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RESEARCH ARTICLE

The *hy*3 Long Hypocotyl Mutant of Arabidopsis Is Deficient in Phytochrome B

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The six long hypocotyl (*hy*) complementation groups of Arabidopsis (*hy*1, *hy*2, *hy*3, *hy*4, *hy*5, and *hy*6) share the common feature of an elongated hypocotyl when grown in white light. The varied responses of these mutants to irradiations of differing wavelengths have suggested that some of the lines may lack elements of the phytochrome signal transduction pathway. We have performed immunoblot and RNA gel blot analyses of the multiple types of phytochrome present in wild-type and mutant Arabidopsis and provide evidence that mutations at the *HY*3 locus cause a specific deficiency in phytochrome B. Using an *Escherichia coli* overexpression system, we have developed and identified monoclonal antibodies that selectively recognize phytochromes A, B, and C from Arabidopsis. In wild-type plants, phytochrome A is highly abundant in etiolated tissue, but rapidly decreases about 200-fold upon illumination. Phytochromes B and C are present at much lower levels in etiolated tissue but are unaffected by up to 24 hr of red light illumination, and together predominate in green seedlings. These data establish that phytochromes B and C are "type 2" or photostable phytochromes. Levels of phytochromes A, B, and C similar to those of the wild type are observed in strains containing mutations at the *HY*4 and *HY*5 loci. In contrast, all four *hy*3 mutant alleles tested here exhibit a modest (twofold to threefold) reduction in *phyB* transcript and a severe (20- to 50-fold) deficiency in *phyB*-encoded protein, relative to levels in wild-type plants. The levels of *phyA*- and *phyC*-encoded mRNA and protein, however, are indistinguishable from the wild type in these mutants. We conclude that the phenotype conferred by *hy*3 is due to the reduced levels of the light-stable phytochrome B.

INTRODUCTION

The complexity and diversity of responses attributable to phytochrome control have long suggested the involvement of more than one species of photoreceptor in the regulation of plant development (Kendrick and Kronenberg, 1986; Furuya, 1987; Smith and Whitelam, 1990). Physiological studies have led to the concept of two functionally distinct pools of phytochrome: (1) a "photolabile" pool characterized by rapid removal of the active, far-red-light-absorbing form (Pfr) from the cell and involvement in such phenomena as the "high irradiance responses" (HIRs) of etiolated seedlings (Kendrick and Kronenberg, 1986; Adamse et al., 1988c; Smith and Whitelam, 1990; Tomizawa et al., 1990) and (2) a "photostable" pool characterized by stability of the Pfr form in the cell and involvement in phenomena such as the "end-of-day far-red" response (Adamse et al., 1988c; Smith and Whitelam, 1990; Tomizawa et al., 1990). A variety of spectroscopic, biochemical, and immunochemical studies have also provided evidence for the existence of at least two operationally defined classes of the photoreceptor: (1) type 1 or "etiolated-tissue" phytochrome, which is the species that is abundant and readily detectable in etiolated tissue, is rapidly degraded as Pfr in the cell, and is the molecule that has been purified and extensively characterized in vitro (Vierstra and Quail, 1986; Cordonnier, 1987; Song, 1988; Furuya, 1989; Quail et al., 1991) and (2) type 2 or "green-tissue" phytochrome, which is of low abundance in etiolated tissue (~1% of type 1) but stable as Pfr in vivo. Therefore, type 2 phytochrome is more readily detectable in light-grown tissue where it is maintained and type 1 phytochrome has been depleted (Vierstra and Quail, 1986; Cordonnier, 1987; Pratt and Cordonnier, 1987; Tokuhisa and Quail, 1987; Furuya, 1989).

Direct evidence that higher plants do indeed contain multiple species of phytochrome encoded by a small family of divergent genes has been provided recently by studies in the dicot Arabidopsis (Sharrock and Quail, 1989; R. Sharrock, unpublished data) and the monocot rice (Dehesh et al., 1991). Five phytochrome-related sequences were detected in genomic DNA of Arabidopsis, and full-length cDNA clones corresponding to three of these genes (*phyA*, *phyB*, and *phyC*) were isolated and characterized

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(Sharrock and Quail, 1989). Recently, the two additional Arabidopsis phytochrome genes, *phyD* and *phyE*, have been cloned, and initial characterization indicates that they are significantly different from each other as well as from *phyA*, *phyB*, and *phyC*, thus confirming that the five phytochrome genes in this plant are divergent and single copy (R. Sharrock, unpublished data).

