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Phytochrome-Deficient *hy*1 and *hy*2 Long Hypocotyl Mutants of Arabidopsis Are Defective in Phytochrome Chromophore Biosynthesis

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The *hy*1 and *hy*2 long hypocotyl mutants of Arabidopsis contain normal levels of immunochemically detectable phytochrome A, but the molecule is photochemically nonfunctional. We have investigated the biochemical basis for this lack of function. When the *hy*1 and *hy*2 mutants were grown in white light on a medium containing biliverdin IXα, a direct precursor to phytochromobilin, the phytochrome chromophore, the seedlings developed with a morphological phenotype indistinguishable from the light-grown wild-type control. Restoration of a light-grown phenotype in the *hy*1 mutant was also accomplished by using phycocyanobilin, a tetrapyrrole analog of phytochromobilin. Spectrophotometric and immunochemical analyses of the rescued *hy*1 and *hy*2 mutants demonstrated that they possessed wild-type levels of photochemically functional phytochrome that displayed light-induced conformational changes in the holoprotein indistinguishable from the wild type. Moreover, phytochrome A levels declined in vivo in response to white light in rescued *hy*1 and *hy*2 seedlings, indicative of biliverdin-dependent formation of photochemically functional phytochrome A that was then subject to normal selective turnover in the far-red-light-absorbing form. Combined, these data suggest that the *hy*1 and *hy*2 mutants are inhibited in chromophore biosynthesis at steps prior to the formation of biliverdin IXα, thus potentially causing a global functional deficiency in all members of the phytochrome photoreceptor family.

INTRODUCTION

Light is one of the most important environmental signals that regulate plant growth and development. Three photoreceptors: phytochrome, a blue/UV-A photoreceptor, and a UV-B photoreceptor, mediate the transduction of the light stimuli that induce such developmental responses (Kendrick and Kronenberg, 1986). Of these three, phytochrome has been characterized the most extensively. This molecule consists of two covalently linked moieties, a polypeptide and a linear tetrapyrrole chromophore (Vierstra and Quail, 1986), and is capable of existing in either of two photointerconvertible forms, the red-light-absorbing (Pr) form and the far-red-light-absorbing (Pfr) form (Kendrick and Kronenberg, 1986). Photoconversion to the Pfr form initiates a large number of diverse biochemical and physiological responses, but the primary induction mechanism is as yet unknown (Kendrick and Kronenberg, 1986; Furuya, 1987).

Although there is an extensive body of data on the physiological, spectrophotometric, and biochemical properties of this regulatory molecule, it has only recently become apparent that phytochrome is actually a family of photoreceptors encoded by multiple divergent genes (Sharrock and Quail, 1989; Dehesh et al., 1991). This gene family consists of at least three differentially regulated members designated *phyA*, *phyB*, and *phyC* that encode prospective holoproteins termed phytochrome A, B, and C, respectively (Sharrock and Quail, 1989; Dehesh et al., 1991; Quail, 1991). By comparing sequenced regions of phytochrome purified from etiolated oat and pea with the predicted amino acid sequences of cloned *phy* cDNAs and genes from these plants (Lagarias and Rapoport, 1980; Hershey et al., 1985; Yamamoto, 1987; Grimm et al., 1988; Sato, 1988; Jones and Quail, 1989), it can be concluded that the type of phytochrome that predominates in etiolated tissue is encoded by *phyA* genes. In retrospect, it is now clear that the vast majority of molecular and biochemical phytochrome research conducted both in vivo and in vitro has involved characterization of phytochrome A (Quail, 1991). In contrast, relatively little is known about the molecular properties of the other phytochrome types. The possibility that the different types of phytochrome possess unique roles in plant development is only beginning to be explored (Adamse et al., 1988; Smith and Whitelam, 1990; Whitelam and Smith, 1991).

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As one approach toward defining the mechanism of phytochrome action, photomorphogenic mutants aberrant in photoreceptor activity have been sought (Koornneef and Kendrick, 1986; Adamse et al., 1988). Plants mutated in a phytochrome structural gene could be utilized in complementation analyses as hosts for transformation with phytochrome coding sequences that have been subjected to site-specific mutagenesis. The *aurea* (*au*^{w}) mutant of tomato and the *hy*1, *hy*2, and *hy*6 long hypocotyl mutants of Arabidopsis have been investigated as candidates for such *phy* gene mutations. For tomato, we have found that the *au*^{w} lesion causes a deficiency in spectrophotometrically and immunochemically detectable phytochrome, presumably phytochrome A (Parks et al., 1987). RNA gel blot and in vitro translation analyses have shown, however, that this mutant possesses wild-type levels of translatable phytochrome mRNA that yields an immunoprecipitable polypeptide, indistinguishable in molecular mass and abundance from the wild-type phytochrome A polypeptide (Sharrock et al., 1988). These and genomic mapping data suggest that the phytochrome deficiency in this mutant is not attributable to a mutation in a *phyA* gene (Sharrock et al., 1988). On the other hand, for Arabidopsis it has been found that, although etiolated *hy*1, *hy*2, and *hy*6 seedlings contain no spectrophotometrically detectable phytochrome, these three mutants each possess immunochemically detectable phytochrome at the same levels and molecular mass as found in etiolated wild-type tissue (Chory et al., 1989; Parks et al., 1989). Our *hy*1 and *hy*2 studies (Parks et al., 1989) used an antibody that has been subsequently shown to be selective for phytochrome A (Somers et al., 1991), thus indicating that these previous data reflect predominantly, if not exclusively, the behavior of phytochrome A. These data showed that the immunochemically detectable 116-kD phytochrome A polypeptide in these two mutants does not yield the diagnostic in vitro proteolytic fragmentation patterns that result from differential cleavage in the Pr and Pfr chromoprotein conformations (Parks et al., 1989). This result demonstrates that the majority of phytochrome A in *hy*1 and *hy*2 is not capable of undergoing conformational changes in response to light.

