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Phytochrome A and Phytochrome B Mediate the Hypocotyl-Specific Downregulation of *TUB1* by Light in Arabidopsis

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Arabidopsis contains six α-tubulin and nine β-tubulin genes that are expressed in a tissue-specific and developmentally regulated manner. We analyzed the effects of light on tubulin mRNA abundance in Arabidopsis seedlings using RNA gel blot hybridizations and gene-specific probes. Transcript levels of all 15 tubulin genes were decreased by continuous white light, although to different degrees. Detailed analysis was performed with the β-tubulin *TUB1* gene. The transcript level of *TUB1* was high in etiolated seedlings and decreased to ~20% of the dark mRNA level after 2 to 6 hr of white light treatment. We showed that this downregulation requires high-irradiance light treatment and that multiple photoreceptors are involved. In particular, using phytochrome mutants and narrow wave band light, we demonstrated that both the phytochrome A (phyA)-mediated far-red light high-irradiance response and the phytochrome B (phyB)-mediated red light high-irradiance response are involved in the downregulation of *TUB1* expression by white light. Histochemical analysis of transgenic plants expressing a *TUB1*-β-glucuronidase chimeric transgene indicated that the downregulation observed only in hypocotyls and not in roots is controlled transcriptionally.

INTRODUCTION

Microtubules (MTs), consisting of heterodimers of α-tubulin and β-tubulin and MT-associated proteins, participate in numerous aspects of plant growth and development. In nondividing plant cells, the cortical array of MTs present beneath the plasma membrane is involved in guiding the deposition of cellulose microfibrils in the cell wall. Because the shape of a plant cell is determined primarily by the orientation of the cellulose microfibrils, MTs play a major, albeit indirect, role in cell elongation and expansion, thereby affecting plant cell morphogenesis (Fosket and Morejohn, 1992; Goddard et al., 1994).

Cortical MTs are usually arranged in a helical pattern, with their predominant orientation perpendicular to the direction of cell elongation. Many investigators have shown that realignment of cortical arrays can be induced by a wide range of factors, such as light, plant hormones, gravity, or wounding (reviewed in Shibaoka, 1992, 1994; Cyr, 1994). In addition, the number of MTs can be concomitantly adjusted to allow for cell elongation or lateral cell enlargement during plant development. The levels of MTs can be regulated by both transcriptional and post-transcriptional processes (Bachurski et al., 1994; Davies and Grossman, 1994). Although in mammalian cells the steady state levels of α-tubulin and β-tubulin mRNAs are mainly autoregulated post-transcriptionally in response to changes in the concentration of unassembled tubulin subunits, it is not yet clear whether mRNA levels of α-tubulin and β-tubulin in plants are similarly regulated.

Although both α-tubulin and β-tubulin are encoded by multigene families in most organisms, plants seem to contain more expressed tubulin genes than do animals (Goddard et al., 1994). Tubulin isotypes have been reported in many plants, including soybean (Guiltinan et al., 1987), maize (Villemur et al., 1994), cotton (Dixon et al., 1994), pea (Brierley et al., 1995), rice (Kang et al., 1994), potato (Taylor et al., 1994), and Arabidopsis (Kopczak et al., 1992; Snustad et al., 1992). It is striking that the small genome of Arabidopsis contains at least six expressed α-tubulin (*TUA*) genes and nine expressed β-tubulin (*TUB*) genes. All 15 Arabidopsis tubulin genes have been cloned and sequenced, and the expression patterns of some of them have been characterized (Kopczak et al., 1992; Snustad et al., 1992). For example, the *TUA1* gene, which encodes the most divergent α-tubulin isotype in Arabidopsis, is expressed almost exclusively in stamens and mature pollen but barely in root hairs or pollen tubes (Carpenter et al., 1992). By contrast, in light-grown Arabidopsis, *TUB1* mRNAs accumulate predominantly in roots, with low levels in flowers and barely detectable levels in leaves (Oppenheimer et al., 1988).

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In etiolated Arabidopsis seedlings, hypocotyl elongation is inhibited by white light (W) as well as by far-red light (FR) and red light (R) (Reed et al., 1994). Analysis of mutants lacking specific functional photoreceptors has demonstrated that both the phytochrome A and B (phyA and phyB) photoreceptors can mediate inhibition of hypocotyl elongation (Casal, 1995; reviewed in Smith, 1995). As yet, there is no molecular marker that can be correlated with this photoreceptor-mediated inhibition. Previous work with other plants has indicated a correlation between the downregulation of tubulin transcript levels and the inhibition of hypocotyl elongation by white light, although the photoreceptors involved have not been identified (Bustos et al., 1989; Colbert et al., 1990; Han et al., 1991). Therefore, we investigated whether there is a similar downregulation of Arabidopsis tubulin gene expression by light. In particular, we addressed the following: (1) which tubulin genes in Arabidopsis are downregulated by light; (2) the light requirements for downregulation; (3) which photoreceptors are involved in downregulation; and (4) whether genes are transcriptionally regulated.

RESULTS

Downregulation of Tubulin Genes by Light

The surprising fact that the genome of Arabidopsis contains six α-tubulin and nine β-tubulin genes (Kopczak et al., 1992; Snustad et al., 1992) raises the question of whether these genes are differentially regulated by light that affects plant cell elongation. To address this question, total RNA isolated from etiolated and light-treated 2-day-old seedlings (2 days after germination) was analyzed. Figure 1 shows typical RNA gel blot results obtained using each of the 15 tubulin gene–specific probes derived from their 3′ noncoding regions (see Methods). The results demonstrate that the mRNA levels of all 15 tubulin genes were decreased, although to varying degrees, in 24 hr of continuous W light (Wc)–illuminated seedlings as compared with etiolated seedlings. After a quantitative analysis using a PhosphorImager, three classes of light-downregulated responses were empirically defined. Figure 1 shows that mRNA levels of *TUA3*, *TUA6*, *TUB1*, and *TUB4* were the most severely decreased (60 to 80% decrease); *TUA2*, *TUA4*, *TUA5*, *TUB5*, *TUB6*, and *TUB9* transcript levels were modestly decreased (~40% decrease), and mRNA levels of *TUA1*, *TUB2*, *TUB3*, *TUB7*, and *TUB8* were only slightly decreased (10 to 20% decrease).