The relationship between the various immunochemically and spectroscopically detected types of phytochrome and these phytochrome genes is incompletely understood. Protein microsequencing data have established that type 1 or etiolated-tissue phytochrome is encoded by *phyA* genes (Lagarias and Rapoport, 1980; Hershey et al., 1985; Yamamoto, 1987; Grimm et al., 1988; Sato, 1988; Jones and Quail, 1989). For consistency in terminology, therefore, we now refer to type 1 phytochrome as phytochrome A. However, the relationship between the remaining phytochrome genes and type 2 or green-tissue phytochrome is yet to be delineated. Two polypeptides immunochemically distinct from each other and from phytochrome A have been detected in green oat extracts with monoclonal antibodies (MAbs) (Pratt et al., 1991; Wang et al., 1991). However, sequence information on these molecular species it not yet available. Preliminary microsequencing data for a preparation of type 2 pea phytochrome (Abe et al., 1989; Furuya, 1989) show two short stretches that are more similar to rice and Arabidopsis *phyB* than to *phyA* or *phyC* (Quail et al., 1991), but definitive identification of the pea protein must await more extensive sequence information.

The recognition that plants contain multiple molecular species of phytochrome has engendered questions as to whether individual members of the photoreceptor family perform discrete physiological functions in the plant and whether each has a distinct mechanism of action (Smith and Whitelam, 1990). Whereas considerable evidence suggests that *phyA* encodes the physiologically defined labile phytochrome, the question of which molecular species of the photoreceptor family corresponds to physiologically stable phytochrome is yet to be answered. One approach to this problem is to examine mutants, which are aberrant in defined phytochrome responses, for deficiencies in specific phytochromes. Using this approach, R.E. Kendrick and colleagues have presented physiological and spectrophotometric evidence that the *aurea* mutant of tomato is deficient in labile phytochrome but is normal for stable phytochrome (Adamse et al., 1988b, 1988c), whereas, conversely, the long hypocotyl (*lh*) mutant of cucumber is normal for labile phytochrome but deficient for stable phytochrome (Adamse et al., 1988a, 1988c). Based on earlier photobiological characterization of the long hypocotyl (*hy*) mutants of Arabidopsis (Koornneef et al., 1980), these authors have also suggested that the *hy*3 mutant of this species, like *lh*, is normal in labile phytochrome but deficient in stable phytochrome (Adamse et al., 1988c). Spectrophotometric and immunoblot analyses have shown that *hy*3 contains wild-type levels of phytochrome A (Koornneef et al., 1980; Parks et al., 1989), but until now it has not been possible to determine the levels of other phytochrome family members. Toward this end, we have monitored the levels of the mRNAs and apoproteins encoded by the *phyA*, *phyB*, and *phyC* genes in *hy*3 and other Arabidopsis long hypocotyl mutants using phytochrome type-selective MAbs and transcript-specific probes.

RESULTS

Seven MAbs Selectively Detect Three Different Phytochrome Apoproteins Expressed in *Escherichia coli*

Three Arabidopsis phytochrome cDNAs, *phyA*, *phyB*, and *phyC* (Sharrock and Quail, 1989), were individually cloned into pET-3c (Rosenberg et al., 1987) and expressed in *E. coli* (Studier and Moffatt, 1986). The inclusion body fractions were solubilized, and overexpressed polypeptides corresponding to sizes near those predicted for each respective phytochrome were obtained and gel purified. The three proteins were recognized with differing affinities by a polyclonal antiserum directed to purified oat phytochrome, indicating in each case that a band corresponding to a phytochrome had been isolated (data not shown). Subsequent reference to these *E. coli*-expressed phytochromes will be as the *phyA*-, *phyB*-, and *phyC*-encoded apoproteins.

The spleens of mice immunized with either the *phyB*- or *phyC*-encoded apoproteins were processed through an electrofusion protocol to obtain a large number of hybridoma clones. Those characterized and used in this study (B1, B7, B8 and C1, C11, C13) were chosen for their strong selective recognition of the appropriate phytochrome antigen and for their unique epitope affinities. Figure 1 shows that the six *phyB*- and *phyC*-directed MAbs strongly recognize their respective immunoblotted antigens and lack any detectable affinity for the other two cloned phytochromes. MAb 073d, originally raised to purified etiolated oat phytochrome (Shanklin, 1988) and previously reported to recognize an etiolated tissue phytochrome in Arabidopsis (Parks et al., 1989), is now shown to selectively recognize the apoprotein encoded by *phyA* (Figure 1).

