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Light Regulation of β-Tubulin Gene Expression during Internode Development in Soybean (*Glycine max* [L.] Merr.)[1]

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ABSTRACT

The relationship between tubulin gene expression and cell elongation was explored in developing internodes of *Glycine max* (L.) Merr., using light as a variable to alter the rate of elongation. First internodes of etiolated seedlings elongated two to three times more rapidly than did those of seedlings growing under a 12 hour diurnal light/dark cycle. Furthermore, light slowed or completely halted internode elongation in the etiolated seedlings, depending upon the age of the seedlings at the time of the light treatment. Steady state levels of β-tubulin mRNA were determined in Northern blots and by solution hybridization of poly(A)$^+$RNA with a probe derived from the coding region of a previously characterized soybean β-tubulin gene. (MJ Guiltinan, DP Ma, RF Barker, MM Bustos, RJ Cyr, R Yadegari, DE Fosket [1987] Plant Mol Biol 10: 171-184). Internodes of light-grown seedlings exhibited levels of β-tubulin mRNA that differed by a factor of three, and varied concomitantly with the elongation rate. Illumination of 10-day-old etiolated seedlings not only stopped first internode elongation, but also brought about a 80% decrease in the steady state level of β-tubulin mRNA over the course of the subsequent 12 hours. This strong down regulation of β-tubulin mRNA occurred without significant changes in the size of the soluble tubulin pool and it was accompanied by a marked increase in chlorophyll *a*/*b* binding protein mRNA.

Microtubules are formed by the regulated self-assembly of the heterodimeric protein tubulin from a soluble tubulin dimer pool, and cytoplasmic microtubules are in a dynamic equilibrium with this tubulin dimer pool (18). Since microtubules can form and persist only when the tubulin dimer concentration exceeds a critical value, maintenance of the tubulin dimer pool at a level above this critical concentration is essential for microtubule formation. Where it has been examined, tubulin synthesis is regulated by a mechanism that is sensitive to changes in the amount of assembled microtubules. In cultured mammalian cells, tubulin synthesis is autoregulated by a post-transcriptional mechanism that is responsive to the size of the tubulin dimer pool (5). In other cases, the tubulin dimer pool is maintained through both transcriptional and post-transcriptional control of tubulin gene expression (1, 17). Little is known about the regulation of tubulin synthesis in plants, but Cyr *et al.* (8) observed changes of several fold in the levels of tubulin protein and mRNA during carrot somatic embryogenesis. Furthermore, Fukuda (11) reported that the rate of tubulin synthesis increased during the formation of cortical microtubules in differentiating tracheary elements. The involvement of cortical microtubules in cell elongation and the increase in tubulin levels and in microtubule assembly which has been shown to accompany cell elongation (9, 29), make elongating tissues attractive as systems for investigating the regulation of tubulin synthesis in higher plants. Here, we show that changes in β-tubulin mRNA steady state levels and internode elongation rates vary concomitantly in light-grown and etiolated soybean seedlings. Internodes of etiolated seedlings exhibited elongation rates that were four to five fold greater than those observed in control seedlings grown under a 12 h light/dark cycle. The rapidly elongating internodes of dark-grown seedlings exhibited proportionally higher steady-state levels of β-tubulin mRNA. Furthermore, subsequent illumination of the dark-grown seedlings brought about a reduction in the rate of elongation and a concomitant decrease in the steady-state level of β-tubulin mRNA.

MATERIALS AND METHODS

Plant Material and Tissue Preparation

Seeds of *Glycine max* [L.] Merr. cv Mitchell (a gift of Ring Around Research Center, Hale Center, Texas), were germinated in moist vermiculite, and seedlings were grown in a controlled environment chamber under a 29°C/24°C, light/dark cycle, with a 12 h photoperiod. Illumination was provided by a combination of fluorescent and incandescent lights. Seedlings were watered with a one quarter strength nutrient solution (24). Dark-grown seedlings were subjected to the same diurnal temperature cycle, but were grown in complete darkness, except during handling when they were exposed to a dim, green safe light, with an emission maximum between 500 and 525 nm. All plant materials were dissected and immediately frozen in liquid nitrogen prior to storage at −70°C, except tissues harvested for tubulin protein determinations which were analyzed immediately without freezing.

Construction of β-Tubulin Coding Sequence Probe

A soybean genomic clone containing the functional β-tubulin gene designated SB1 was the source of the tubulin

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probe used in this study (14, 15). A 1.6 kb *Xho*I-*Bam*HI fragment contained entirely within the transcription unit of the SB1 gene, was subcloned into the vector pSP65 (21) and labeled either by nick-translation, or transcribed *in vitro* using SP6 RNA polymerase to give the probe designated SB1-CD.

