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# Overexpression of Phytochrome B Induces a Short Hypocotyl Phenotype in Transgenic Arabidopsis

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**The photoreceptor phytochrome is encoded by a small multigene family in higher plants. *phyA* encodes the well-characterized etiolated-tissue phytochrome. The product of the *phyB* gene, which has properties resembling those of "green tissue" phytochrome, is as yet poorly characterized. We have developed a phytochrome B overexpression system for analysis of the structure and function of this protein. Using newly generated polyclonal and monoclonal antibodies that are selective for phytochrome B, we have demonstrated high levels of expression of full-length rice and Arabidopsis phytochrome B under the control of the cauliflower mosaic virus 35S promoter in transgenic Arabidopsis. The overexpressed phytochrome is spectrally active, undergoes red/far-red-light-dependent conformational changes, is synthesized in its inactive red light-absorbing form, and is stable in the light. Overexpression of phytochrome B is tightly correlated with a short hypocotyl phenotype in transgenic seedlings. This phenotype is strictly light dependent, thus providing direct evidence that phytochrome B is a biologically functional photoreceptor. Based on similarities to phenotypes obtained by overexpression of phytochrome A, it appears that phytochromes A and B can control similar responses in the plant.**

## INTRODUCTION

Plants perceive the intensity, direction, and quality of light, as well as the length of the photoperiod, and utilize this information to optimize photosynthesis, to sense the density of neighboring plants, and to regulate the timing of flowering and germination. Phytochrome is the best characterized of the photoreceptors involved in these light-dependent responses (Kendrick and Kronenberg, 1986). From physiological, biochemical, and mutant analyses, evidence has mounted that multiple pools of phytochromes might control such diverse responses. Initially, physiological data led to speculation on the heterogeneity of the phytochrome pool (Hillman, 1967). In vivo spectral analysis of oat (Jabben and Holmes, 1983) pointed toward two pools of phytochrome: a light-labile (or type 1) pool predominantly present in etiolated tissue and a light-stable (or type 2) pool present in green tissue. Biochemical analysis of light-grown oat identified, in addition to the light-labile species, at least one spectrally and immunologically distinct type of phytochrome that predominated in extracts from green plants (Shimazaki et al., 1983; Tokuhisa and Quail, 1983, 1987, 1989; Shimazaki and Pratt, 1985, 1986; Tokuhisa et al., 1985; Cordonnier et al., 1986; Pratt et al., 1991; Wang et al., 1991). Light-grown dicot (pea) plants (Abe et al., 1985, 1989; Konomi et al., 1987), on the other hand, contained an immunologically but not spectrally distinct type of phytochrome in green plant extracts. Data from analysis of the responses of photomorphogenic long hypocotyl mutants were also consistent with lesions in either the light-labile (*aurea* mutant of tomato) or the light-stable (*lh* mutant of cucumber, *hy*3 mutant of Arabidopsis) types of physiologically active phytochrome (Adamse et al., 1988a; Furuya, 1989; Smith and Whitelam, 1990; Tomizawa et al., 1990). The light-labile type of phytochrome has been implicated in the "high-irradiance response" of etiolated seedlings, whereas the light-stable type is necessary for the "end-of-day far-red response," where increases in hypocotyl length are observed when a far-red light pulse is given prior to the dark period (Smith and Whitelam, 1990).

The discovery of a multigene family encoding phytochrome in Arabidopsis (*phyA*, *phyB*, *phyC*, *phyD*, and *phyE*, Sharrock and Quail 1989; R. Sharrock, personal communication) and rice (*phyA*, *phyB*, and *phyC*, Dehesh et al., 1991; K. Dehesh and P. Quail, unpublished data) and the subsequent isolation of the individual phytochrome genes provide the opportunity for the molecular characterization of the distinct classes of the photoreceptor

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described above. Comparison of the *phyA*-derived amino acid sequences with protein sequences from proteolytic fragments of phytochrome purified from etiolated tissue indicates that *phyA* encodes the well-characterized light-labile or type 1 phytochrome (Lagarias and Rapoport, 1980; Hershey et al., 1985; Yamamoto, 1987; Grimm et al., 1988; Sato, 1988; Jones and Quail, 1989). This phytochrome species shows an apparent molecular mass of 116 to 127 kD depending on the plant species (Vierstra et al., 1984) and occurs as a dimer (Jones and Quail, 1986). The holoprotein has a covalently attached linear tetrapyrrole chromophore (Rüdiger and Scheer, 1983; Vierstra and Quail, 1986). Irradiation with red (660 nm) light converts the molecule from the inactive, red light-absorbing (Pr) form to the active, far-red-light-absorbing (Pfr) form. This process can be reversed by irradiation with far-red (730 nm) light.

The amino acid sequence derived from *phyB* is more conserved between the monocot rice and the dicot Arabidopsis (73%) than that of *phyA* between the same two species (63%). Yet *phyA* compared with *phyB* in rice shows only 50% identity at the amino acid level, as does *phyA* compared with *phyB* in Arabidopsis (Sharrock and Quail, 1989; Dehesh et al., 1991). In addition, the mRNA for phytochrome B is constitutively expressed in white light in contrast to that of *phyA*, which is down regulated (Sharrock and Quail 1989; Dehesh et al., 1991). Limited N-terminal sequences of proteolytic fragments from type 2 phytochrome of pea show higher homology to the *phyB*-derived amino acid sequences of rice and Arabidopsis than to those of the other reported *phy* genes. Thus, it has been an attractive hypothesis that *phyB* encodes a light-stable phytochrome. Because of the potentially central role of light-stable phytochrome in fully green plants, we are interested in understanding the structural properties, biological function, and mechanism of action of phytochrome B. One approach to these questions is to overexpress *phyB* sequences in transgenic plants and monitor for an altered phenotype. This approach has been used successfully for phytochrome A (Boylan and Quail, 1989, 1991; Kay et al., 1989; Keller et al., 1989; Cherry et al., 1991; Nagatani et al., 1991).

Overexpression is useful for a number of purposes. First, it provides the opportunity to examine whether the *phyB* sequences that have been cloned do indeed encode functionally active photoreceptors. Second, overexpression allows determination of whether phytochrome A and phytochrome B elicit similar, distinct, or overlapping phenotypes as an initial indication of whether the individual phytochromes are capable of regulating similar or different cellular processes. Third, the overexpression system can be used for dissection of functionally important domains in the polypeptide by in vitro mutagenesis and subsequent analysis for activity in transgenic plants. To this end, we report here the overexpression of rice and Arabidopsis B phytochromes in transgenic Arabidopsis and examine the phenotypic consequences of this overexpression.

## RESULTS

### *Arabidopsis* and Rice *phyB* Constructs Induce Short Hypocotyls in Transgenic Arabidopsis

To study phytochrome B by overexpression in Arabidopsis, clones encoding full-length copies of either rice or Arabidopsis phytochrome B polypeptides were generated. For introduction into Arabidopsis and expression under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the cointegrate vector pMON316 was chosen. This vector also carries the neomycin phosphotransferase II gene for selection of transformants on kanamycin (Rogers et al., 1987).