Comparative epitope mapping of the six new MAbs was performed by limited V8 proteolysis of the *phyB* and *phyC* apoproteins (Cleveland, 1983; Tokuhisa et al., 1985) and immunoblot analysis of the peptide fragments. The six new clones each displayed a different characteristic recognition pattern for the peptide fragments (data not shown), demonstrating that each bears an affinity for a unique region of its respective phytochrome antigen.

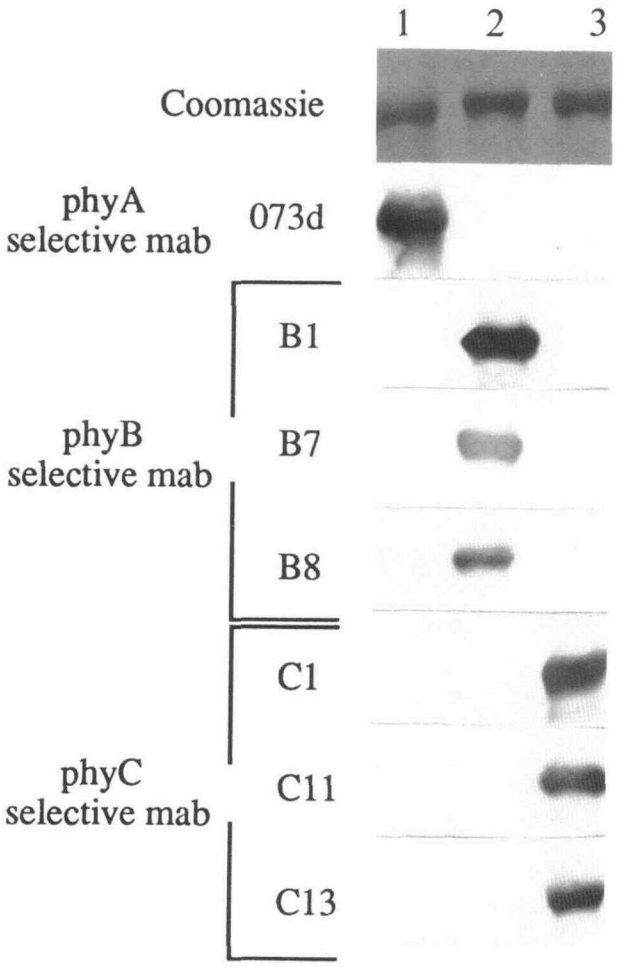

Figure 1. Selective Recognition by Seven MAbs of *phyA*-, *phyB*-, and *phyC*-Encoded Apoproteins Produced in *E. coli*.

The *phyB*- (B1, B7, and B8) and *phyC*- (C1, C11, and C13) selective MAbs were directed against the respective cloned phytochrome apoprotein expressed in *E. coli*. MAb 073d was raised to purified etiolated oat phytochrome (Shanklin, 1988). Approximately 300 ng of the appropriate gel-purified apoprotein was loaded per lane (lane 1, *phyA*; lane 2, *phyB*; lane 3, *phyC*) and subjected to immunoblot analysis. Identical replicate blots were probed separately with the MAb indicated. "Coomassie" indicates the Coomassie Brilliant Blue R 250-stained gel demonstrating the amount of the appropriate phytochrome apoprotein in each lane.

Three Different Proteins from Plant Extracts Are Uniquely Recognized by Three Phytochrome Type-Selective MAb Pools

To increase the detection level of phytochromes on electroblots of plant extracts, the *phyB* and *phyC* type-selective antisera were used as two pools of three MAbs each. B1, B7, and B8 were mixed and used at a 1:100 dilution each (*phyB* MAb pool) to selectively detect the protein encoded by *phyB*. C1, C11, and C13 were similarly diluted to obtain a *phyC* MAb pool. The MAb 073d "pool," used at a 1:200 dilution, was sufficiently sensitive alone to recognize the *phyA*-encoded protein. All subsequent uses of the seven MAbs on immunoblots were in these combinations.