RNA Extraction and Analysis

Total cellular RNA was isolated by a phenol-chloroform extraction procedure as previously described (8). Poly(A)$^+$RNA was prepared by selection on oligo(dT)-cellulose (Collaborative Research, type III) by binding at 20°C and eluting at 42°C. Northern analysis was conducted using standard methods (19). After prehybridization the blots were hybridized to either nick-translated DNA probes, or to *in vitro* transcribed, antisense RNA probes in 50% formamide +4 × SSC + 4× Denhardt's solution (see ref. 19 for the composition of SSC and Denhadt's solutions), with 100 μg/mL denatured salmon sperm DNA and 50 μg/mL yeast tRNA, for 18 to 24 h, at the temperature indicated in the figure legends, and then washed with three changes of 1 × SSC + 0.1% SDS at the hybridization temperature for 1.5 h. When RNA probes were used, the blots were washed an additional time in 0.1 × SSC + 0.1% SDS. Autoradiographs were made using Kodak AR X-ray film, at −70°C, with Cronex Plus (DuPont) intensifying screens. The use of RNA probes reduced the required exposure times and increased the sensitivity of the analysis by a factor of ten. The RNA fractions also were analyzed by solution hybridization in the presence of a large excess of radiolabeled probe, essentially using the protocol of Melton *et al.* (21). The hybridization of the ^{32}P-labeled antisense RNA SB1-CD probe (1–2 × 10^5 cpm) with poly(A)$^+$RNA was conducted in 40 mM Pipes (pH 6.7), 80% formamide, 0.4 M NaCl and 1 mM EDTA at 85°C for 5 min, followed by incubation at 45°C for 16 to 36 h. The β-tubulin mRNA content of the tissues was determined by extrapolation from a standard curve prepared by hybridizing the labeled SB1-CD probe with known quantities of unlabeled, sense-strand SB1-CD RNA synthesized *in vitro* from the SB1-CD DNA cloned in a pSP64 vector.

Protein Extraction and Radioimmunoassay

Fresh internode tissues were homogenized in a glass-teflon tissue homogenizer in tubulin extraction buffer (8), but without added calcium. The homogenates were centrifuged at 48,000*g* for 10 min, and their protein concentrations were determined by the Bradford (4) method. Tubulin was quantified by a direct-binding, dot-blot immunoassay, using a polyclonal antiserum raised against taxol-assembled soybean tubulin, and ^{125}I-labeled protein A (8). A standard curve was obtained with purified soybean tubulin which exhibited a correlation coefficient of 0.996 in the linear binding region. After background subtraction, soluble and tubulin levels of the experimental samples were computed by linear regression analysis using the standard binding data.

Recombinant DNA Techniques

All recombinant DNA techniques and nucleic acid hybridizations not described above were performed according to Maniatis *et al.* (19).

RESULTS

Internode Elongation in Etiolated and Light-Grown Seedlings

Biometric analysis was used to characterize the elongation of the soybean first internode in etiolated seedlings and in seedlings growing under a 12 h light/dark cycle, and to characterize the action of light on internode elongation in etiolated seedlings. The first internode of light-grown seedlings exhibited sigmoidal growth. The elongation rates increased progressively from the point at which measurements were first made (four days after sowing), reached a maximum of 15 mm/d between eight and nine days after sowing, and declined thereafter (Fig. 1A). Internode extension was not isometric, however, and the lower half of the internodes ceased growth between the eighth and ninth day after sowing, whereas the upper portion continued to elongate for another three to five days (Fig. 1A). Internode elongation was greater in the etiolated seedlings (Fig. 1B). Exposure to light reduced the growth rate of etiolated internodes to that of the light-grown seedlings, or halted it altogether, depending on when the dark-grown seedlings were illuminated (Fig. 1B). The most rapid light-induced inhibition of growth occurred when seedlings were illuminated after nine days in the dark.