The initial possibility that this lack of photochemical activity in *hy*1 and *hy*2 results from a lesion in the *phyA* gene itself has been eliminated by mapping data that show that *phyA* (Chang et al., 1988), *hy*1, and *hy*2 (Koornneef et al., 1983) loci are all located on different chromosomes. Thus, the possibility has been raised that *hy*1 and *hy*2 are mutated in genetic components involved in biosynthesis or availability of phytochromobilin, the phytochrome tetrapyrrole chromophore (Parks et al., 1989). This possibility has important implications because it suggests that all members of the phytochrome family may be functionally impaired in these mutants. Such chromophore-deficient mutants, while not being suitable as gene-transfer recipients for phytochrome polypeptide mutagenesis studies, would be extremely useful for investigating chromophore biosynthesis and for examining the physiological consequences of deficiencies in all phytochrome family members (Smith and Whitelam, 1990). Previous biochemical studies that used inhibitors of chromophore synthesis have established that biliverdin IXα is the probable direct precursor to phytochromobilin in vivo (Elich et al., 1989). Here we have exploited this observation to test the hypothesis that the *hy*1 and *hy*2 lesions result in a deficiency in the production or availability of phytochromobilin. We have grown these mutants in the presence of natural and nonnatural tetrapyrrole chromophore precursors and have monitored for the restoration of photochemically and biologically functional phytochrome.

RESULTS

Tetrapyrroles Restore Normal Photomorphogenesis in the *hy*1 and *hy*2 Mutants

Initially, we tested whether exogenously supplied crude biliverdin could restore the wild-type light-grown phenotype in the *hy*1 and *hy*2 mutants through rescue of photochemically and biologically functional phytochrome. Crude biliverdin is a readily available mixture of biliverdin isomers, including biliverdin IXα as one constituent (McDonagh and Palma, 1980; Elich and Lagarias, 1987). This specific isomer is the proposed direct precursor to phytochromobilin (Elich and Lagarias, 1987). Figure 1 compares the structures of these and the other tetrapyrroles referred to in this study.

Figure 2 shows that when the *hy*1 and *hy*2 mutants were germinated and grown in white light in the presence of crude biliverdin, hypocotyl elongation decreased with increasing biliverdin concentration. For this same concentration range, the length of the hypocotyl was unaffected for the wild type and the *hy*3, *hy*4, and *hy*5 long hypocotyl mutants that all possess photochemically functional phytochrome A (Chory et al., 1989; Parks et al., 1989). This result indicates that the effect of this biliverdin mixture was specific with respect to the lesions of both *hy*1 and *hy*2. Visual examination of biliverdin-treated *hy*1 and *hy*2 seedlings revealed that the cotyledons also appear more green than the same untreated mutant seedlings (data not shown). Thus, two observable phytochrome-regulated responses (hypocotyl elongation and chlorophyll production) appear to be restored in these two mutant lines. Figure 3 shows a quantitative analysis of hypocotyl lengths for seedlings grown in darkness or white light on different biliverdin concentrations. Biliverdin had no effect on the hypocotyl length of the wild type or any of the *hy* mutants when these plants were grown in complete darkness (Figure 3). However, the hypocotyl lengths for *hy*1 and *hy*2 were progressively shorter with increasing biliverdin

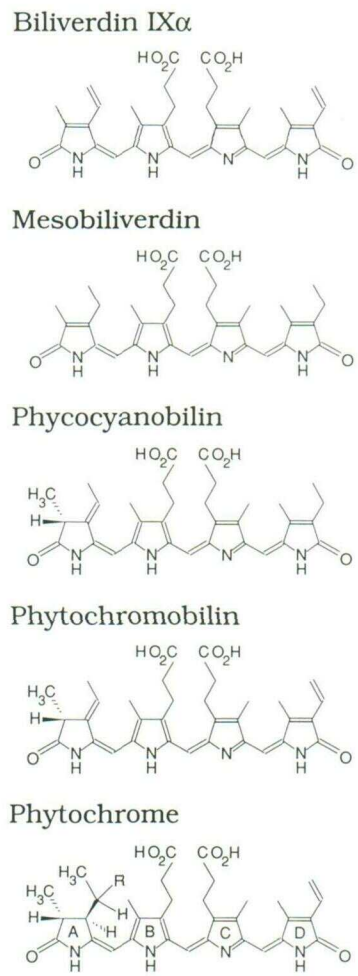

Figure 1. Structures of Linear Tetrapyrroles.

Biliverdin, biliverdin IXα, mesobiliverdin, and phycocyanobilin were used in experiments where specified. The compounds were obtained as described in Methods. The proposed structure for phytochromobilin is shown for comparison (Rüdiger and Scheer, 1983). Pyrrole ring designations are displayed in the last structure at the bottom of the figure. This last structure also shows a thioether linkage between phytochromobilin and a cysteine residue at or near position 321 of the phytochrome polypeptide (R) depending on the plant species (Lagarias and Rapoport, 1980; Hershey et al., 1985; Sharrock et al., 1986).

concentration when these two mutants were grown in the light (Figure 3). Therefore, the effect of biliverdin on the hypocotyl length of the *hy*1 and *hy*2 mutants was contingent upon a light signal, which is consistent with the restoration of a biologically active photoreceptor. The extent of light-induced inhibition of hypocotyl elongation was not as great in the *hy*2 as in the *hy*1 mutant over the range of biliverdin concentrations tested (Figure 3). This result suggests that the *hy*2 lesion may affect a step that either precludes total restoration of functional phytochrome using exogenously supplied biliverdin or has pleiotropic effects such that complete rescue of the fully