Based on transformation to kanamycin resistance, 29 transgenic Arabidopsis lines carrying the rice *phyB*

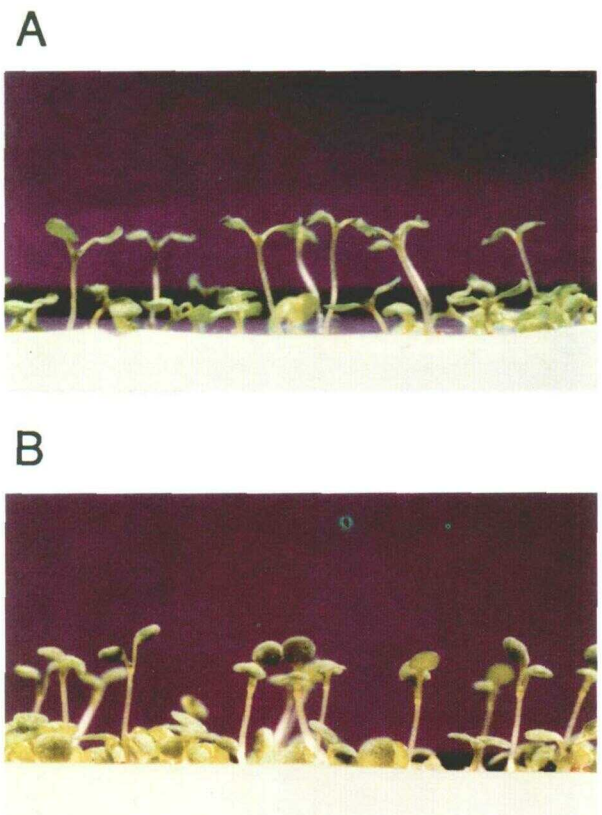


**Figure 1.** Segregation of Transgenic $R_1$ Arabidopsis Seedlings for a Short Hypocotyl Phenotype.

$R_1$ seedlings were derived from selfed Arabidopsis lines transformed with the rice *phyB* construct (RBO line) or the Arabidopsis *phyB* construct (ABO line).

**(A)** Seven-day-old RBO seedlings segregate for a short hypocotyl phenotype.

**(B)** Seven-day-old ABO seedlings segregate for a short hypocotyl phenotype.

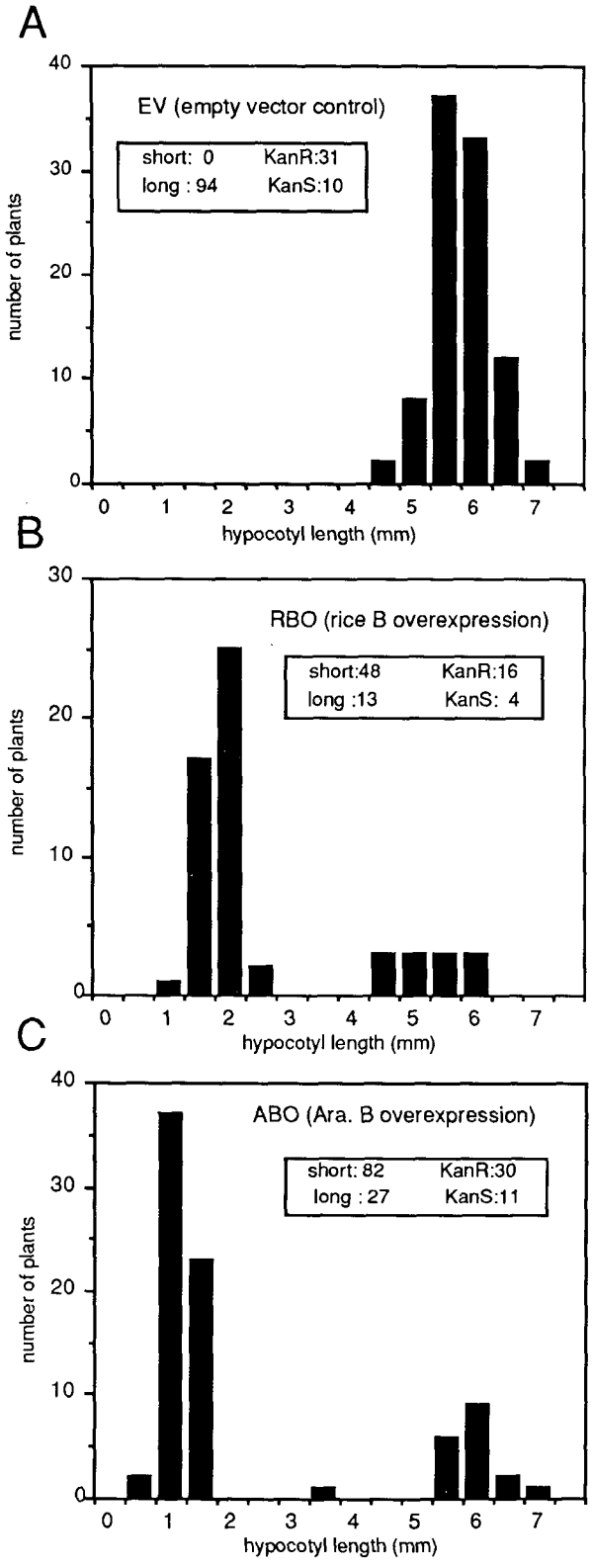


**Figure 2.** Hypocotyl Length Distribution of 7-Day-Old Seedlings of Three Transgenic Arabidopsis Lines.

construct and 10 lines carrying the Arabidopsis *phyB* construct were selected for further characterization. For each construct, all lines segregating for kanamycin resistance in the $R_1$ generation also segregated for a pronounced short hypocotyl phenotype when grown without kanamycin selection. When the short and tall plants of the $R_1$ generation of each line were transferred to plates containing kanamycin, the short plants showed resistance and the tall plants were sensitive. Figure 1A shows the $R_1$ progeny of a selfed transformant carrying the rice *phyB* construct (referred to as RBO, for rice phytochrome B overexpression). The seedlings segregated for short and long hypocotyls after 7 days of growth in the light. Figure 1B shows that plants of the same age carrying the Arabidopsis *phyB* construct (ABO, for Arabidopsis phytochrome B overexpression) also segregated for the short hypocotyl phenotype. The ABO line exhibited even shorter hypocotyls than the RBO line. This more pronounced phenotype was found in all ABO lines tested so far (data not shown). Hypocotyl lengths of 7-day-old seedlings of both lines depicted in Figure 1 were compared with a line transformed with the "empty" vector (pMON316 without insert). The hypocotyl length of the empty vector control averaged 5.5 mm, as shown in Figure 2A. However, both the RBO and ABO lines showed a bimodal distribution of hypocotyl length with about 75% of the plants clustered around 2 and 1 mm, respectively, and about 25% of the plants averaging 5.5 mm similar to the control (Figures 2B and 2C). The values obtained for the short (<4.0 mm) to long segregation as well as for kanamycin resistance to sensitivity are shown in the insets. For all three lines, the ratios found are close to 3:1.

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**(A)** Hypocotyl length distribution of the transformation control line. An Arabidopsis line transformed with the empty pMON316 vector (no insert, [EV]) segregates 3:1 ($\chi^2$ = 0.008) for kanamycin resistance to sensitivity (see inset), respectively. This line has hypocotyl lengths of >4.0 mm when grown without kanamycin selection. Ninety-four seedlings were scored.

**(B)** Hypocotyl length distribution of the RBO line. The RBO line shown in **(A)** segregates 3:1 for kanamycin resistance ($\chi^2$ = 0.266) and shows bimodal distribution of hypocotyl length with approximately 75% (48) of the seedling heights significantly <4.0 mm and 25% (13) >4.0 mm when grown without kanamycin selection. The $\chi^2$ for 3:1 hypocotyl length segregation is 0.44.

**(C)** Hypocotyl length distribution of the ABO line. The ABO line shown in **(B)** segregates 3:1 for kanamycin resistance ($\chi^2$ = 0.070) and shows bimodal distribution of hypocotyl length with 75% (82) of the seedling heights significantly <4.0 mm and 25% (27) >4.0 mm when grown without kanamycin selection. The $\chi^2$ for 3:1 hypocotyl length segregation is 0.003.

Absolute numbers of short and long hypocotyls and of kanamycin-resistant (KanR) and kanamycin-sensitive (KanS) seedlings were determined in separate experiments and are boxed.