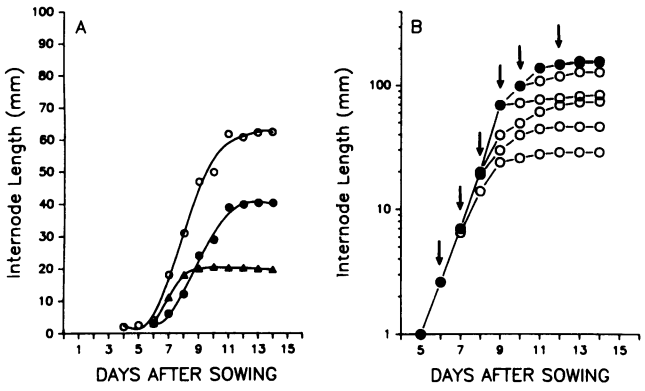

Figure 1. Growth kinetics of soybean first internodes in the light and in darkness. A, The growth kinetics of the first internodes of light-grown soybean seedlings were determined. Four days after sowing, transverse marks were made with India ink at 1 mm intervals on seedlings growing under a 12 h light/dark cycle. The distance between the marks was determined each day during the subsequent 11 d and the change in length was plotted against time for the entire internode (O——O) and for the lower (▲——▲) and upper (●——●) halves of the internode. B, Light inhibition of first internode elongation in etiolated soybean seedlings. Groups of 10 to 15 seedlings were germinated in total darkness. At intervals thereafter groups of plants were transferred to another growth chamber with the same temperature cycle, but with a 12 h light/dark cycle. The plants were transferred at the beginning of a light cycle and internode length was measured at daily intervals thereafter. Each point is an average of ten measurements.

Levels of Tubulin mRNA are Modulated During Internode Growth

Northern blot and solution hybridization of poly(A)$^+$RNA isolated from first internodes of light-grown seedlings at different stages of their growth cycle, with the β-tubulin coding sequence probe (SB1-CD), demonstrated that changes in steady state levels of β-tubulin mRNA and in elongation rates paralleled each other in their timing (Fig. 2). The peak elongation rate was observed on the 9th d after sowing, at which time these tissues exhibited their highest level of β-tubulin mRNA. Both the elongation rate and the β-tubulin mRNA level declined after d 9. The lowest and highest measured values for the elongation rate differed by a factor of two, and for β-tubulin mRNA levels by a factor of three.

Light Reduced Tubulin mRNA Levels in Internodal Tissues

The dramatic inhibition of etiolated internode growth by light provided an experimental system to test the hypothesis that elongation rate influences tubulin gene expression. Seedlings were germinated and grown in the dark, and illuminated at d 10 for an additional 2 d. Northern analysis of total cellular RNA or poly(A)$^+$RNA with either the β-tubulin SB1-CD probe or with a Cab (Chl *a/b* binding protein) probe demonstrated that light brought about a drastic decrease in the steady state level of β-tubulin mRNA (compare lanes 1 and 2 on Fig. 3A) and a substantial increase in Cab mRNA (lanes 1 and 2, Fig. 3B). These two genes are regulated in opposite ways during the dark-to-light transition, but both are expressed in light-grown plants (lane 3, Fig. 3, A and B).

Time Course of the Light Induced Decrease in β-Tubulin mRNA Levels

Changes in steady state levels of β-tubulin mRNA and tubulin protein were determined as a function of time after exposure to light. Seedlings germinated and grown 10 d in the dark were illuminated. RNA and soluble proteins were extracted from samples taken at intervals thereafter and analyzed as described in "Materials and Methods." The β-tubulin mRNA level began to decrease as soon as the plants were