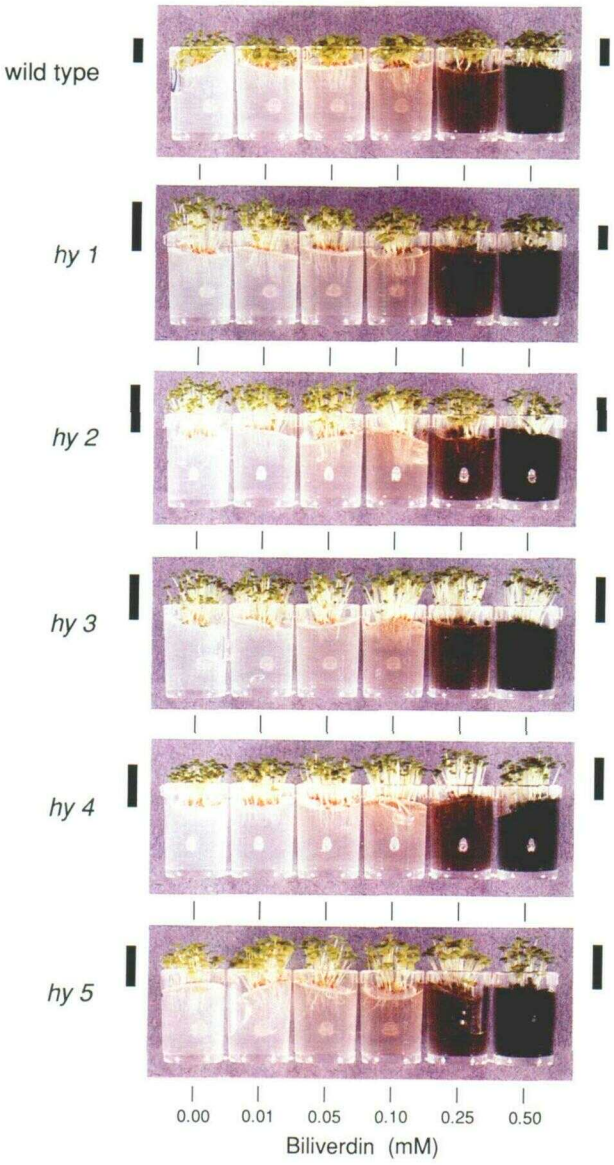

Figure 2. Effect of Biliverdin on Light-Grown Wild-Type and Mutant Arabidopsis Seedlings.

The wild type and the *hy* mutants (*hy*1, *hy*2, *hy*3, *hy*4, and *hy*5) were germinated on various concentrations of biliverdin and grown for 4 days in continuous white light as described previously in Methods. Solid black bars on the left and right side of the figure represent the mean height of the seedling hypocotyls in the absence of biliverdin and at the highest biliverdin concentration, respectively.

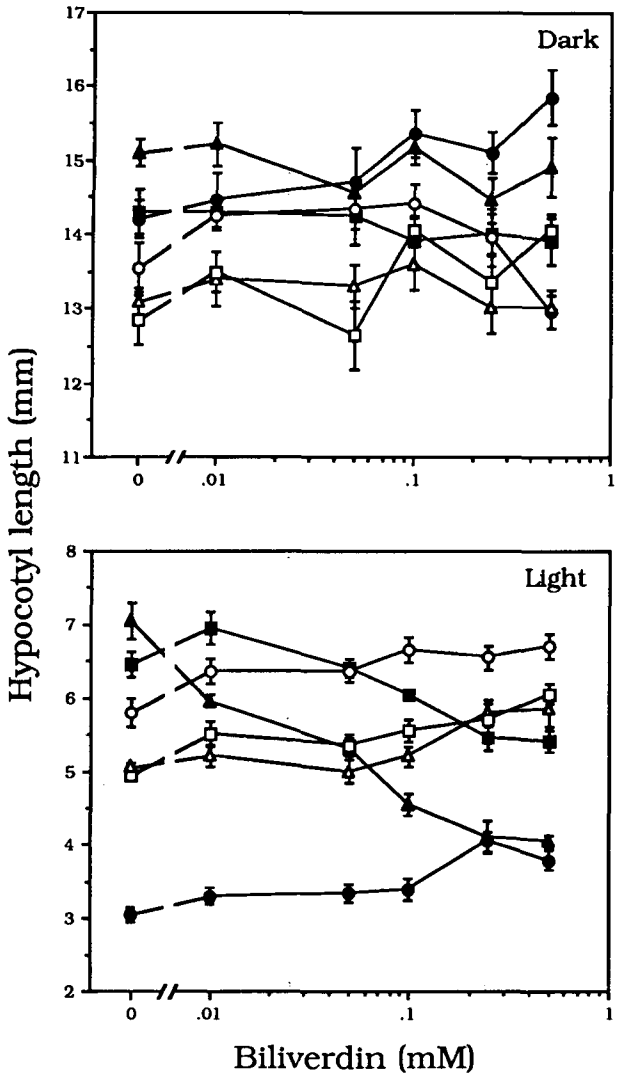

Figure 3. Dose-Response Curves for the Effect of Biliverdin on Hypocotyl Elongation of Dark- and Light-Grown Wild-Type and *hy* Mutants of Arabidopsis.

The wild type and the five *hy* mutants were germinated and grown for 4 days in white light or darkness on various concentrations of biliverdin as described in Methods. The mean values are plotted for 10 seedlings at each point along the curves. Bars represent 1 standard error of the mean. ●, wild type; ▲, *hy*1; ■, *hy*2; ○, *hy*3; △, *hy*4; □, *hy*5.

functional photoreceptor only partially alleviates the effects of this lesion.