### Newly Generated Antibodies Are Selective for Phytochrome B

The rice *phyB* construct was overexpressed in *Escherichia coli* using the T7 expression system (Studier and Moffat, 1986). The *phyB*-encoded product was purified by band isolation after acrylamide gel electrophoresis and used as an antigen to immunize mice. Polyclonal serum as well as several monoclonal cell line supernatants that recognize phytochrome B selectively were obtained. The two monoclonal antibodies (MAbs) used in this study did not detect *E. coli*-produced, purified Arabidopsis A and C phytochromes in immunoblot analysis, as shown in lanes 1 and 2 of Figure 3 in the top (MAb 1) and center (MAb 2) panels. In contrast, both MAbs reacted well with *E. coli*-produced, purified rice and Arabidopsis B phytochromes (lanes 3 to 8). MAb 1 reacted less strongly (twofold, data not shown) with the Arabidopsis than with the rice phytochrome B (compare lanes 3 and 4) and was used for most of the analysis. MAb 2 showed similar apparent affinity for both B phytochromes. On the Coomassie Brilliant Blue R 250-stained gel, only phytochromes A and C, which were loaded at 10-fold higher concentrations than phytochrome B, could be visualized (Figure 3, bottom panel, lanes 1 and 2). Both antibodies were mapped to the first 266 amino acids of phytochrome B by expressing deleted derivatives of rice *phyB* in *E. coli* (data not shown). However, MAb 2 recognizes a different epitope than MAb 1 (based on reactivity with additional phytochrome fragments upon in vitro proteolysis; data not shown) and was used for the proteolysis analysis discussed below.

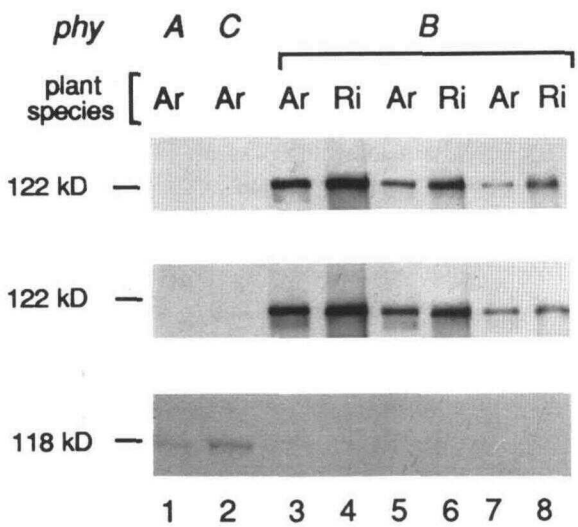


**Figure 3.** Selectivity of MAbs toward Phytochrome B.

The top panel shows an immunoblot probed with MAb 1. Three hundred nanograms each of *E. coli*-produced, gel-purified Arabidopsis (Ar) phytochrome A and phytochrome C were loaded onto lane 1 and lane 2, respectively. Lanes 3 through 8 show decreasing amounts of *E. coli*-produced, gel-purified Arabidopsis (Ar, odd-numbered lanes) and rice (Ri, even-numbered lanes) B phytochromes. Amounts of phytochrome B loaded were 30 ng (lanes 3 and 4), 20 ng (lanes 5 and 6), and 10 ng (lanes 7 and 8) for both proteins. The apparent molecular mass of Arabidopsis phytochrome B (122 kD) is indicated. The center panel shows an immunoblot probed with MAb 2. The loading was identical to the top panel. The bottom panel shows a Coomassie blue-stained gel. The loading was identical to the top panel. The apparent molecular mass of Arabidopsis phytochrome A (118 kD) is indicated.

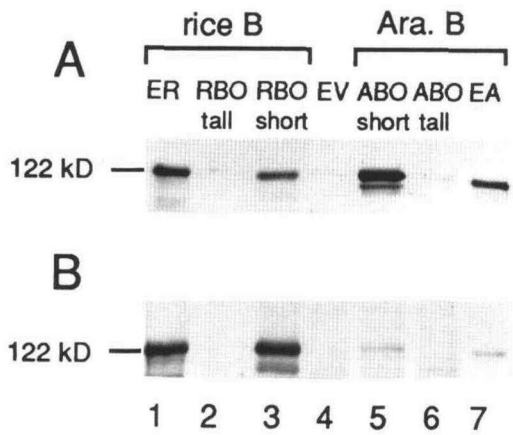


**Figure 4.** Immunoblot Analysis of Phytochrome B Polypeptides Produced by Homozygous RBO, ABO, and Control Lines.

**(A)** Immunoblot probed with MAb 1. Crude protein extracts (50 μg per lane) were prepared from homozygous, 7-day-old, light-grown seedlings (lanes 2 to 6). Homozygous short RBO and ABO seedlings were assayed in lanes 3 and 5, respectively. Lane 4 contains extracts of the homozygous control plants (EV). Homozygous siblings of the RBO and ABO lines that segregate for wild-type seedling height (tall) were tested in lanes 2 and 6, respectively. Lanes 1 and 7 contain 30 ng of *E. coli*-produced, gel-purified rice (ER) and Arabidopsis (EA) B phytochromes, respectively.

**(B)** Immunoblot probed with polyclonal phytochrome B-selective ascites depleted of Arabidopsis phytochrome B-reactive antibodies. The lanes are identical to those in **(A)**.

### Short Hypocotyl Phenotype Is Correlated with Overexpression of Phytochrome B

Phytochrome B levels were quantitated by immunoblot analysis of crude extracts from light-grown homozygous individuals of the control, RBO, and ABO lines discussed above, as well as from siblings of the RBO and ABO lines that segregate for wild-type height (tall). Figure 4 shows that the tall siblings and the control line had barely

detectable levels of phytochrome B. In contrast, both overexpressing lines showed high levels of phytochrome B (Figure 4, lanes 3 and 5). Overexpression of either rice or Arabidopsis *phyB* yielded a full-length polypeptide, as determined by comparison with the corresponding *E. coli*-produced, gel-purified protein (Figure 4A). The overexpressed Arabidopsis phytochrome B also comigrated with endogenous Arabidopsis phytochrome B (barely visible here; data not shown). We estimate that in 50 $\mu$g of crude Arabidopsis protein loaded, 14 ng of overexpressed protein was present in the extract of RBO seedlings (Figure 4, lane 3), and 82 ng of overproduced protein was present in the ABO-derived extract. These estimates are based on densitometric determination of concentration by comparison with 30 ng each of *E. coli*-produced, gel-purified rice phytochrome B (lane 1) and Arabidopsis phytochrome B (lane 7) in Figure 4, as well as by serial dilution comparisons of both the *E. coli*-produced proteins and the extracts derived from RBO, ABO, and control lines (data not shown). The levels of overexpression are comparable with those seen in tomatoes overexpressing oat phytochrome A (Boylan and Quail, 1989). Compared with the protein levels reportedly expected for type 2 phytochrome (Furuya, 1989) and to the endogenous levels of Arabidopsis phytochrome B (data not shown), these levels represent increases of from threefold to fivefold (RBO lines) to 18- to 30-fold (ABO lines).

Because the MAb 1 (Figure 4A) reacted with both rice and Arabidopsis B phytochromes, it was possible that, in the RBO line tested here, we simply detected increased levels of endogenous Arabidopsis phytochrome B caused by the introduction of the rice *phyB* construct. To exclude this possibility, polyclonal antiserum reactive to both B phytochromes was depleted of the Arabidopsis reactive antibodies by incubation with *E. coli*-produced Arabidopsis phytochrome B and subsequent centrifugation. The depleted antiserum reacted only very weakly with the Arabidopsis phytochrome B, as shown in Figure 4B (lanes 5 and 7). However, it reacted strongly with rice phytochrome B (Figure 4B, lanes 1 and 3). This result indicates that the protein recognized by MAb 1 in lane 3 is rice phytochrome B. Thus, elevated levels of the *phyB* gene product are present in the RBO and ABO plants. These levels correlated strictly with the short hypocotyl phenotype, showing that both rice and Arabidopsis *phyB* encode functional phytochromes.