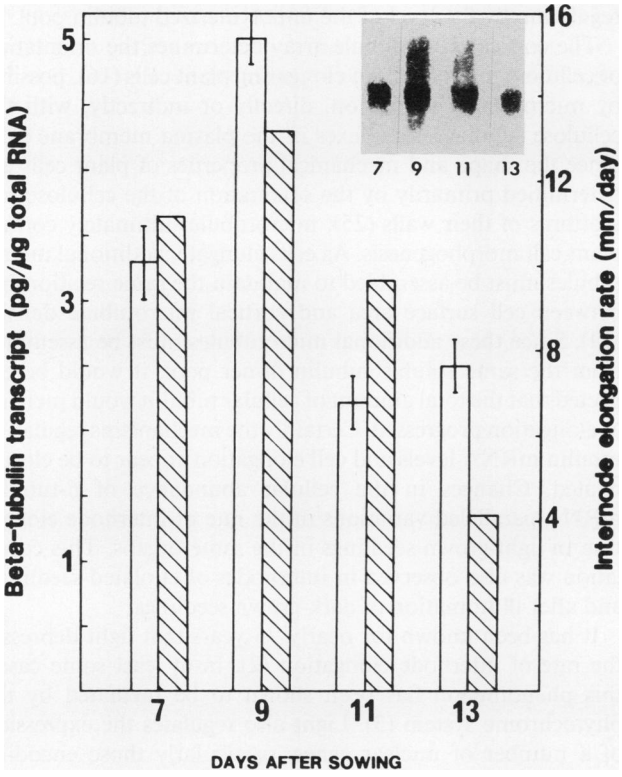

Figure 2. Steady state levels of β-tubulin mRNA and elongation rates of soybean first internodes. Soybean seedlings were germinated under a 12 h light/dark cycle. Elongation rates were determined from internode length measurements made at daily intervals on 15 seedlings. The mean elongation rate for each time point is presented as an *open bar*, together with the standard error of the mean. Quantitative determinations of β-tubulin mRNA levels, represented by the *cross-hatched bars*, were done by solution hybridization (see "Materials and Methods"), from first internode harvested at 2 d intervals. A Northern blot from the same samples is shown in the upper right corner.

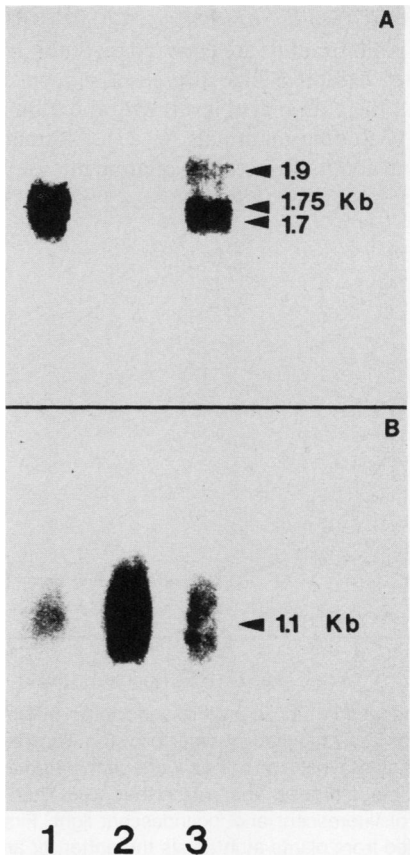

Figure 3. Northern analysis of poly(A)$^+$ RNA from soybean first internodes grown under various light conditions. Gel blots of poly(A)$^+$ RNA prepared from seedlings grown under three different conditions were hybridized with the soybean β-tubulin coding sequence RNA probe, SB1-CD (A), or with a nick-translated 1.1 kb *Hin*dIII fragment from the plasmid pCab 1.1 Chl *a/b* binding protein probe (B). The growth conditions were: 10 d in continuous darkness (lane 1); 10 d in continuous darkness plus an additional 48 h with continuous light (lane 2); 12 d under a 12 h. light/dark cycle (lane 3).

exposed to light. The decrease was exponential during the initial postillumination period, with an apparent half-life of 3.5 h. The minimum value was attained 12 h after the onset of illumination, after which no further change occurred (Fig. 4). In contrast with the rapid down-regulation of tubulin mRNA, the amount of soluble tubulin protein remained constant during the first 18 h postillumination, and only began a modest decrease between 18 and 24 h after exposure to light (Fig. 4). Hybridization with the Cab probe showed, as expected, that light induction of the Chl *a/b* binding protein mRNA was rapid, and it reached a maximum level within six h after the start of illumination (Fig. 3).

DISCUSSION

Higher plant tubulin has been isolated and partially characterized (22). Antibodies raised against SDS-PAGE purified plant tubulin monomers, as well as ligand binding studies, have shown that plant tubulins have some unique biochemical properties, but share many of the characteristics of the better characterized vertebrate tubulins (10). Analysis of the sequences of β-tubulin genomic clones isolated from soybean (15) and from *Arabidopsis thaliana* (20, 23) have demonstrated that plant tubulins are encoded by multi-gene families, and that they exhibit a high degree of sequence homology (over 80% at the amino acid level) with the tubulins of other eukaryotes, including mammals (6, 27). Although relatively little is known about the factors regulating microtubule assembly in higher plant cells, given the high degree of similarity between eukaryotic tubulins, it is likely that the assembly process will be similar to that observed in mammalian cells where the assembled microtubules have been shown to be in a dynamic equilibrium with a pool of soluble tubulin dimers (18). In cultured mammalian cells tubulin synthesis is autoregulated by a mechanism that is sensitive to the size of the soluble tubulin dimer pool (5). As a result, factors that stimulate or inhibit microtubule assembly change the amount of tubulin in the soluble tubulin dimer pool, and thereby alter the rate of tubulin synthesis. This autoregulatory mechanism is post-transcriptional and is triggered by the first four codons of the tubulin mRNA (12). Tubulin synthesis in *Chlamydomonas* also has been shown to be closely coordinated with microtubule assembly (30), but in this organism the regulation is both transcriptional and post-transcriptional (1, 17). The experiments described here do not allow us to distinguish between transcriptional and post-transcriptional events in the control of tubulin gene expression. However, the observation that the amount of soluble, unpolymerized tubulin present in soybean internodes remains unchanged during the initial 18 h postillumination period is not consistent with the notion that the decrease in tubulin mRNA is a result of feedback regulation by the level of the unpolymerized tubulin pool.