Oppenheimer et al. (1988) reported previously that *TUB1* mRNA accumulates preferentially in root tissues of adult Arabidopsis plants. In our study, however, we found that *TUB1* transcripts are also abundant in etiolated young seedlings and are strongly downregulated by light (~80% decrease). We considered the possibility that the *TUB1* gene responds differently to light in different organs or at various developmental stages. Therefore, among the four tubulin genes that are strongly downregulated by light (*TUA3*, *TUA6*, *TUB1*, and *TUB4*; Figure 1), *TUB1* was chosen for further analyses.

Kinetics of *TUB1* Downregulation by Light

Because mRNA levels of *TUB1* decreased markedly after 24 hr of Wc (Figure 1), RNA was analyzed by gel blot hybridization over a period of 24 hr. Figure 2 shows that during 2 to 6 hr of light treatment, *TUB1* mRNA levels decreased rapidly (~80% decrease). These new levels decreased only slightly upon extended light treatment. In general, control seedlings grown in the dark for an additional 24 hr also showed some decrease in *TUB1* mRNA abundance (~30% decrease). This decrease may have been due to developmental downregulation of the *TUB1* gene (see below). In the experiment shown in Figure 2, *TUB1* mRNA levels at 0.5 hr of light treatment appeared to be reduced as compared with those at 1 and 2 hr.

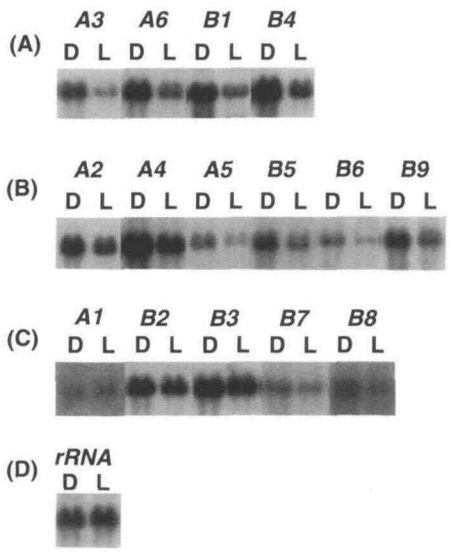

Figure 1. RNA Gel Blot Analysis of Tubulin mRNA Accumulation in Etiolated and Deetiolated Arabidopsis Seedlings.

One-day-old etiolated seedlings were either exposed to Wc (L) or kept in the dark (D) for 24 hr. Equal amounts of total RNA (10 μg) were loaded in each lane, and 16 identical blots were hybridized to 15 tubulin gene–specific probes (*TUA1* to *TUA6* and *TUB1* to *TUB9*) or to the 18S rDNA probe as a control. Blots were exposed overnight for autoradiography, except in the case of *TUA1* and *TUB8* probes, for which blots were exposed for 7 days. The levels of tubulin mRNA were measured using a PhosphorImager, and three classes of responses were empirically defined.

(A) A 60 to 80% decrease for *TUA3*, *TUA6*, *TUB1*, and *TUB4* (*A3*, *A6*, *B1*, and *B4*, respectively).

(B) A 40% decrease for *TUA2*, *TUA4*, *TUA5*, *TUB5*, *TUB6*, and *TUB9* (*A2*, *A4*, *A5*, *B5*, *B6*, and *B9*, respectively).

(C) A 10 to 20% decrease for *TUA1*, *TUB2*, *TUB3*, *TUB7*, and *TUB8*.

(D) rRNA control.

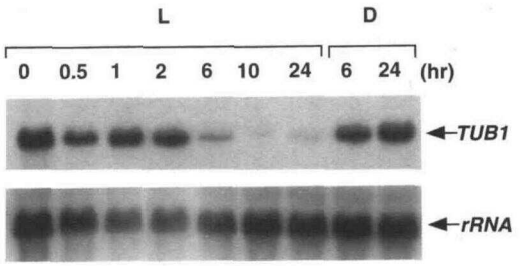

Figure 2. Time Course of Light Downregulation of *TUB1* Gene Expression.

RNA was extracted from 1-day-old seedlings (0 hr) or seedlings after Wc treatment (0.5, 1, 2, 6, 10, or 24 hr; L). Control RNA from seedlings that were kept in the dark (D) for 6 or 24 hr are shown at right. The blot was rehybridized with the 18S rDNA for loading controls. Each lane contains 10 μg of RNA.

However, this decrease was not detected in four other experiments, in which the *TUB1* mRNA levels remained relatively steady during the first 2 hr of light treatment.