To compare the levels of overexpressed phytochrome B protein in several different RBO and ABO lines, crude extracts were prepared from light-grown seedlings of five independent lines for each construct. The data for three representative lines of each are shown in Figure 5. All three RBO lines (lanes 1 to 3) segregated for a single locus of T-DNA insertion, as did two of the ABO lines (lanes 5 and 6). The third ABO line segregated for two independent T-DNA loci (lane 7). The control extract is shown in lane 4. The levels of overproduced protein were similar among

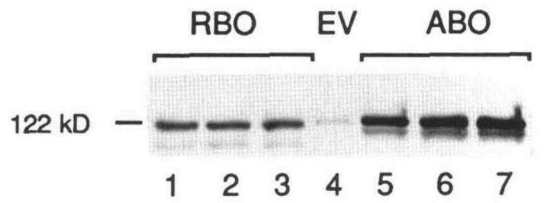


**Figure 5.** Similar Levels of Overexpressed Phytochrome B in Three Independent RBO and ABO Lines.

Crude extracts were prepared from light-grown $R_1$ seedlings of three independent RBO and ABO lines. Forty-eight micrograms of crude protein were loaded in each lane. Lanes 1 to 3 show the RBO lines and lanes 5 and 6 are the ABO lines segregating for a single locus of T-DNA insertion; lane 7 is an ABO line that segregates for two independent T-DNA insertion loci. Lane 4 represents the empty vector (EV) control, which also segregates for a single locus of T-DNA insertion. Lanes 1 and 5 show the RBO and ABO lines used throughout this study.

all RBO lines tested and among all ABO lines tested, yet the levels of the RBO lines were consistently lower than those of the ABO lines. The higher protein levels correlated in all cases tested with larger decreases in hypocotyl length.

### Overexpressed Phytochrome B Is Stable in Light

Because endogenous green tissue phytochrome is stable in both red and white light, we tested the behavior of the overexpressed protein under those conditions. Figure 6 displays the immunologically detectable levels of the overexpressed phytochromes in homozygous seedlings grown for 7 days in darkness, in darkness followed by 6 hr of red light exposure, and with 16-hr light/8-hr dark cycles. In the overexpressing lines (RBO, lanes 4, 5, and 6; ABO, lanes 7, 8, and 9), the level of phytochrome B present did not change with either light treatment (Figure 6A). Thus, the behavior of the overexpressed protein is similar to that of green tissue phytochrome in that it is stable in the light. In contrast, the endogenous phytochrome A levels probed with a phytochrome A-specific MAb 073D (Shanklin, 1988; Somers et al., 1991) dropped below the level of detection during both the red light and white light treatments (Figure 6B). Phytochrome A was present at equal levels in the overexpressing (RBO and ABO) and the control lines in the dark (Figure 6B, lanes 1, 4, and 7), indicating that phytochrome B overexpression does not affect the synthesis of photochemically functional phytochrome A.

When the dark- and red light-grown plants were assayed for spectral activity (Figure 6C), crude extracts from dark-grown tissue of the ABO line showed a substantial increase in $\Delta\Delta A$ levels when compared with extracts of the control line. The spectral activity in the control line

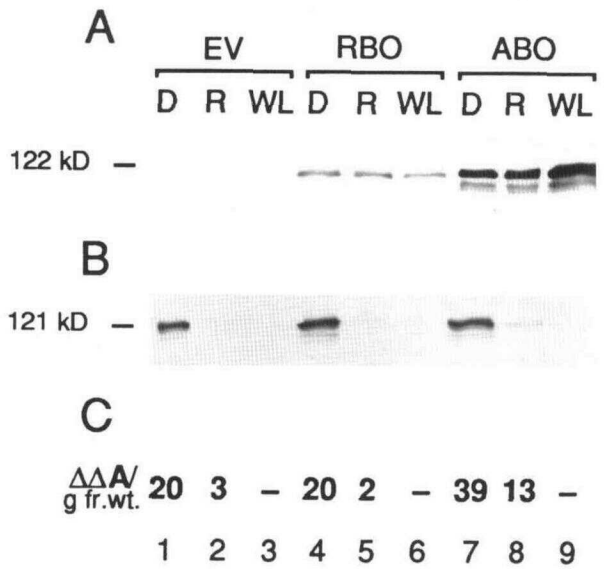


**Figure 6.** Light Stability of Overexpressed Rice and Arabidopsis B Phytochromes in Transgenic Arabidopsis.

**(A)** Immunoblot of crude extracts of the control (EV) as well as of RBO and of ABO seedlings grown under different light conditions. The plants were grown for 7 days either in the dark (D), in darkness followed by a 6-hr treatment with red light (R), or under white light conditions (WL, 16-hr day/8-hr night). Forty-five microliters of crude extract (= 30 mg fresh weight of tissue) for each treatment was loaded per lane. Lanes 1 to 3 show the control seedlings; lanes 4 to 6, RBO seedlings; lanes 7 to 9, ABO seedlings. The blot was probed with MAb 1.

**(B)** Immunoblot of ABO, RBO, and control seedlings probed with a phytochrome A-selective antibody (073D). The lanes are as given in **(A)**.

**(C)** Spectral activity of crude extracts from etiolated and red light-treated ABO, RBO, and control lines. $\Delta\Delta A$ values are arbitrary units. The dash in lanes 3, 6, and 9 indicates "not determined."

decreased to background levels after 6 hr of continuous red light irradiation (Figure 6C, lane 2), as did the immunologically detectable light-labile protein (Figure 6B, lane 2). However, increased spectral activity was maintained after 6 hr of red light illumination in the ABO line (Figure 6C, lane 8). The remaining measurable spectral activity is likely exclusively due to the overexpressed protein because the difference in activity observed between dark and red light treatments (Figure 6C, lanes 7 and 8) corresponds approximately to the loss of endogenous spectral signal (Figure 6C, lanes 1 and 2), which is presumably primarily attributable to phytochrome A. These data indicate that the overexpressed Arabidopsis *phyB*-encoded protein is photochemically active and light stable in the cell. Crude extracts from the RBO line, on the other hand, showed no increase in spectral activity relative to the control line in either darkness or after 6 hr of red light exposure. Because the RBO line showed a sixfold lower level of immunochemically detectable phytochrome B than the ABO line (see Figures 4 and 5), the expected increase in spectral activity associated with this amount of additional *phyB*-encoded protein would be below the level of detectability of the spectrophotometric assay used in these experiments. This finding together with the phenotype observed (Figure 1A) suggests that only relatively minor changes in the level of phytochrome B can lead to pronounced phenotypic consequences.