Because a crude mixture of biliverdin isomers was used in the above experiments, we wished to examine more precisely the chemical structure requirements for restoration of normal photomorphogenesis in *hy*1. Three purified tetrapyrroles were used: biliverdin IXα, phycocyanobilin, and mesobiliverdin (Figure 1). Phycocyanobilin is the naturally occurring chromophore for C-phycocyanin, a light-harvesting pigment involved in photosynthesis in many algal species (Glazer, 1988). It is structurally identical to phytochromobilin, with the exception of an ethyl side group substitution for the vinyl group on the D-ring of the chromophore. Previous reports have shown that phycocyanobilin can substitute for phytochromobilin in the synthesis of a photochemically functional phytochrome holoprotein in extracts and explants of inhibitor-treated oat tissue (Elich and Lagarias, 1989; Elich et al., 1989). In addition, phytochrome apoprotein synthesized by in vitro transcription and translation has been reported to self-assemble with this substitute chromophore, suggesting that covalent attachment occurs without modification of the analog structure (Lagarias and Lagarias, 1989). Mesobiliverdin was chosen as a potential negative control. It possesses vinyl to ethyl group substitutions on rings A and D, distinguishing this tetrapyrrole from biliverdin IXα. The ethyl group on ring A renders mesobiliverdin stable against modification, thus potentially precluding its conversion in vivo to the ethylidene structure on the A-ring necessary for the formation of the thioether linkage with a cysteine residue in the phytochrome apoprotein (see phytochromobilin, Figure 1).

We tested these three tetrapyrroles in addition to the crude biliverdin preparation for their ability to rescue light-regulated inhibition of hypocotyl elongation in the *hy*1 mutant. *hy*2 was not tested in these particular feeding experiments due to the limited availability of the specific tetrapyrrole isomers. Figure 4 shows that none of these tetrapyrroles significantly altered the elongation of the hypocotyl in the wild type, *hy*1, or *hy*3 when these plants were grown in darkness. Similarly, no significant effect on hypocotyl elongation was observed for the wild type and *hy*3 when these two lines were grown in white light. Although data for the effects of biliverdin IXα on the *hy*3 mutant are lacking, *hy*4, another long hypocotyl mutant that also possesses photochemically functional phytochrome A, was not affected by biliverdin IXα when this mutant line was grown in darkness or in white light (data not shown). Crude biliverdin, biliverdin IXα, and phycocyanobilin each restored normal photomorphogenesis in *hy*1 when this line was grown in white light (Figure 4). For the same light conditions, however, mesobiliverdin was not effective. These results suggest that specific structural configurations of the tetrapyrrole, expected to permit covalent attachment to the phytochrome apoprotein, are necessary to establish phenotypic rescue in the *hy*1 and probably the *hy*2 mutant lines.

Biliverdin Restores Normal Spectrophotometric and Conformational Properties to Phytochrome in the *hy*1 and *hy*2 Mutants

To determine directly whether phenotypic rescue is associated with restoration of a functional photoreceptor, we

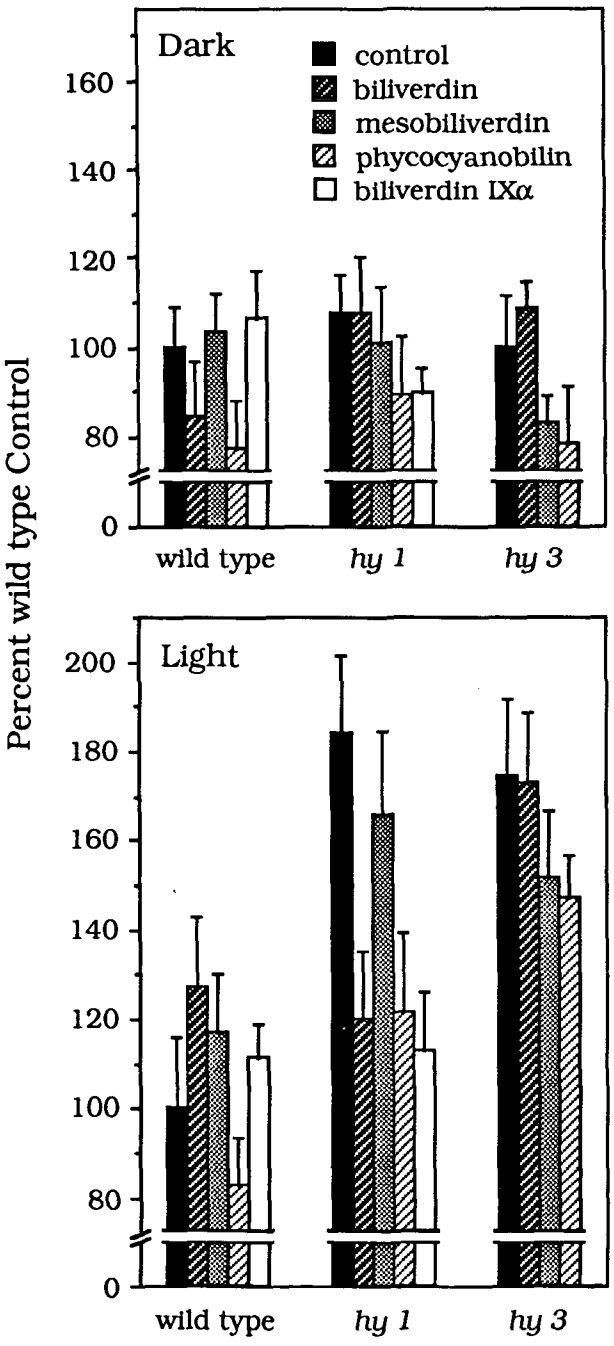

Figure 4. Effect of Various Tetrapyrroles on Hypocotyl Elongation of the Dark- and Light-Grown Wild-Type, *hy*1, and *hy*3 Arabidopsis Seedlings.