Downregulation of *TUB1* Requires Continuous Illumination

To examine whether the downregulation of *TUB1* mRNA levels induced by Wc (Figures 1 and 2) is mediated by phytochromes, we first performed a typical R/FR reversibility test. Figure 3 shows that an R pulse is not sufficient to downregulate the *TUB1* gene. By contrast, the *CAB* gene (which encodes the chlorophyll *a/b* binding protein) is induced by the same R pulse treatment, and this induction can be reversed by a subsequent FR treatment. Whereas *CAB* expression is a well-characterized low-fluence response mediated by phytochromes (Thompson and White, 1991), it is evident that the downregulation of *TUB1* expression in Arabidopsis by light, if mediated by phytochromes, cannot be brought about by such a low-fluence R pulse (total fluence of ~2000 μmol m^{-2} sec^{-1}). Multiple (three or six) R pulses given during a 6-hr time period were still ineffective in reducing *TUB1* mRNA levels (data not shown). By contrast, a saturating R pulse induces 40 and 55% decreases in β-tubulin mRNA levels in oats and barley, respectively (Colbert et al., 1990). We concluded that the downregulation of *TUB1* by light in Arabidopsis requires a high-irradiance treatment. Therefore, continuous illumination was used in all subsequent experiments in this study.

Continuous R (Rc) and FR (FRc) treatments were provided separately to test their effects on *TUB1* downregulation in etiolated seedlings of wild-type Arabidopsis (Landsberg *erecta*). In a time course analysis, we found that *TUB1* mRNA levels were decreased after 6 hr of treatment with either Rc or FRc (data not shown). Wc appeared to be more effective than Rc or FRc alone; this may have been due to the higher fluence rate of Wc used in our study (see Methods). Because FRc is thought to activate primarily phyA and Rc to activate primarily phyB and other stable phytochromes, our results suggest that both phyA and phyB may be involved in regulating *TUB1* expression. To obtain additional evidence on this point, we examined the responses of Arabidopsis photoreceptor mutants *phyA-201* and *phyB-5* to Wc light. Figure 4 shows that the downregulation of *TUB1* was still detected in both mutants. These results suggest that no single photoreceptor is absolutely required for the Wc-mediated downregulation of *TUB1*; instead, multiple photoperception pathways, together or independently, may be involved.

Roles of phyA and phyB

Several recent reports have suggested that the FR high-irradiance response (FR-HIR) is specifically mediated by phyA, whereas the prolonged R response (R-HIR) is mainly mediated by phyB (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Therefore, the downregulation of *TUB1* mRNA levels by both FRc and Rc suggests that both phyA and phyB may be involved in the response. To confirm this notion, we tested the downregulation of *TUB1* expression by using different narrow wave bands of light in different photoreceptor mutants. If phyA were involved, then a *phyA* mutant would not respond to FRc treatment because it is "blind" to FR light. Similar experiments to examine the role of phyB were performed using a *phyB* mutant exposed to Rc. Figure 4 shows that in the *phyA-201* mutant, the *TUB1* mRNA level was downregulated in Rc as compared with the dark control; however, FRc was completely ineffective. Similarly, in the *phyB-5* mutant, downregulation of the *TUB1* mRNA level was normal in FRc but was severely impaired in Rc. The slight repressive

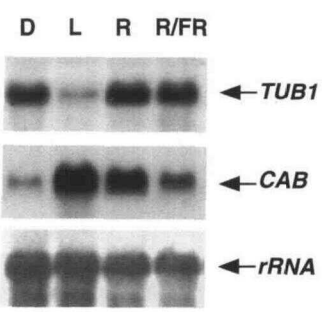

Figure 3. R/FR Reversibility Test.

Two-day-old etiolated seedlings were treated under the following conditions: 6 hr of dark (D); 6 hr of Wc (L); an R pulse followed by 6 hr of dark (R); and an R pulse followed immediately by an FR pulse and then 6 hr of dark (R/FR). The same blot was hybridized with the *TUB1* probe, then with the *CAB* probe, and finally with the 18S rDNA probe. Each lane contains 10 μg of RNA.

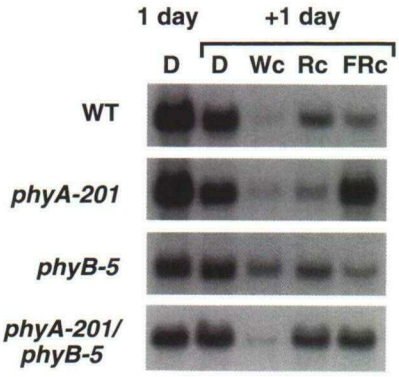

Figure 4. *TUB1* Transcript Levels in Photoreceptor Mutants Treated with W, R, and FR Light.

One day after germination in the dark, Arabidopsis wild-type (WT; Landsberg *erecta*), *phyA-201*, *phyB-5*, or *phyA-201 phyB-5* seedlings were harvested (1 day; D) or were kept in the dark for an additional day or illuminated with Wc, Rc, or FRc for 1 day before harvesting (+1 day; D, Wc, Rc, and FRc). Each lane contains 10 μg of RNA. The blot was also hybridized with the 18S rDNA probe as a control (data not shown).

effect of Rc in *phyB-5* seedlings might have resulted from the presence of phyA that was photoconverted to the active Pfr form (PfrA) by Rc. However, the effect was slight because PfrA is light-labile and is degraded quickly by the ubiquitin-dependent proteolytic pathway (Jabben et al., 1989).

The results with *phyA* and *phyB* mutants were confirmed using the *phyA-201 phyB-5* double mutant. The responses of this double mutant to both Rc and FRc were severely impaired (Figure 4). To extend these studies, we also examined *TUB1* mRNA levels in these photoreceptor mutants exposed to Wc. Because phyA is labile under Wc treatment, phyB is expected to play a major role under Wc light condition. Figure 4 shows that *phyB-5* was much less responsive to Wc than were the wild type and *phyA-201*. However, it is clear that *TUB1* mRNA levels in the *phyA-201 phyB-5* double mutant were still partially repressed by Wc (a 30 to 70% decrease in three independent measurements, compared with an ~80% decrease in the case of the wild type), indicating that photoreceptors other than phyA and phyB also contribute to *TUB1* downregulation by Wc treatment. Interestingly, the extent of *TUB1* downregulation by Wc in the *phyA phyB* double mutant appears to be equal to or even higher than that in *phyA* or *phyB* single photoreceptor mutants. These results imply that other photoreceptors might be able to compensate more effectively for the function of phyA and phyB when both phytochromes are absent.