### Overexpressed Arabidopsis Phytochrome B Is Spectrally Similar to Phytochrome A

Figure 7 shows difference spectrum analysis of concentrated green tissue extracts derived from the RBO and ABO overexpressing lines as well as from the control line. Both overexpressing lines showed reproducibly

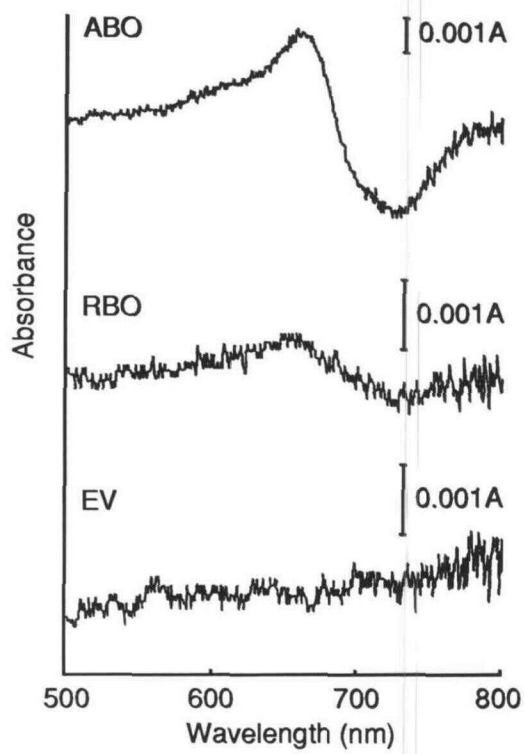


**Figure 7.** Spectral Activity of Overexpressed Rice and Arabidopsis B Phytochromes in Transgenic Arabidopsis.

Difference spectra (red minus far-red) of extracts derived from green tissue of the ABO (top), RBO (center), and control (EV, bottom) seedlings were recorded from 500 to 800 nm. Bars indicate absorbance differences. All samples were concentrated 10-fold from the crude extracts and depleted of chlorophyll (see Methods).

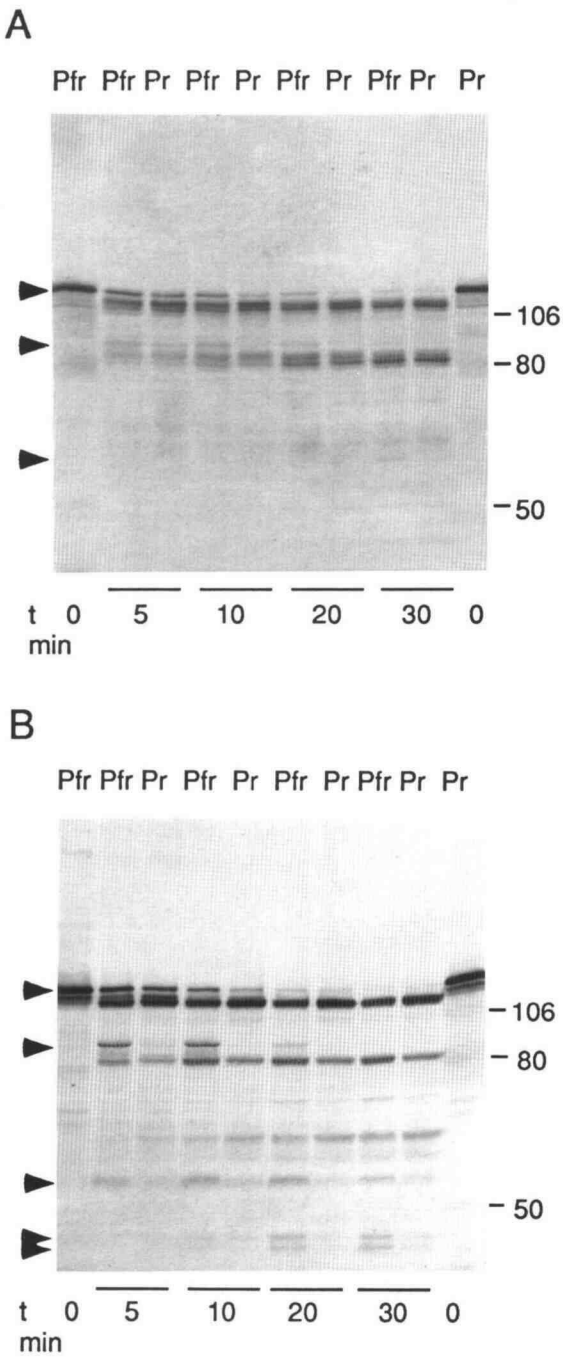


**Figure 8.** Photoinduced Conformational Changes in Overexpressed Rice and Arabidopsis B Phytochromes.

**(A)** Immunoblot showing differential in vitro proteolysis of overexpressed rice phytochrome B. Separate aliquots of a crude extract from the RBO line were irradiated with far-red or red light to convert the phytochrome pool to the Pr and Pfr forms, respectively. Chymotrypsin was added to 1.2 $\mu$g/mL and digestion was allowed to proceed for 0 to 30 min at 23°C. Fifty micrograms of crude protein was loaded per lane. The Pfr and Pr samples were run in alternating lanes for each time point. The blot was probed with MAb 2. Proteolytic fragments retained or appearing preferentially in the Pfr form are marked by arrows. Sizes of marker standards (in kilodaltons) are indicated.

**(B)** Immunoblot showing differential in vitro proteolysis of overexpressed Arabidopsis phytochrome B. Aliquots of a crude extract of the ABO line were irradiated as given in **(A)**. Chymotrypsin was added to 1.6 $\mu$g/mL and digestion was allowed to proceed as given in **(A)**. Twenty-two micrograms of crude protein was loaded per lane; the order of loading was as given in **(A)**. The blot was probed with MAb 2, and fragments retained or appearing preferentially in the Pfr form are marked with arrows. Sizes of marker standards (in kilodaltons) are indicated.

measurable spectral activity with this procedure. At the same concentration, however, green tissue extracts of the control line showed no detectable activity. Thus, the observed difference spectra appear to represent exclusively the overexpressed B phytochromes. The spectral activity in the ABO line was about sixfold higher (0.006 $\Delta A$) than that of the RBO line (0.001 $\Delta A$), paralleling the difference observed in levels of overexpressed protein immunochemically detected in both lines (Figures 5 and 6 and discussion above).

We estimate the absorbance maxima and minima for overexpressed Arabidopsis phytochrome B from the difference spectrum to be at about 664 and 729 nm, respectively. The peak positions for Arabidopsis phytochrome A have been reported at 665 and 730 nm, respectively (Parks et al., 1989). Thus, phytochrome A and phytochrome B may have very similar spectral properties in Arabidopsis. No estimate of peak positions was attempted for rice phytochrome B because of the low signal in this analysis.

It is well established that phytochrome A is synthesized in the Pr form in the cell (Vierstra and Quail, 1986). To determine in which form phytochrome B is synthesized in transgenic Arabidopsis, we performed spectral analysis of crude extracts from dark-grown seedlings of the ABO line. In three independent measurements, we could not detect significant changes in absorbance after initial far-red irradiation of the extract, whereas red irradiation yielded the same absorbance changes as obtained after successive far-red/red irradiations (data not shown). These results establish that all of the phytochrome in dark-grown plants, including the additional overexpressed phytochrome B, was present in the Pr form.

### Overexpressed Phytochrome B Undergoes Photoconversion-Induced Conformational Changes

The biological activity of phytochrome A is dependent on the attachment of the linear tetrapyrrole chromophore. Only the holoprotein undergoes reversible conformational changes in a red/far-red-light-dependent manner. These

conformational changes can be demonstrated by differential susceptibility to cleavage at several sites by exogenous proteases (Vierstra et al., 1984; Lagarias and Mercurio, 1985). After testing several different proteases, chymotrypsin was found most suitable for the analysis of phytochrome B. Figure 8 shows that chymotrypsin treatment of crude extracts derived from homozygous RBO (Figure 8A) and ABO (Figure 8B) lines yielded different proteolytic fragments when phytochrome B was in the Pfr or Pr form. These data provide direct evidence that the phytochrome B polypeptide undergoes light-induced conformational changes in the holoprotein.

### Short Hypocotyl Phenotype Is Light Dependent

To determine directly whether the short hypocotyl phenotype does indeed result from the photobiological activity of the overexpressed B phytochromes as opposed to an unrelated property of the transgenic lines, we examined the phenotypic behavior of seedlings grown in darkness and in the light. Seedlings from each of the three homozygous lines (control, RBO, and ABO) were grown under 16-hr light/8-hr dark cycles or in complete darkness. Figure 9 shows that the short hypocotyl phenotype was strictly light dependent. This result establishes that phytochrome B is a functionally active photoreceptor. Moreover, because we have determined that overexpressed phytochrome B is synthesized in the Pr form (see above) and undergoes reversible photoconversion-induced absorbance changes in the red and far-red regions of the spectrum, it may be deduced that phytochrome B, like phytochrome A, can exist in interconvertible inactive Pr and active Pfr forms.

