBA4404 by triparental conjugation, using the helper plasmid pRK2013 (Rogers *et al.*, 1986). The SR1 strain of *Nicotiana tabacum* was transformed by the leaf disk method and plants were regenerated from the transformed leaf cells (Horsch *et al.*, 1985).

### *Primer extension*

An 18 mer synthetic oligonucleotide with the sequence 5'-TAATGCATCCATTGAAAG-3', representing the region from position 533 to 550 of the genomic clone MG-2 containing the *tubB*1 gene (Guiltinan *et al.*, 1987), was end-labeled with T4 polynucleotide kinase (Promega) and [$\gamma$-$^{32}$P]ATP (Amersham). Total RNA (50 μg) from 7-day-old light- or dark-grown soybean seedlings was denatured at 80°C for 5 min with 1.0 pmol of the radiolabeled oligonucleotide primer in 10 mM Pipes buffer (pH 6.4) containing 0.4 mM NaCl, and then hybridized for 3 h at 37°C. The RNA–primer hybrid was precipitated with ethanol and resuspended in a 50 mM Tris–HCl buffer (pH 8.5) containing 30 U reverse transcriptase (AVM), 10 mM dNTPs, 60 mM KCl, 10 mM $MgCl_2$ and 20 U RNasin (Promega). The reaction was stopped with EDTA, digested with RNase, phenol/chloroform treated, and the product was analyzed on a polyacrylamide sequencing gel.

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