Transcriptional and Organ-Specific Regulation Mediated by the *TUB1* Promoter

To investigate whether the light downregulation of *TUB1* expression is controlled at the level of transcription, a chimeric reporter gene was constructed by fusing a 2-kb fragment containing *TUB1* 5′ upstream sequences to an *Escherichia coli* promoterless β-glucuronidase (*GUS*) coding sequence. This transcriptional fusion construct was then introduced into Arabidopsis (ecotype Columbia) by Agrobacterium-mediated Ti transformation. Homozygous T_3 progenies from four independent transgenic lines were analyzed. Because the GUS protein is very stable, the enzyme activity cannot be expected to reflect accurately the rapid decrease of mRNA levels in response to illumination. Therefore, GUS activities in etiolated seedlings and seedlings grown in Wc were compared. Figure 5 shows that in 2-day-old etiolated seedlings, strong *TUB1–GUS* expression was detected in both hypocotyls and roots and was only slightly detected in cotyledons. By contrast, Wc-grown seedlings showed severely decreased GUS staining in hypocotyls, although GUS activity in the roots remained high.

Our results are similar to those of Tonoike et al. (1994), who have shown previously that in the soybean β1-tubulin gene, a 2-kb promoter fragment is sufficient for transcription in etiolated elongating hypocotyl tissues and for the downregulation of its transcription by W. However, in their study, the photoreceptors responsible for this light regulation of β-tubulin gene expression were not identified. To address this question for the Arabidopsis *TUB1* gene, we grew the *TUB1–GUS* transformed seedlings under Rc and FRc. These seedlings have longer hypocotyls than Wc-grown seedlings, probably due to the lower fluence rate used in these treatments (see Methods). Nevertheless, it is clear that GUS activities in the hypocotyls were drastically decreased by both Rc and FRc (Figure 5). These results, together with those presented in Figure 4, provide evidence that the *TUB1* gene is downregulated by FRc and Rc via phyA and phyB, respectively, at the transcriptional level, although post-transcriptional mechanisms cannot be

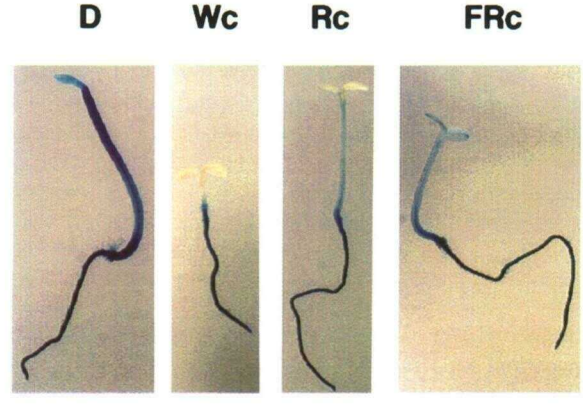

Figure 5. *TUB1–GUS* Chimeric Gene Expression in Etiolated Arabidopsis Seedlings or Seedlings Grown under Wc, Rc, or FRc.

Transgenic Arabidopsis seedlings carrying the chimeric *TUB1–GUS* construct (pBI2000) were grown in the dark (D) or under Wc, Rc, or FRc for 4 days (2 days old, if counted from germination). Blue indicates GUS staining.

ruled out (Figure 4). In older etiolated seedlings, GUS activity was weaker in the lower parts of hypocotyls; however, it is still detectable in the top parts of hypocotyls as well as in roots (data not shown). A qualitatively similar expression pattern was found in four independent transgenic lines and in all the seedlings analyzed (10 to 15 seedlings per line for each light condition).

When the length of the *TUB1* 5′ upstream fragment was decreased to 570 bp, no substantial GUS activity could be detected in transgenic plants (data not shown). A constitutively active cauliflower mosaic virus 35S–*GUS* construct was used as a control. Transgenic seedlings carrying the 35S–*GUS* transgene showed GUS activity mainly in roots, transition zones, and cotyledons in light-grown seedlings, as described by Benfey et al. (1989). Essentially the same *GUS* expression patterns were observed in etiolated seedlings and in light-grown seedlings (data not shown). These results demonstrate that the light/dark regulation of *TUB1* expression is controlled mainly at the level of transcription, and the *cis*-acting element(s) involved in the light response is located within the 2-kb upstream region of the *TUB1* gene. Furthermore, >570 bp of 5′ sequences is necessary for strong expression of the *GUS* reporter gene, suggesting the presence of one or more enhancer elements between the −2000 and −570 region.

It is important to emphasize that the light-mediated downregulation of *TUB1* expression was organ specific and confined mainly to the hypocotyl. GUS activity in cotyledons is already low in dark-grown seedlings; conversely, in roots it remains consistently high and is not affected by light (Figure 5). To confirm this observation, RNA was prepared from dissected organs of 3-day-old seedlings (hypocotyls of 3-day-old seedlings are usually ~10 mm in length and thus easier for dissection). Figure 6 shows that although the *TUB1* transcript levels were generally threefold lower in such 3-day-old seedlings than in 1-day-old seedlings (see results below in Figure 7), a clear decrease (~50%) by 6 hr of Wc treatment could still be detected in hypocotyls and cotyledons. Conversely, in roots, the *TUB1* transcript level remained the same in light and dark conditions. Because we generally used whole seedlings for RNA analysis, the extent of light downregulation of *TUB1* mRNA levels in the hypocotyls is actually underestimated. Moreover, in dark-grown seedlings, the *TUB1* mRNA levels seemed to be slightly higher in cotyledons and hypocotyls than in roots.