Seeds were germinated and grown in continuous white light or darkness for 4 days. All tetrapyrroles were tested at 0.1 mM. Values are the mean of 10 individual seedlings and are plotted as a percent of the wild-type control (no added tetrapyrrole) in continuous light or darkness. Bars represent 1 standard deviation.

assayed for photochemically functional phytochrome directly in biliverdin-grown *hy*1 and *hy*2 seedlings. Table 1 shows that, whereas *hy*1 and *hy*2 contained no detectable levels of spectrally active phytochrome when grown without biliverdin, the same mutants grown with biliverdin contained wild-type levels of photochemically detectable phytochrome. The levels of spectrophotometrically detectable phytochrome in wild-type seedlings were not affected by biliverdin treatment. In addition, biliverdin in buffer alone did not generate a measurable spectral signal (data not shown), indicating that the values measured were due specifically to the recovery of photochemically functional phytochrome in the biliverdin-grown *hy*1 and *hy*2 mutants. The relatively small absorption changes obtained with the etiolated Arabidopsis tissue precluded accurate estimates of phytochrome absorption peak positions through difference spectra analysis (Parks et al., 1989). The result for *hy*2 is particularly noteworthy inasmuch as it suggests that the incomplete rescue of light-regulated hypocotyl inhibition in this mutant (Figure 3) did not result from only partial restoration of functional phytochrome under those growth conditions. The data indicate instead that the effect of the *hy*2 lesion is more probably pleiotropic, affecting other unknown parameters involved in the response as well as the photoreceptor.

The two light-absorbing forms of phytochrome (Pr and Pfr) represent two conformationally distinct forms of the holoprotein. These conformational differences are revealed by distinctive polypeptide profiles generated when parallel aliquots containing Pr or Pfr are proteolytically degraded in vitro (Vierstra and Quail, 1982; Parks et al., 1987, 1989). Thus, differential susceptibility of the two phytochrome forms to proteolysis not only provides an additional means to assay for the photochemical integrity of the molecule, but also permits an assessment of the fidelity of conformational changes in the holoprotein. This method had established previously that the immunochemically detectable phytochrome A in *hy*1 and *hy*2 does not exhibit the

Table 1. Spectrally Detectable Phytochrome in Crude Extracts of Etiolated Arabidopsis Seedlings

	$\Delta\Delta A_{730-800}$ ($A \times 10^{-4}$)	
Seedlings	−BV[a]	+BV[b]
Wild type	17.3	16.7
*hy*1	ND[c]	18.9
*hy*2	ND	16.4

Crude extracts of 4-day-old, dark-grown wild-type, *hy*1, and *hy*2 seedlings were prepared, and phytochrome was measured spectrophotometrically as described in Methods.

[a] Without biliverdin.

[b] With biliverdin.

[c] None detected.

in vitro degradation pattern normally generated by the functional Pr and Pfr forms from wild-type tissue (Parks et al., 1989). Using this same diagnostic assay, we examined here the pattern of in vitro proteolysis exhibited by phytochrome A extracted from both *hy*1 and *hy*2 seedlings grown in the presence of biliverdin. Figure 5 shows that this pattern is identical to that observed for phytochrome A extracted from wild-type tissue, and contrasts with the pattern detected on immunoblots of proteins extracted from untreated *hy*1 and *hy*2 seedlings. Immunoblots of far-red-irradiated aliquots of crude extracts from biliverdin-grown *hy*1 and *hy*2 seedlings (phytochrome A primarily as Pr) show that the major degradation product is a 111-kD polypeptide fragment. In contrast, a 64-kD polypeptide fragment is the primary degradation product in red-irradiated aliquots of these same extracts (phytochrome A primarily as Pfr). Growth in the presence of biliverdin did not affect the proteolytic pattern for phytochrome A extracted from the wild-type seedlings (Figure 5). A direct comparison of the wild type and the rescued mutants showed that when wild-type and *hy*1 tissues, each grown in the presence of biliverdin, were homogenized together and checked for differential proteolysis of Pr and Pfr, the fragmentation pattern of phytochrome A from the *hy*1 mutant was not different from that of the wild type (data not shown), further supporting the conclusion that phytochrome in rescued mutant seedlings is now structurally equivalent to the wild type.

Biliverdin Restores In Vivo Molecular Properties to Phytochrome in *hy*1 and *hy*2

To further examine the fidelity of the molecular behavior of phytochrome A in rescued *hy*1 and *hy*2 seedlings, we monitored the stability of the molecule in response to light in vivo. Phytochrome A is much less stable in vivo as Pfr than as Pr with a half-life of approximately 1 hr in the Pfr form (Colbert et al., 1983). This difference presumably reflects a difference in recognition of the two pigment forms by the endogenous protein turnover system (Shanklin et al., 1987). Figure 6 shows that for light-treated Arabidopsis seedlings, where phytochrome A is present as the less stable Pfr form, direct extracts of wild-type tissue contained strongly reduced levels of immunochemically detectable phytochrome A compared to dark-grown plants whether grown with or without biliverdin. In contrast, light-treated *hy*1 and *hy*2 seedlings grown without biliverdin still contain considerable immunochemically detectable phytochrome A, albeit partly reduced by comparison to the dark-grown tissue. These lower levels suggest that a fraction of phytochrome A in these mutants may be photochemically functional, and thus susceptible to protein turnover as Pfr. This result is qualitatively consistent with previous observations where analysis of differential proteolysis of phytochrome A in red- and far-red-irradiated protein