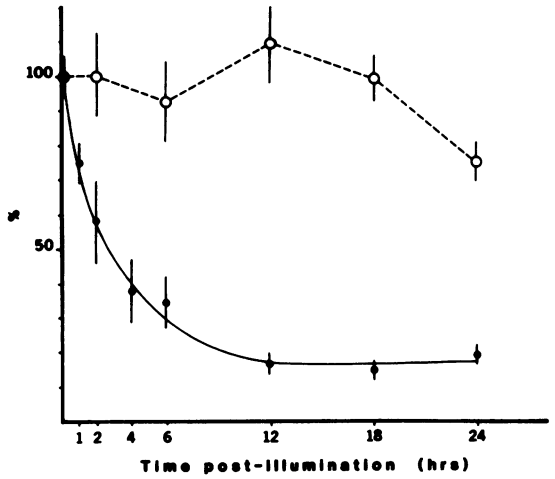

Figure 4. Changes in tubulin protein and tubulin mRNA in soybean first internodes as a function of time following illumination of dark-grown seedlings. Soybean seedlings were germinated and grown for 10 d in complete darkness, after which they were illuminated with a combination of fluorescent and incandescent light. First internodes were harvested from plants at intervals thereafter for analysis. Total soluble protein was determined by the Bradford (4) method while soluble tubulin protein content was determined by means of a solid state radioimmunoassay (8). The tubulin protein content (O—O) of the tissue was determined at each time point and is expressed as a percentage of the value found at the start of illumination. The β-tubulin mRNA (●—●) was determined by solution hybridization of extracted poly(A)$^+$ RNA with the SB1-CD RNA probe and also is expressed as a percentage of the level observed at the start of illumination.

The cortical microtubule array determines the orientation of cellulose microfibrils in elongating plant cells (16), possibly by microtubule interaction, directly or indirectly, with the cellulose synthase complexes in the plasma membrane (13). Since the shape and mechanical properties of plant cells are determined primarily by the orientation of the cellulose microfibrils of their walls (25), microtubules ultimately control plant cell morphogenesis. As cells elongate, additional microtubules must be assembled to maintain the same relationship between cell surface area and cortical microtubule density (29). Since these additional microtubules must be assembled from the same soluble tubulin dimer pool, it would be expected that the total amount of cellular tubulin would increase as elongation progressed. Certainly the mechanisms regulating tubulin mRNA levels and cell elongation appear to be closely related. Changes in the cellular abundance of β-tubulin mRNA paralleled variations in the rate of internode elongation in light-grown seedlings in the same organs. This correlation was also observed in internodes of etiolated seedlings, and after illumination of dark-grown seedlings.

It has been known for nearly 75 years that light depresses the rate of internode elongation (2). In at least some cases, this phenomenon has been shown to be mediated by the phytochrome system (3). Light also regulates the expression of a number of nuclear genes, particularly those encoding chloroplast proteins (28). For example, the genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean are positively regulated at the transcriptional level by light and this effect is mediated by the phytochrome system (26). In most cases light leads to enhanced gene expression, rather than the down regulation exhibited by the β-tubulin genes in the experiments described here. There are a few exceptions to this, the most remarkable of which is the phytochrome gene itself which is autoregulated (7). Red or white light treatment of etiolated oat seedlings has been shown to

bring about a 90% decrease in phytochrome mRNA abundance over a 5 h period (7). The rate at which tubulin mRNA disappeared in the etiolated tissues after illumination was comparable to the rate of decrease of transcripts of light-regulated genes such as the small subunit of ribulose-1,5 biphosphate carboxylase, after light grown soybean plants are placed in the dark (26). The observed induction of Cab mRNA expression, argues against the possibility of the down-regulation of tubulin mRNA being a consequence of an overall shut-down of RNA transcription or a generalized reduction in mRNA stabilities. It will be of interest to see if the light induced down regulations of tubulin and phytochrome utilize the same mechanism.

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