As a control for the purity of tissue preparations, we hybridized the same blot to a *CAB* probe, because this gene is known to be drastically induced by light only in the aerial organs in Arabidopsis seedlings (Millar et al., 1992). Figure 6 shows that, as expected, *CAB* mRNA was present only in hypocotyls and cotyledons, and expression was clearly up regulated by light. Oppenheimer et al. (1988) reported previously that *TUB1* mRNA accumulates predominantly in roots. However, in etiolated seedlings, we observed similar, if not higher, levels of *TUB1* expression in hypocotyls as compared with roots (Figures 5 and 6). This difference in results is probably explained by the use of light-grown seedlings in the experiments of Oppenheimer et al. (1988). Consequently, the *TUB1* mRNA level in hypocotyls

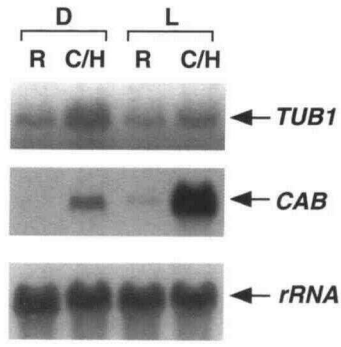

Figure 6. Tissue-Specific Downregulation of *TUB1* mRNA.

Dark-grown Arabidopsis seedlings (3 days old) were illuminated with Wc (L) or kept in the dark (D) for 6 hr. Cotyledons and hypocotyls (C/H) were dissected from roots (R) and collected separately. The same blot was hybridized with the *TUB1* probe, then with the *CAB* probe, and finally with the 18S rDNA probe. Each lane contains 10 μg of RNA.

was decreased, whereas in roots that were not exposed to light, the level remained high. That light can inhibit hypocotyl elongation but has no obvious effect on the extension of root (Figure 5) is consistent with this notion. This observation suggests that the control of *TUB1* expression may be related to the control of cell elongation. Both *TUB1* expression and cell elongation are downregulated by light in hypocotyls but not in roots.

TUB1 Transcript Levels and Hypocotyl Elongation

Figure 7A shows that the hypocotyl elongation rate of dark-grown Arabidopsis seedlings was very high, ~5 mm per day, during the first day after emergence from seed coats, but gradually decreased to only ~0.5 mm per day on day 5. To examine further the correlation between hypocotyl elongation and *TUB1* expression, we performed parallel measurements of hypocotyl elongation rate and *TUB1* transcript levels using seedlings of different ages. Consistently, the amount of *TUB1* mRNA decreased from 100% (arbitrarily defined as the highest observed expression level) at day 1 to 63, 30, 27, and 20% at days 2, 3, 4, and 5, respectively (Figure 7B). This result shows a good correlation between *TUB1* mRNA levels and the rate of hypocotyl elongation.

We found that irrespective of seedling age, hypocotyl extension ceased rapidly whenever etiolated seedlings were exposed to continuous light (at most, they elongated an additional 1 to 2 mm; Figure 7A). Similarly, under the same conditions, *TUB1* mRNA levels were drastically decreased by light to basal levels (20 to 30% of the dark control), irrespective of the age of the seedlings used (Figure 7B).

Because hypocotyl elongation in Arabidopsis resumed when light-grown seedlings were transferred back to the dark (data not shown), we measured *TUB1* transcript levels to determine whether they would be concomitantly elevated, that is, induced

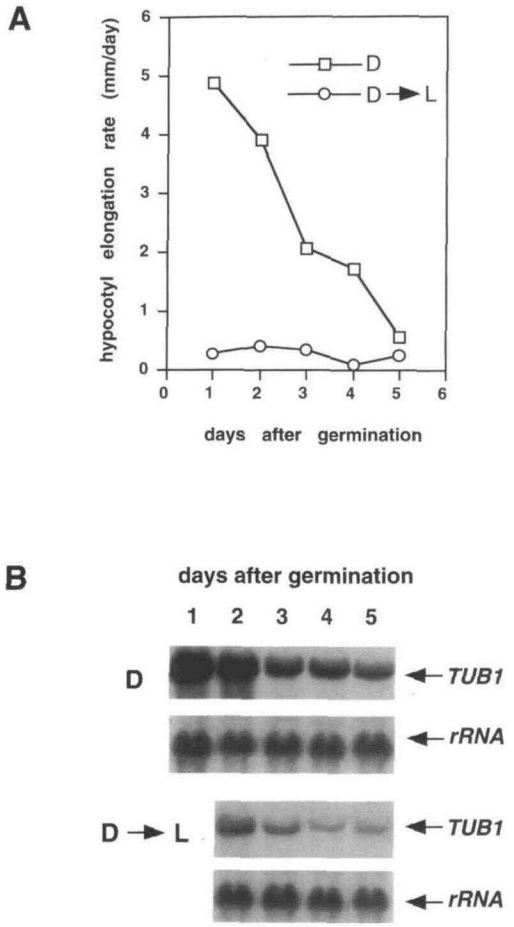

Figure 7. Hypocotyl Elongation Rate and Changes in *TUB1* mRNA Levels during Seedling Development.

(A) Daily elongation rate of hypocotyls. Seedlings were grown in the dark for 1 to 5 days after germination and were then shifted to Wc for 24 hr or left in the dark for an additional 24 hr. At the end of this period, hypocotyls were measured using projected photographs of seedlings. Growth rate was calculated by comparing average hypocotyl length (n = 40) with that from the previous day (n = 40). Open square, growth rate in the dark (D); open circle, growth rate of dark-grown seedlings on the first day of the light shift (D→L).