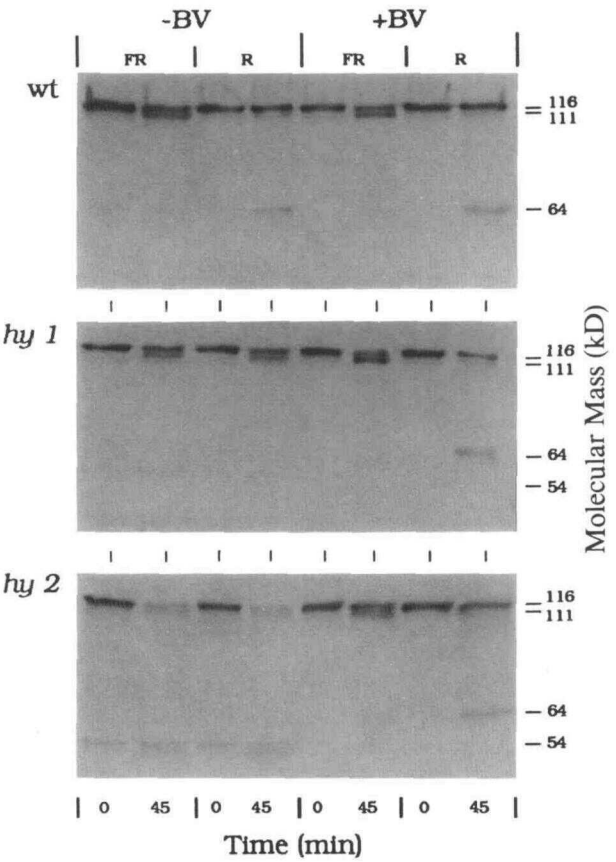

Figure 5. Effect of Biliverdin on In Vitro Proteolysis of Wild-Type, *hy*1, and *hy*2 Arabidopsis Phytochrome A.

Crude extracts prepared from etiolated seedlings of the wild type (wt), *hy*1, or *hy*2, grown in the absence (−BV) or presence (+BV) of biliverdin, were divided in half and irradiated for 1.5 min at 0°C with either red (R) or far-red (FR) light (Vierstra and Quail, 1982). An aliquot was removed for the zero time point, added to an equal volume of sample buffer (125 mM Tris, pH 6.8, 1.4 M 2-mercaptoethanol, 4% SDS, 7.5% glycerol, 0.02% bromphenol blue), and rapidly frozen at −80°C. Subtilisin (0.05 mg/mL in extraction buffer) was added to the balance of the extracts at a dilution of 1:30 (protease:extract). Proteolysis at 25°C was terminated by the addition of an equal volume of sample buffer followed by rapid freezing to −80°C. All samples were boiled for 3 min prior to loading on the gel. Fifty microliters (approximately 200 μg of total protein) of each of these samples was loaded onto an acrylamide gel, subjected to electrophoretic separation, and electroblotted onto nitrocellulose. The blot was then probed with monoclonal antibody 073d. The sizes of the various polypeptide species detected by the antibody are shown to the right of the figure. All seedlings used for this experiment were generated by growth on medium supplemented with gibberellin A_3 at 0.1 mM (see Methods). Gibberellin A_3 improves germination in darkness but does not affect the proteolysis of phytochrome A in vitro irrespective of biliverdin treatment (data not shown).

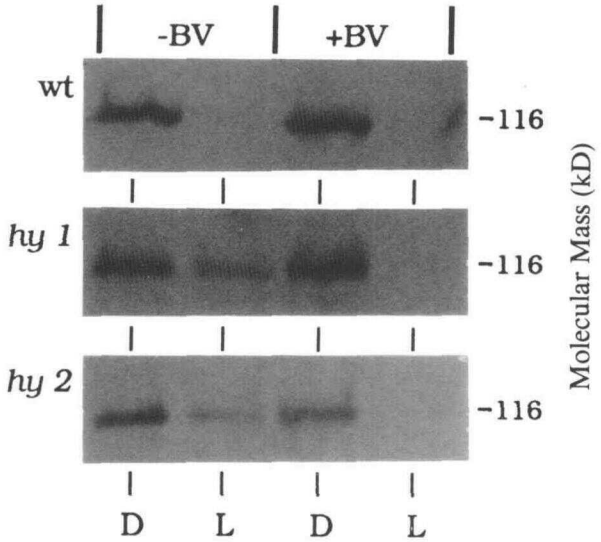

Figure 6. Effect of Biliverdin on the Levels of Immunochemically Detectable Phytochrome A in Direct Extracts of Wild-Type, *hy*1, and *hy*2 Arabidopsis Seedlings Grown in White Light or Darkness.

Seedlings were grown for 6 days in darkness or 5 days darkness plus 1 day continuous white light with (+BV) or without (−BV) 0.5 mM biliverdin as described in Methods. Direct extracts were prepared from seedlings of the wild type (wt), *hy*1, or *hy*2 as described in Methods. Boiled samples were loaded at 23, 40, and 25 μL for each set of lanes for the wild-type, *hy*1, and *hy*2 tissue extracts, respectively, representing 7.6, 13.3, and 8.3 mg fresh weight of tissue, respectively. Separated proteins were transferred to nitrocellulose and probed with monoclonal antibody 073d as described in Methods. The size of the polypeptide detected by the antibody is shown to the right of the figure. L, light-grown; D, dark-grown.

extracts of *hy*1 and *hy*2 indicated that a minor fraction of the phytochrome A in these two mutants was photochemically functional (Parks et al., 1989). However, when *hy*1 and *hy*2 plants grown with biliverdin were light-treated, direct extracts from these tissues contained strongly reduced levels of immunochemically detectable phytochrome A comparable to those observed for the light-grown wild type. These results further indicate that the phytochrome in *hy*1 and *hy*2 tissues grown in the presence of biliverdin possesses molecular characteristics in the living plant cell that reflect restoration of normal photoreceptor properties.