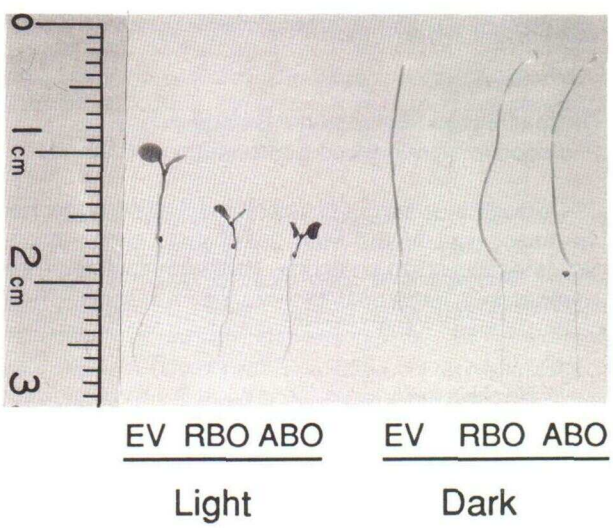


**Figure 9.** Light Dependence of the Short Hypocotyl Phenotype in Transgenic Phytochrome B Overexpressers.

Seven-day-old homozygous Arabidopsis seedlings of the empty vector (EV) control, RBO, and ABO transgenic lines grown in the light (16-hr day/8-hr night cycles) are on the left and those grown in the dark are on the right. The arrowhead indicates the hypocotyl-root junction.

### Exogenous Gibberellin Does Not Restore Wild-Type Phenotype in Overexpressers

Gibberellins have been implicated in many responses that are also controlled by phytochrome, such as lettuce seed germination (Frankland and Taylorson, 1983) and pea epicotyl elongation (Kende and Lang, 1964). Therefore, we wished to determine whether the short hypocotyl phenotype caused by phytochrome B overexpression could be reversed by treatment with gibberellic acid ($GA_3$). The effect of $GA_3$ treatment on hypocotyl elongation was investigated in the control, RBO, and ABO transgenic lines (data not shown). In all lines, the effect of very low ($10^{-7}$ M) $GA_3$ concentrations was indistinguishable from untreated controls. At $10^{-5}$ M $GA_3$, all three lines showed a similar increase in hypocotyl length (-fold length increase over untreated plants: control, 1.25; RBO, 1.43; ABO, 1.48). However, further increases in the $GA_3$ concentration yielded increases in height only for the control plants, suggesting that the response in the phytochrome B overexpressing lines was saturated. None of the $GA_3$ concentrations used restored the hypocotyl length in the RBO and ABO lines to that of the wild type.

## DISCUSSION

Transgenic homozygous Arabidopsis seedlings transformed with the Arabidopsis and rice *phyB* coding sequences (referred to as ABO and RBO, respectively) were shown to overexpress full-length Arabidopsis and rice phytochromes B that reacted specifically with newly produced phytochrome B-selective monoclonal and polyclonal antibodies (Figure 4). The overexpressed phytochrome B exhibits several properties indicative of photochemically active phytochrome, by comparison with those established previously in extensive studies of phytochrome A in etiolated seedlings (Pratt, 1983; Rüdiger and Scheer, 1983; Vierstra and Quail, 1986). First, difference spectra of green tissue extracts from both rice and Arabidopsis phytochrome B-overexpressing homozygous lines provide direct evidence that both phytochromes are spectrally active in the transgenic plants (Figure 7). Second, spectrophotometric analysis also shows that the overexpressed phytochrome B is synthesized in the Pr form in dark-grown transgenic plants (data not shown), as has been established for phytochrome A (Quail et al., 1973). Third,

both B phytochromes were cleaved differentially by exogenous protease after exposure to red or to far-red light. These results indicate that the overexpressed phytochrome B has formed a holoprotein in the plant and that it undergoes conformational changes in a red/far-red-light-dependent manner. Photoinduced conformational changes detectable by differential proteolytic sensitivity of phytochrome A have been shown to be dependent on holoprotein formation (chromophore attachment) in vitro and in vivo (Lagarias and Lagarias, 1989; Parks and Quail, 1991) and result from the transition from the inactive Pr to the active Pfr form. Thus, we conclude that *phyB* encodes a photochemically active photoreceptor that undergoes reversible, photoconversion-induced conformational changes analogous to those of phytochrome A.

The observation that, in all lines tested, overexpressed phytochrome B was stable in the light (Figure 6) is consistent with the properties of type 2 phytochrome. Moreover, because Arabidopsis phytochrome B is stable in transgenic Arabidopsis, the data demonstrate that the stability of this protein is not simply the result of expression of a foreign (i.e., monocot) polypeptide that is less susceptible to specific degradation in a heterologous host. On the other hand, the data presented here leave open the possibility that stability is the result of CaMV 35S promoter-driven expression of the protein in cell types that do not contain the degradative machinery responsible for selective Pfr turnover. The extent of such "ectopic" expression is currently unknown but can be assessed when the expression pattern and localization of the endogenous phytochrome B are better characterized. However, because recent independent experiments have shown that endogenous Arabidopsis phytochrome B is stable in the light (Somers et al., 1991), the observed stability of the overexpressed molecule is most likely primarily due to determinants residing within the polypeptide.

Light-stable phytochrome from oat showed spectral properties different from those of light-labile phytochrome in the same species in that the Pr absorption maximum was shifted 15 nm to a shorter wavelength (Tokuhisa and Quail, 1987, 1989; Pratt et al., 1991). In contrast, light-stable phytochrome showed similar spectral properties to light-labile phytochrome in pea (Konomi et al., 1987; Abe et al., 1989). Using transgenic Arabidopsis plants overexpressing Arabidopsis phytochrome B, we estimated absorption maxima and minima in difference spectra similar to those of Arabidopsis phytochrome A. Because phytochrome B appears to be a major component of the light-stable phytochromes thus far examined in Arabidopsis (Somers et al., 1991), it may follow that in dicots the light-labile and the predominant light-stable phytochrome have similar spectral properties. We propose to determine the spectral properties of overexpressed rice (monocot) phytochrome B upon further purification to assess whether it shows a similar shift toward shorter wavelength, as has been demonstrated for light-stable phytochrome from oat.

Several lines of evidence indicate that overexpressed rice or Arabidopsis phytochrome B regulates seedling photomorphogenesis in transgenic Arabidopsis. First, transgenic lines show a tight correlation between transformation with the *phyB* constructs and a short hypocotyl phenotype (Figures 3 and 5), inasmuch as all positive (kanamycin-resistant) transformants exhibit the short hypocotyl phenotype. Second, all short hypocotyl lines tested show increased levels of phytochrome B. Conversely, neither siblings that segregate for wild-type height nor the transformation control shows elevated levels of phytochrome B. Third, the short hypocotyl phenotype is strictly light dependent because elevated levels of phytochrome B have no effect on plants grown in the dark (data not shown). These data show that the phenotype is the result of altered photomorphogenesis, providing compelling evidence that it results directly from the overexpression of a biologically active photoreceptor. In conjunction with the finding that phytochrome B is synthesized in the Pr form (data not shown), we conclude that the Pfr form of phytochrome B is the biologically active form of the photoreceptor. In addition, because the phenotype observed in response to overexpression of Arabidopsis and rice B phytochromes is qualitatively similar, we conclude that the observed phenotype is due to elevated levels of the phytochrome B photoreceptor, rather than overexpression of a foreign (i.e., monocot) molecule in the plant.