(B) Developmental and light-induced downregulation of *TUB1* mRNA levels. RNA was isolated from seedlings of different ages (1 to 5 days old) that were dark grown (D) or had been exposed to 24 hr of Wc prior to harvesting (D→L). The blots were hybridized with the *TUB1* probe. Duplicate blots were hybridized with the 18S rDNA probe as a control. Each lane contains 10 μg of RNA.

by dark. Figure 8 shows that in light-grown seedlings that were dark-adapted for 1 day (L→D), the *TUB1* mRNA level was higher than that of seedlings grown continuously under light (L) but was only slightly elevated when compared with the level of 1-day-old light-grown seedlings. Because *TUB1* mRNA levels can also be decreased by aging (Figure 7), the dark-inducible effect, if it exists, could possibly be masked by an age-related downregulation of *TUB1*. We also measured hypocotyl re-elongation ability and *TUB1* dark-inducibility in older seedlings (5 days old, data not shown). However, both phenomena were barely detectable in seedlings at this developmental stage. Taken together, our data establish a good correlation between hypocotyl elongation rate and the level of *TUB1* expression, suggesting that the inhibition of cell elongation by light may act, at least partially, through the regulation of *TUB1* expression.

DISCUSSION

In this study, we showed that in etiolated Arabidopsis seedlings, transcript levels of all 15 tubulin genes are decreased by light, albeit to different extents. Among these genes, the response of *TUB1* to light was investigated in some detail. We found that a pulse of R light cannot decrease *TUB1* transcript levels but that Wc, Rc, and FRc are all effective. Therefore, this response can be categorized as an HIR requiring the prolonged presence of the Pfr form of phytochromes. Because *phyA*, *phyB*, and *phyA phyB* photoreceptor mutants examined in this study still exhibit downregulation of *TUB1* when exposed to Wc, our results suggest that multiple photoreceptor pathways, either alone or in combination, control *TUB1* gene expression. Further analyses of the photoreceptor mutants irradiated with narrow wave band light demonstrated that both the phyA-mediated FR-HIR and the phyB-mediated R-HIR are involved in the downregulation of *TUB1* by light.

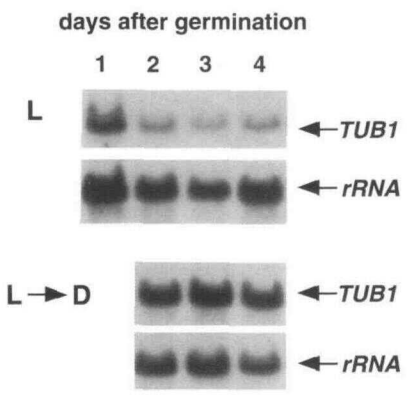

Figure 8. *TUB1* Transcript Levels in Dark-Adapted Light-Grown Seedlings.

Arabidopsis seedlings were germinated in the light for 1 day and then transferred to the dark and harvested on the second, third, or fourth day (L→D). Control seedlings were grown under Wc and harvested on the first, second, third, or fourth day after germination (L). The blots were hybridized first with the *TUB1* probe and then with the 18S rDNA probe. Each lane contains 10 μg of RNA.

Molecular Evidence for Distinct Roles of phyA and phyB in Photoperception

The deetiolation responses of plants to R and FR illumination are mediated mainly by two pools of phytochromes, type I (represented by phyA) and type II (represented by phyB), distinguished by the stability of protein in their Pfr form (reviewed in Smith, 1995). Recently, the availability of Arabidopsis that is either overproducing or deficient in specific phytochromes has provided invaluable tools for assigning roles of individual phytochromes in light-mediated responses (reviewed in Whitelam and Harberd, 1994; Quail et al., 1995; Smith, 1995). Accumulated evidence based on analysis of hypocotyl elongation has led to the notion that phyA is necessary and probably sufficient for FRc perception (FR-HIR). By contrast, Rc is mediated mainly by phyB and in part by phyA, because the latter is degraded quickly under Rc. Thus, although both FRc and Rc treatments inhibit hypocotyl elongation in etiolated seedlings, they act through different photoreceptors. Such distinct photosensory roles of phyA and phyB in light perception are further supported by the molecular analysis of light-regulated *TUB1* expression presented here. In particular, the inability of the *phyA* mutant to respond to FRc suggests that phyB and other photoreceptors play no significant role in FR-HIR (Figure 4).

The special function of phyA in mediating such a FR-HIR may be due entirely to the abundance of the phyA molecule (50- to 100-fold greater than the amount of phyB in etiolated tissue). Therefore, the low ratio of active PfrA photoconverted under FRc (calculated Pfr/total phytochrome ~2%, 10 μmol m^{-2} sec^{-1}; Mancinelli, 1994) might be enough to trigger a common phototransduction pathway. Alternatively, phyA might be coupled to a unique signal transduction pathway not shared by any other photoreceptors. To address these possibilities, it would be interesting to examine the FR-HIR in transgenic *phyA* mutant seedlings overproducing phyB. This would allow us to distinguish whether an increased level of phyB could substitute for phyA in mediating the FR-HIR.

An HIR Response

In Arabidopsis, downregulation of *PHYA* mRNA requires prolonged light treatment, and studies using a *PHYA–GUS* transgene have suggested the involvement of multiple photoreceptors (Somers and Quail, 1995). Therefore, *TUB1* and *PHYA* appear to display a very similar mode of response to light. However, our studies here have further distinguished and defined the roles of phyA and phyB in the control of *TUB1* downregulation by light.