DISCUSSION

All six of the known long hypocotyl mutants of Arabidopsis (*hy*1 through *hy*6) have been shown to contain levels of immunochemically detectable phytochrome A in etiolated tissue that are comparable to the wild-type parent (Chory et al., 1989; Parks et al., 1989). The deficiency in spectrophotometrically detectable phytochrome observed in the *hy*1, *hy*2, and *hy*6 mutants indicated that aberrant photomorphogenesis in these three mutants results from a nonfunctional photoreceptor. We have suggested that there are at least two classes of lesions that could directly affect phytochrome function without altering its level of synthesis (Parks et al., 1989). First, a phytochrome apoprotein structural gene could contain a mutation that would alter the chromophore binding site or otherwise affect the proper photochemical activity of the chromoprotein. As argued in the Introduction, physical and genetic mapping data have rendered this possibility unlikely. Second, a mutation could disrupt chromophore biosynthesis and/or its ligation to the phytochrome protein. The data presented here, demonstrating that a precursor to the phytochrome chromophore (biliverdin IXα) can rescue a light-regulated response in the *hy*1 and *hy*2 mutants with reinstatement of normal levels of fully functional phytochrome, are consistent with the conclusion that the genes at these two loci are important for chromophore biosynthesis. The alternative possibility that phytochromobilin is synthesized but somehow unavailable for attachment to the apoprotein in the mutants is eliminated by these data because conversion of the exogenous biliverdin to phytochromobilin by the endogenous biosynthetic machinery is required to enable the observed holoprotein formation (Elich and Lagarias, 1989). Thus, phytochromobilin is available for attachment once synthesized in these mutants.

The lack of complete rescue despite apparent full restoration of photochemically functional phytochrome A by biliverdin in the *hy*2 mutant implies that the lesion affects other cellular functions in addition to the activity of the photoreceptor itself. The basis for this apparent pleiotropic effect is unknown, but one possibility is that *hy*2 represents a mutation very early in the chromophore biosynthetic pathway. Because this pathway branches from the heme pathway (Brown et al., 1990), a lesion upstream of this branch point could potentially affect the levels of heme or other critical cellular components, including chlorophyll. Deficiency in such components might inhibit full restoration of the wild-type photomorphogenic response.

The discovery that the *hy*1 and *hy*2 loci are important for chromophore biosynthesis has provided a tool to probe a number of questions that are pertinent to the understanding of phytochrome function. First, a phytochrome deficiency resulting from aberrant chromophore production opens the possibility that the *hy*1 and *hy*2 mutants might be deficient in all types of phytochrome, and would thus contrast them from other mutants such as *hy*3 where only one phytochrome type appears to be affected (Somers et al., 1991). A deficiency in all phytochrome types may assist studies of blue light receptor-induced responses, which appear unaffected in *hy*1 and *hy*2 (Koornneef et al., 1980), by minimizing potentially complicating effects of

phytochrome activation (Gaba and Black, 1987; Mancinelli, 1989). In this regard, the phytochrome-deficient *hy*6 mutant (Chory et al., 1989) has been utilized very recently to assist in photobiological studies of prospective *blue light-insensitive long hypocotyl mutants* (Liscum and Hangarter, 1991). Second, the ability to restore an active pool of phytochrome in the two mutants by chromophore precursor feeding as described here permits the design of experiments in which the levels of the functional holoprotein might be manipulated at any time during the plant life cycle. Third, these chromophore-deficient mutants provide the opportunity to isolate genes that are important for tetrapyrrole synthesis upstream of biliverdin IXα, thereby assisting in attempts to understand the regulation of this important biosynthetic pathway (Brown et al., 1990). Finally, phenotypic rescue with biliverdin provides a rapid and simple test that can be incorporated into future mutant selection schemes designed to identify or eliminate additional chromophore synthesis-deficient lines.

Our results represent an important contribution to a strategy directed at understanding the mechanism of phytochrome action through the synthesis and assembly of a biologically functional photoreceptor in a nonplant system. Previous work using tetrapyrrole synthesis inhibitors demonstrated that the readily available phycocyanobilin can be substituted for the natural phytochromobilin, both in vivo (Elich et al., 1989) and in vitro (Elich and Lagarias, 1989), in the production of a photochemically functional holoprotein. Difference spectra for phytochrome produced under these experimental conditions suggested that phycocyanobilin is attached to the apoprotein directly without prior chemical modification to phytochromobilin. The results presented here extend these previous reports by demonstrating that a phycocyanobilin-phytochrome polypeptide adduct exhibits normal regulatory properties in plant tissue (Figure 4). The gross conformational changes that are necessary for biological function are apparently maintained in this synthetic holoprotein. This conclusion applied in conjunction with the finding that phytochrome holoprotein assembly is probably autocatalytic (Lagarias and Lagarias, 1989) implies that attempts to synthesize and assemble a phytochrome holoprotein in a nonplant system such as yeast should yield a photoreceptor with potential for normal photoregulatory function. Thus, it now becomes reasonable to expect that a functional phytochrome transduction pathway can eventually be assembled and manipulated in a foreign nonplant system.