The relatively small (threefold to fivefold) increase in immunochemically detectable phytochrome B over endogenous levels observed in the rice *phyB*-expressing lines (Figures 4 and 5 and data not shown) as well as the small increase in spectral activity (Figure 7) result, nonetheless, in a 70% reduction in hypocotyl length when compared with the transformation control (data not shown). Phytochrome B may be, therefore, highly active or, alternatively, the plant response system may be very sensitive to small changes in the photoequilibrium of phytochrome B. In contrast, the larger (18- to 30-fold) increases in phytochrome B in the Arabidopsis *phyB*-overexpressing lines lead to disproportionately small additional effects on hypocotyl elongation, i.e., a decrease in hypocotyl length to 15% of that of the transformation control. These results may indicate a saturation of the photomorphogenic response system with respect to levels of phytochrome B photoreceptor.

The pronounced inhibition of hypocotyl elongation in light-grown seedlings overexpressing rice or Arabidopsis phytochrome B is similar to the short hypocotyl phenotype observed in Arabidopsis plants overexpressing oat phytochrome A (Boylan and Quail, 1991). Hence, both phytochromes A and B can regulate hypocotyl elongation in transgenic Arabidopsis. These overexpression phenotypes complement the long hypocotyl phenotypes observed in photomorphogenic mutants deficient in either phytochrome A or phytochrome B. The long hypocotyl *aurea* mutant of tomato is deficient in light-labile phytochrome A

(Parks et al., 1987) but retains normal levels of light-stable phytochrome (Adamse et al., 1988c). Conversely, based on photobiological studies, it has been proposed that the *lh* long hypocotyl mutant of cucumber (Adamse et al., 1987, 1988b) and the *hy*3 long hypocotyl mutant of Arabidopsis (Koornneef et al., 1980) are deficient in light-stable phytochrome but have normal levels of light-labile phytochrome (Adamse et al., 1988a). The *hy*3 mutant has now been shown to be deficient in phytochrome B, the predominant light-stable phytochrome thus far examined in Arabidopsis (Somers et al., 1991). The observation that overexpression of either phytochrome A or phytochrome B leads to exaggerated shortening of hypocotyl length, in conjunction with the evidence from the mutant analyses that both phytochromes are necessary for correct hypocotyl length, suggests that the combined total amounts of active phytochrome A and B in the plant can determine the rate of hypocotyl elongation. This notion is supported by the finding that only slight increases in the level of phytochrome B in the overexpressing seedling (RBO, see above) cause a pronounced reduction of hypocotyl elongation. Thus mutant, wild type, and overexpressers may represent a continuum of phenotypic expression in response to increasing levels of phytochrome (Boylan and Quail, 1989). However, because the pattern of expression of phytochromes A and B may be altered spatially and temporally under the regulation of the CaMV 35S promoter in overexpressers, it is also possible that the effect in transgenic Arabidopsis is the result of "ectopic" expression per se rather than a simple reflection of the amount of phytochrome present.

Phytochrome function through regulation of phytohormone levels has been suggested by several authors (Frankland and Taylorson, 1983; Cosgrove, 1986). Gibberellin-deficient and gibberellin-insensitive mutants in Arabidopsis (Koornneef and van der Veen, 1980; Koornneef et al., 1985) show phenotypes potentially similar to phytochrome B-overexpressing transgenic Arabidopsis lines. Therefore, we tested whether the short hypocotyl phenotype caused by phytochrome B overexpression could be reversed by treatment with exogenous $GA_3$. None of the $GA_3$ concentrations used could restore the hypocotyl length in the overexpressing lines to that of the control line. Hence, our data provide no evidence in support of the hypothesis that $GA_3$ mediates the phenotype caused by overexpression.

In conclusion, transgenic Arabidopsis plants overexpressing phytochrome B have provided (1) a direct demonstration that this gene family member encodes a photochemically and biologically functional photoreceptor, (2) information on the spectral and physiological properties of the chromoprotein in vivo, and (3) support for the notion that phytochromes A and B may be capable of regulating similar cellular processes based on the similar phenotypes obtained when either is overexpressed in Arabidopsis. However, different photosensory roles have been proposed for light-labile and light-stable phytochromes: the high-irradiance response has been hypothesized to be mediated by light-labile phytochrome, whereas the shade avoidance and end-of-day far-red responses are attributed to the light-stable phytochrome pool (Smith and Whitelam, 1990). Arabidopsis seedlings overexpressing phytochrome B may prove useful in determining the properties of a light-stable phytochrome through analysis of the response of the transgenic lines under various light regimes. Comparison with the behavior of phytochrome A overexpressers may help to determine whether the different phytochromes can have discrete physiological effects in the plant. In addition, the overexpression system described here provides a powerful tool for further analysis of the biochemical and molecular properties of the phytochrome B photoreceptor. As we have demonstrated, transgenic Arabidopsis can be used to determine the spectral properties of low-abundance phytochromes. This approach may be helpful in the spectral characterization of other members of the gene family, alleviating the need for extensive purification, which in the past has led to partial degradation and resulted in changes of the spectral properties of the protein (Pratt et al., 1991). Finally, this Arabidopsis overexpression system can be used for rapid analysis of the functional domains of phytochrome B. In contrast to tobacco and tomato, Arabidopsis has a very short generation time, and the transformation procedure is both rapid and simple. In vitro mutagenesis of the coding sequence of phytochrome B and subsequent analysis in transgenic Arabidopsis are thus greatly facilitated.

## METHODS

### Construction and Cloning of Full-Length Rice and Arabidopsis *phyB* Coding Sequences

Full-length rice *phyB* was generated from a central 2586-bp BglII-EcoRI fragment of a cDNA clone and an upstream genomic clone as well as a downstream polymerase chain reaction (PCR)-generated clone (Dehesh et al., 1991). An SnaBI site was introduced 51 bp upstream of the ATG start codon by site-directed mutagenesis (Kunkel, 1985). Similarly, a KpnI site was introduced 52 bp downstream of the TGA stop codon. The plant transformation vector pMON316 (Rogers et al., 1987) was linearized at the ClaI site (blunted) and then digested with KpnI. In one ligation reaction, the linearized vector, the 5′ SnaBI to BglII fragment, the central BglII to EcoRI region, and the 3′ EcoRI to KpnI fragment were included to yield the final transformation vector pMRB316. For overexpression in *Escherichia coli*, the full-length rice *phyB* was reconstructed in plasmid pPO9 (Rottman et al., 1991). An XbaI site was introduced 4 bp 3′ to the ATG start codon. The XbaI-BglII (genomic), BglII-EcoRI (cDNA), and EcoRI-KpnI (PCR from RNA) fragments were ligated into pPO9 digested with NheI and KpnI. The full-length construct, pRB6, is a perfect translational fusion.

The full-length Arabidopsis *phyB* clone was generated by PCR from a cDNA clone of *phyB* in λ EMBL3 (Somers et al., 1991). Both primers contained KpnI sites. This clone was subcloned into pBluescript KS−. The fidelity of approximately 75% of the PCR-generated clone was verified by sequence analysis (Tabor and Richardson, 1987), and the full-length clone was transferred into pMON316 to yield the final transformation vector pMAB316. This clone contains none of the original 5′ and 3′ untranslated leader sequences. The correct orientation in pMON316 was ascertained by sequence analysis and restriction mapping. For overexpression in *E. coli*, the Arabidopsis *phyB* clone was introduced into the plasmid pet-3c (Somers et al., 1991).

### Arabidopsis Transformation

*Arabidopsis thaliana* (ecotype NO-O) seedlings were grown in sterile culture for 3 weeks on growth medium (GM, Valvekens et al., 1988). The cointegrate vector was transferred into *Agrobacterium tumefaciens* by triparental mating as described by Rogers et al. (1986). Roots were infected with *A. tumefaciens* carrying pMON316 alone or pMRB316 or pMAB316. The root transformation procedure was according to Valvekens et al. (1988).