Traditionally, the HIR is defined and analyzed at the phenotypic and physiological levels, for example, seed germination, hypocotyl elongation, and photoperiodism. Our studies provide the basic characterization of a molecular event that has the characteristics of an HIR, and this molecular event is correlated with an HIR at the morphological level, namely, hypocotyl elongation (see discussion below). Therefore, *TUB1* may be used as a marker gene for examining the HIR. However, a detailed analysis of the dose–response relationship (both for fluence and fluence rate) and the efficiency of various wavelengths of light should be made. The *TUB1*–reporter transgene system, demonstrated to be light responsive (see Results), would provide a convenient tool for such a quantitative analysis.

Tissue-Specific Photoregulation of *TUB1* Promoter Activity

Hypocotyls of transgenic Arabidopsis seedlings containing the chimeric *TUB1–GUS* gene exhibit strong GUS activity in the dark. However, GUS activity in the hypocotyls is drastically decreased in seedlings germinated under Wc, Rc, or FRc (see Figure 5). This result demonstrates that the downregulation of *TUB1* expression by light is controlled mainly at the level of transcription. The *cis* elements within the 2-kb promoter fragment of *TUB1* are sufficient to confer this downregulation. Moreover, the decrease of *TUB1–GUS* expression by light is detected only in hypocotyls, in contrast with the consistently high GUS activity observed in roots, and the low level of expression seen in cotyledons. Such organ-specific photoregulation might result from a light-responsive repressor that is absent in roots; alternatively, a positive transcription factor promoting *TUB1* expression in the dark might be present only in hypocotyls. Somers and Quail (1995) have reported that the Arabidopsis *PHYA* gene also exhibits hypocotyl-specific photoregulation. Whether the same regulatory mechanism operates in both cases remains to be investigated.

An element responsible for negative light regulation was first identified in a transient expression assay of the oat *PHYA3* gene (Bruce et al., 1991). This element, which contains the sequence CATGGGCGCGG, was designated "repressor element 1." Similar sequence motifs also have been found in the 5′ region of *PHYA* genes from both monocot and dicot plants, including Arabidopsis. All these motifs contain a conserved core sequence, ATGGG (Dehesh et al., 1994). Interestingly, two copies of such a core sequence, centered at −75 and −111, are also found on the opposite strand of the 5′ region of the *TUB1* gene. Future mutagenesis studies are necessary to verify the role, if any, of these putative elements in the negative light regulation of *TUB1*.

Compared with light-upregulated genes, few light-downregulated genes have been investigated. The most detailed analyses were performed with the genes encoding protochlorophyllide reductase in barley (Mösinger et al., 1988), asparagine synthase in pea (Tsai and Coruzzi, 1990), and phytochrome from pea, oat, and rice (reviewed in Thompson and White, 1991). Transcript levels of each of these genes are decreased by a pulse of R, and this decrease is reversible by a pulse of FR. Thus, all three genes mentioned above are regulated by a

low-fluence response mediated by phytochromes. By contrast, downregulation of the *PHYA* gene of tomato and Arabidopsis (Somers and Quail, 1995) and of the Arabidopsis *TUB1* gene reported here all exhibit an HIR. To date, all of the genes positively regulated by phytochrome require only low-fluence light treatment. Recent analyses demonstrated that these genes are activated by phytochrome via three phototransduction pathways: one mediated by cGMP, another by calcium, and the third by both small molecules (Neuhaus et al., 1993; Bowler et al., 1994a, 1994b). It is important to determine whether the same phototransduction pathways are used to turn off expression of downregulated genes, in particular the Arabidopsis *TUB1* gene.

Correlation between *TUB1* Transcript Levels and Cell Elongation Rate

In oat, the inhibition of mesocotyl elongation as well as the downregulation of β-tubulin transcript levels are induced, at least in part, by R light treatments in the very low fluence range (<1° μmol m^{-2} sec^{-1}; Colbert et al., 1990). Conversely, in Arabidopsis, both phenomena require high-fluence illumination (Smith, 1995; this study). These data suggest a correlation between hypocotyl elongation and *TUB1* gene expression. Several additional lines of evidence support this notion: (1) in Wc, the *TUB1–GUS* transgene is highly expressed in the upper region of etiolated hypocotyls but is decreased in the lower part of hypocotyls, where cell elongation has ceased (data not shown); (2) root elongation is not repressed by light, and no decrease of *TUB1* transcript by light is detectable in roots (Figure 6); and (3) both the rate of hypocotyl elongation and the level of *TUB1* transcripts decrease with seedling age (Figures 4 and 7). Hypocotyls of young seedlings resume elongation when shifted from light to dark growth; under the same conditions, *TUB1* transcript levels also remain high, if not elevated (Figure 8).

Although *TUB1* mRNA levels appear to correlate with Arabidopsis hypocotyl length, it is clearly important to establish whether the TUB1 protein level is also downregulated by light. The unavailability of a TUB1 isotype–specific antibody has precluded us from addressing this issue.

Many Arabidopsis photomorphogenic mutants were identified based on their abnormal hypocotyl lengths in complete darkness or under specific light conditions (reviewed in Millar et al., 1994; Quail et al., 1995). It was therefore of interest to examine the *TUB1* expression levels in these mutants. The relatively high *TUB1* levels in the *phyA-201* mutant under FRc or the *phyB-5* mutant under Wc and Rc are consistent with their abnormally long hypocotyl phenotypes under such conditions (see Figure 4). Similarly, the deetiolated *det1* mutant, which constitutively displays light-grown characteristics such as short hypocotyls (Chory et al., 1989), contains only ~10% of *TUB1* transcripts in dark-grown seedlings, and no further decrease is seen upon illumination (W.-M. Leu, unpublished results). Also, both etiolated and light-grown *diminuto* mutants, distinguished by cells that are severely decreased in length, contain only ~25% of *TUB1* transcripts as compared with the wild type (Takahashi et al., 1995). These observations provide additional evidence that *TUB1* transcript levels are correlated with Arabidopsis hypocotyl length.