METHODS

Linear Tetrapyrroles

Biliverdin was purchased from Sigma. Biliverdin IXα was a generous gift from Dr. J. Clark Lagarias (Department of Biochemistry and Biophysics, University of California, Davis). Phycocyanobilin was prepared by methanolysis of purified *Gastroclonium coulteri* C-phycocyanin and was a generous gift from Dr. Alexander N. Glazer (Department of Microbiology, University of California, Berkeley). Mesobiliverdin was prepared by oxidation of mesobilirubin (Porphyrin Products, Inc., Logan, UT) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (McDonagh, 1979) and purified using thin-layer chromatography with 70:30 chloroform:methanol as the solvent. All tetrapyrroles, with the exception of biliverdin, were quantitated by absorption spectrophotometry using the following molar absorption coefficients (McDonagh, 1979): biliverdin IXα, 14,400 M^{-1} cm^{-1} at 666 nm in methanol; phycocyanobilin, 12,300 M^{-1} cm^{-1} at 600 nm in methanol; mesobiliverdin, 15,800 M^{-1} cm^{-1} at 631 nm in chloroform. All tetrapyrroles were stored dried under nitrogen at −20°C in darkness. Fresh stock solutions of all linear tetrapyrroles were always prepared in methanol just prior to use in a given experiment.

Plant Material

Four- to 6-day-old wild-type *Arabidopsis thaliana* cv Landsberg and mutant (*hy*1, *hy*2, *hy*3, *hy*4, and *hy*5) seedlings were used in the reported experiments (Koornneef et al., 1980). Seeds were soaked and sterilized for 30 min in white light in a solution containing 20% hypochlorite and 0.2% SDS, washed with six changes of sterile water, and then sown onto a sterile nylon screen (250-μm pore size, Small Parts, Inc., Miami, FL) on top of buffered agarose (0.7% low melting temperature agarose [NuSieve GTG, FMC Bioproducts, Rockland, ME], 15 mM Hepes, pH 7.4, 2.16 g/L Murashige and Skoog salts [Gibco, Grand Island, NY]) in plastic microtiter wells or Petri dishes. Various concentrations of fresh linear tetrapyrrole stock solutions prepared in methanol were diluted 1:50 into melted buffered agarose prior to pouring to attain the appropriate working concentrations. Buffered agarose without linear tetrapyrroles was supplemented with a volume of methanol equivalent to that added for experimental wells and dishes. Seeds sown on wells and dishes were incubated at 4°C in darkness for at least 2 days to promote germination and growth at 25°C in darkness or in fluorescent white light (10 W/m^2). Only where specifically stated were plates supplemented with gibberellin A_3 at 0.1 mM to promote germination in darkness. Tissues harvested for protein extraction were blotted gently with tissue paper and weighed prior to rapid freezing at −80°C. This tissue harvesting, the generation of nondenatured protein extracts (see below), and all subsequent protocols that used these particular protein extracts, with the exception of SDS-PAGE and immunoblot analysis, were performed under green safelights, with samples maintained at or below ice temperatures.

Tissue Extracts

Extraction under Denaturing Conditions

Frozen Arabidopsis seedlings were rapidly homogenized in dilute SDS-containing sample buffer (60 mM Tris, pH 6.8, 0.7 M 2-mercaptoethanol, 2% SDS, 4% glycerol, 0.01% bromphenol blue) supplemented with phenylmethylsulfonyl fluoride at 2 mM to inhibit proteolysis and maintained at 100°C. Hot buffer was used at a ratio of 3 mL of buffer to 4 g fresh weight of frozen tissue.

The brei was then centrifuged at 16000*g* for 5 min, and the supernatant was used directly for SDS-PAGE. For simplicity, extracts generated by this protocol are periodically referred to as "direct extracts" throughout the text.

Extraction under Nondenaturing Conditions

Frozen Arabidopsis seedlings were rapidly homogenized under red light in a cold mortar in the presence of extraction buffer (100 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.6, 50% ethylene glycol, 5 mM Na_4EDTA, 56 mM 2-mercaptoethanol) at a ratio of 1 mL of buffer to 1 g fresh weight of tissue. The brei was then centrifuged at 48000*g* for 10 min, and the supernatant was retained for analysis. Extracts generated by this procedure are periodically referred to as "crude extracts" throughout the text.

Spectrophotometry

Phytochrome in crude extracts of etiolated Arabidopsis was measured spectrophotometrically using a custom-built dual-wavelength spectrophotometer as described previously (Tokuhisa et al., 1985). To increase light scatter, $CaCO_3$ was added at a ratio of 0.4 g to 0.6 mL of extract just prior to measurement (Butler and Norris, 1960). All crude extracts of Arabidopsis used for spectrophotometric measurements were generated as described above, except that tissues were ground in buffer at a ratio of 3 mL to 4 g of fresh frozen tissue. The 0.8 mL of generated supernatant from each tissue extract was loaded onto a 3-mL spin column of packed G-50 Sephadex that was equilibrated with extraction buffer. The column was then centrifuged at 5000*g* for 4 min. This step removes free biliverdin from the crude extract supernatant.

Immunochemical Detection of Electroblotted Proteins

Proteins in direct and crude extracts of Arabidopsis were separated in 6% polyacrylamide gels using SDS-PAGE (Laemmli, 1970) followed by electroblotting and subsequent immunochemical detection of separated proteins as described previously (Parks et al., 1987). Culture supernatants containing primary mouse monoclonal antibodies from the cell line 073d (Shanklin, 1988) were used for immunochemical detection of Arabidopsis phytochrome at a 1:200 dilution in 0.4% milk buffer (Johnson et al., 1984). A reporter consisting of alkaline phosphatase conjugated to secondary antibodies directed against mouse immunoglobulins (Promega, Madison, WI) was used at a 1:5000 dilution in 0.4% milk buffer.

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