### Plant Material and Extraction

Seeds were sterilized for 10 min in 30% bleach and 0.02% Triton X-100, plated onto GM, and incubated at 4°C for 3 days. Kanamycin sensitivity was assayed on GM plates using 50 μg/mL kanamycin. Otherwise, plants were grown for 7 days without kanamycin selection in the light (16-hr day/8-hr night cycles). Alternatively, plants were grown on identical medium but in darkness for 7 days or in darkness followed by 6 hr of red light irradiation (0.2 $W/m^2$). Crude extracts were prepared essentially as described in Parks et al. (1987). The 2 × extraction buffer (100 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.6, 50% ethylene glycol, 5 mM EDTA, 56 mM mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride) was adjusted to 1 μg/mL aprotenin, pepstatin, and leupeptin just before use. Tissue (400 mg) was ground in 300 μL of 2 × extraction buffer, and after 10-min centrifugation at 48,000*g*, the supernatant was mixed 1:1 with 2 × sample buffer (Laemmli, 1970) and frozen in liquid nitrogen. Crude protein concentrations were determined by Bradford analysis (Bradford, 1976). All extractions were performed in dim green safelight. Green plant extracts for difference spectrum analysis were prepared essentially as described in Pratt et al. (1991). To the extraction buffer aprotenin, pepstatin, and leupeptin were added to 1 μg/mL. By scaling down the extraction procedure, the tissue could be handled faster and spectral activity could be measured within 1 hr of the start of extraction. The ammonium sulfate pellet was routinely resuspended in resuspension buffer at one-tenth of the volume of the crude extract. For the gibberellin analysis, plants were germinated on GM plates containing $10^{-7}$, $10^{-5}$, $10^{-4}$, and $10^{-3}$ M $GA_3$ (Sigma). Stock solutions of $GA_3$ were prepared at 100 mM in DMSO.

### Production and Purification of Rice and Arabidopsis B Phytochromes in *E. coli*

Overexpression of pRB6 and Arabidopsis *phyB* in pet-3c in BL21 (DE3) was as described in Studier and Moffat (1986). Inclusion bodies were isolated as described in Nagai and Thøgersen (1987) with the following modifications. For the DNase treatment, $MnCl_2$ was omitted and the DNase concentration was raised to 0.5 mg/mL. Resuspension of the final pellet was in 1:100 to 1:50 of the original volume in 50 mM Tris, pH 7.8, 0.5 mM DTT, 1 mM EDTA, 6 M urea, and 1% SDS. The proteins were further purified by electrophoresis on a 3.0-mm preparative 6% acrylamide gel. Bands were visualized with $CuCl_2$ staining (Lee et al., 1987), excised, and electroeluted into 0.01% SDS, 190 mM glycine, 25 mM Tris, pH 7.8, at 100 V for 24 hr.

### Antibody Preparation

Four mice (Swiss Webster and BALB/c) were used for antibody production. Polyclonal ascites were produced essentially as described in Hunter et al. (1990). The antigen was *E. coli*-produced, gel-purified rice phytochrome B. Ten micrograms of antigen emulsified in 2 × reconstituted adjuvant (RIBI Adjuvant System, RIBI Immunochemical Research Incorporated, Hamilton, MT) was used for four consecutive immunizations. Polyclonal ascites were obtained after 6 weeks.

The monoclonal cell line supernatants were obtained essentially as described in Karu (1991). After hyperimmunization, the spleen of a high-titer mouse was removed, and electrofusion between myeloma and spleenocytes was performed. Hybridoma supernatants were transferred to immunological assay plates using a robotic sampling system (Karu et al., 1985). In ELISA-based screens, 300 positive cell lines were identified that reacted with the rice phytochrome B antigen. These were further tested for phytochrome B selectivity on ELISA microtiter plates and immunoblots. Crude epitope mapping was performed by testing *E. coli*-produced deletion clones of rice phytochrome B. Cell lines that recognized the same deletion-defined region of the polypeptide were tested further for differential recognition of proteolytic patterns of rice phytochrome B obtained with V8 protease (Cleveland, 1983) and other proteases. Six phytochrome B selective hybridoma lines were subcloned to obtain monoclonal cell lines.

### Immunoblotting

Proteins from crude extracts were separated by SDS-PAGE (Laemmli, 1970). The acrylamide gels consisted of 1.5-mm thick, 6% resolving, and 4.5% stacking gels except after proteolysis, where 8% to 5% resolving gradient gels with 4% stacking gels were used. After blotting onto nitrocellulose membranes (600 mA for 2 hr), the remaining binding sites were saturated with 4% milk buffer (powdered milk dissolved in 20 mM Tris, pH 7.5, 0.15 mM NaCl, 0.05% Nonidet P-40). Both monoclonal cell supernatants were diluted 1:100 in this buffer. The polyclonal antiserum was depleted of Arabidopsis phytochrome B-reactive antibodies by incubation with one-half volume of *E. coli*-overproduced Arabidopsis *phyB*-encoded protein at 4°C overnight with stirring. The antibody-antigen complexes were removed by centrifugation in a microcentrifuge for 20 min. The supernatant was diluted 1:200 in the milk buffer for use on immunoblots. After 3-hr incubation with the primary antibodies, the blots were washed 3 × 10 min with the milk buffer and incubated for 1 hr with anti-mouse Ig alkaline phosphatase conjugate (Promega) at 1:5000 dilution in milk buffer. After washing as above, the blots were developed in 100 mM Tris, pH 8.9, 100 mM NaCl, 5 mM $MgCl_2$ solution, to which 75

mg/mL 5-bromo-4-chloro-3-indolyl phosphate and 150 mg/mL nitroblue tetrazolium were added. Video imaging was used for densitometric analysis of immunoblots to determine the relative concentration of phytochrome in different samples.

### In Vitro Proteolysis

Crude extracts were prepared as above except aprotenin was omitted from the extraction buffer. After extraction, the protease inhibitors were removed by 1-min centrifugation through a packed Sephadex G-50 spin column, which had been equilibrated with 1 × extraction buffer without proteases. Crude extract (600 μL) was loaded on a column with a 3-mL bed volume and centrifuged at 5000*g* for 1 min. Samples were pooled, divided in half, and irradiated for 5 min with red or far-red light (on ice). Time 0 time points were taken immediately after the irradiation. Chymotrypsin (Sigma) aliquots (prepared at 0.5 mg/mL in 1 × extraction buffer without protease inhibitors and stored at −80°C) were thawed on ice, diluted 1:10 with 1 × extraction buffer, and added to a final concentration of 1.6 μg/mL for the ABO line (0.56 μg/mL crude protein) or 1.2 μg/mL for the RBO line (1.1 μg/mL crude protein). Digestion was stopped after 5, 10, 20, and 30 min by removal of aliquots and addition to boiling sample buffer, followed by freezing in liquid nitrogen.

### Spectral Analysis

Crude extract supernatants (as described above) were prepared from 7-day-old dark-grown seedlings or after irradiation of such seedlings for 6 hr with red light. Spectrally active phytochrome ($\Delta\Delta A$) was measured with 730- and 800-nm measuring beams in a dual-wavelength spectrophotometer (Pratt et al., 1985) using $CaCO_3$ as a scattering agent, and the resulting values were presented in arbitrary units per gram fresh weight of tissue. Difference spectra were measured in clear, chlorophyll-depleted, concentrated green extracts (prepared as described above to yield a resuspended ammonium sulfate precipitate 10-fold concentrated relative to the crude extract) in a Shimadzu dual-wavelength, double-beam recording spectrophotometer (model UV-3000, Shimadzu Corporation, Kyoto, Japan).

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