METHODS

Plant Materials and General Growth Conditions

Mutants *phyA-201* (Nagatani et al., 1993), *phyB-5* (Reed et al., 1993), and *phyA-201 phyB-5* (Reed et al., 1994) were used. These mutants are in the *Arabidopsis thaliana* Landsberg *erecta* genetic background. Seeds (15 mg) were routinely soaked and sterilized for 10 min in a 100% hypochlorite solution containing 0.1% (v/v) Tween 20, washed with four changes of sterile water, and then sown on Murashige and Skoog (JRH Biosciences, Lenexa, KS) plates containing 3% sucrose.

Light Treatments

To induce germination, seeds were exposed to continuous white light (Wc; 30 μmol m^{-2} sec^{-1}) provided by cool-white fluorescent bulbs (GRO-LUX F96T12/GRO; Sylvania Co., Danvers, MA) for 2 hr. Germination of seeds in the dark on vertically oriented plates usually takes 2 to 2.5 days. After germination, seedlings having hypocotyls 1 to 2 mm in length (called 1-day-old seedlings) were treated with the following light regimes for different times before RNA isolation: Wc (as described above for the induction of seed germination); continuous red light (Rc) (10 to 12 μmol m^{-2} sec^{-1}; output of red light bulbs [660 F48T12/2364/VHO, SR No. 5954; Sylvania Co.] filtered through one layer of Plexiglas [No. 2793; Atohaas North America Inc., Philadelphia, PA]); and continuous far-red light (FRc) (8.5 μmol m^{-2} sec^{-1}; output of light bulbs [F48T12/232/VHO, SR No. 5976, Sylvania Co.] filtered through one layer of Plexiglas [No. 067894; West Lake Plastics, Lenni, PA]).

For R/FR reversibility tests, an R pulse was given by cadmium-coated red light bulbs for 2 min (total fluence of ~2000 μmol m^{-2} sec^{-1}; F20T12R; General Electric Co., Cleveland, OH). For a brief FR irradiation, a narrow wave band of light was applied for 10 min. R intensities were measured by a quantum sensor (model No. LI-189 in connection with LI-190SA quantum sensor; Li-Corp. Inc., Lincoln, NE). FR intensities were measured by a radiometer (model No. IL-1400A with filter FS750; International Light Inc., Newburyport, MA).

RNA Extraction and Gel Blot Analyses

Seedlings were harvested under a dim green safelight and immediately frozen in liquid nitrogen. RNA was isolated using RNA Matrix (Bio-101, Inc., Richmond, CA), according to the manufacturer's instructions. RNA blot hybridizations were performed as described by Oppenheimer et al. (1988). All DNA fragments used as gene-specific probes for detection of α-tubulin and β-tubulin genes (*TUA1* to *TUA6* and *TUB1* to *TUB9*) were synthesized by polymerase chain reaction using plasmids containing 3′ noncoding sequences of each tubulin gene as templates (Kopczak et al., 1992; Snustad et al., 1992). Com-

mercial M13/pUC sequencing primers 1233 and 1224 (New England Biolabs Inc., Beverly, MA) were used as 5′ and 3′ primers. The polymerase chain reaction products were gel purified using a GeneClean kit (Bio-101, Inc.) or a Qiaex DNA gel extraction kit (QIAGEN Inc., Chatsworth, CA). *CAB* DNA was a 527-bp AvaII fragment from pAB180 plasmid containing *CAB1* genomic DNA (Millar and Kay, 1991). ^{32}P-labeled DNA probes were prepared by a random primer protocol (Megaprime DNA labeling kit; Amersham Life Science Co.). Intensity of hybridized mRNA bands was quantified by a PhosphorImager (model No. 400E; Molecular Dynamics, Sunnyvale, CA).

Promoter–Reporter Constructs and Plant Transformation

Plasmid pBI2000 was constructed by replacing the cauliflower mosaic virus 35S promoter of plasmid pBI121 with a 2000-bp restriction fragment from the 5′ flanking region of an Arabidopsis *TUB1* genomic clone. The original *TUB1* genomic clone in the EcoRI site of pUC119 was digested with EcoRI-BspHI and blunted with mung bean nuclease. The blunt-ended fragment was cloned into the HincII site of pUC119. Cleavage at the BspHI site 3 bp upstream of the *TUB1* ATG translation start triplet and correct orientation of the blunted fragment were verified by sequencing after insertion of the fragment into HincII-cut pUC119. The HindIII-XbaI restriction fragment excised from pUC119 was inserted into the HindIII and XbaI acceptor sites of plasmid pBI121 after the removal of the 35S promoter. The resulting plasmid, designated pBI2000, was introduced into *Agrobacterium tumefaciens* AGL1 by electroporation. AGL1 containing the pBI2000 construct was used to transform root explants of Arabidopsis ecotype Columbia following the protocol of Valvekens et al. (1988) as modified by Carpenter et al. (1992). At least four independent transgenic Arabidopsis lines were obtained for each construct.

Histochemical GUS Assays

The procedures were modified from protocols developed by Jefferson (1987). Whole seedlings were vacuum infiltrated with a solution containing 1 mg/mL X-gluc, 0.05% (v/v) Triton X-100, and 0.02% (v/v) β-mercaptoethanol in 50 mM phosphate buffer, pH 7.5. After incubation overnight in the staining solution at 37°C, the seedlings were destained with 70% ethanol before photography.

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