Figure 2A shows that each of the MAb pools detects a single, uniquely sized protein in phytochrome-enriched extracts from etiolated tissue. The 118-kD band detected by 073d (Figure 2A, lane 1) was shown by Parks et al. (1989) to be a phytochrome, based on differential susceptibility to proteolysis in response to red and far-red irradiation. In addition, the plant protein migrates identically with the *phyA*-encoded apoprotein (data not shown). Figure 2B (row phyA) illustrates the well-established rapid disappearance of phytochrome A in response to red light (Colbert et al., 1983).

Each of the two plant proteins detected by the *phyB* and *phyC* MAb pools (Figure 2A, lanes 2 and 3) migrates identically with its respective apoprotein (data not shown). Their apparent molecular masses (122 and 120 kD, respectively) are consistent with those predicted from the *phyB* (129 kD) and *phyC* (122 kD) cDNAs. In contrast to *phyA*, the *phyB*- and *phyC*-encoded protein levels remain relatively unchanged after up to 24 hr of red light illumination (Figure 2B, rows phyB and phyC). These results are consistent with the reported properties of light-stable, green-tissue phytochrome in oat and pea (Abe et al., 1985; Shimazaki and Pratt, 1985; Tokuhisa et al., 1985).

Based on two criteria, we concluded that each of the MAb pools recognizes on immunoblots an endogenous phytochrome that differs from that detected by the other two pools. First, each of the *phyA*, *phyB*, and *phyC* MAbs selectively detects its respective *E. coli*-expressed apoprotein (Figure 1). Second, on immunoblots of plant extracts, the MAb pools recognize single bands that differ from each other in regard to both apparent molecular mass and change in abundance in response to light (Figures 2A and 2B). For example, the bands detected by the *phyC*- and *phyA*-selective MAb pools, although similar in size, differ in the greater light stability of the *phyC*-encoded protein. On the other hand, the *phyB*- and *phyC*-selective MAb pools recognize proteins that are both light stable but differ significantly in migration. We will refer here to the proteins detected in tissue extracts by the MAb pools as phytochromes A, B, and C.

An approximate measure of the affinity of each type-selective MAb pool for its endogenous phytochrome was obtained by using the band-purified apoproteins encoded by *phyA*, *phyB*, and *phyC* that were overexpressed in *E. coli*. An electroblot of a serial dilution of each of the phytochrome apoproteins was probed with the appropriate MAb pool, and the sensitivity of detection was assessed visually. The *phyB* and *phyC* pools recognized their

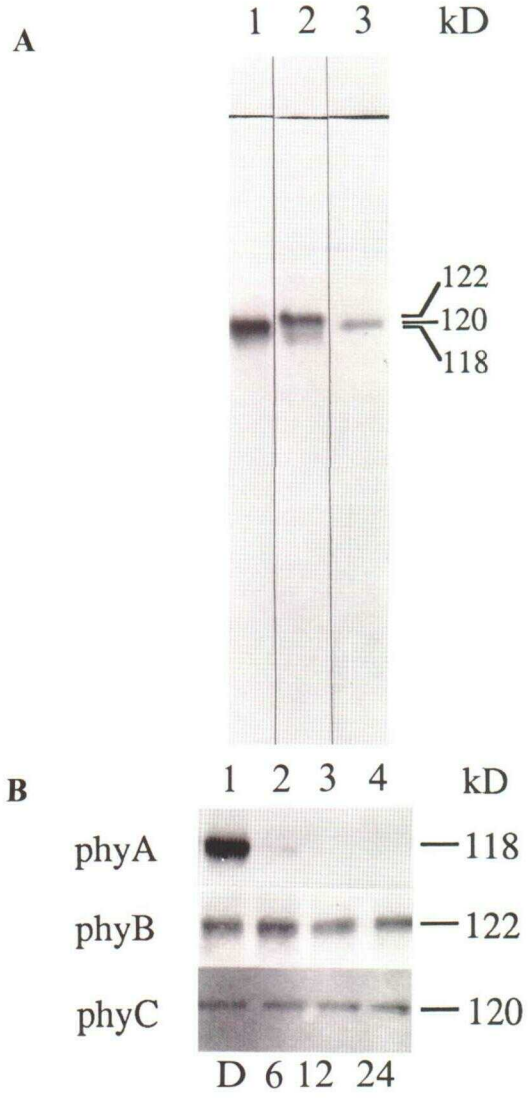

Figure 2. Selective Recognition of Three Endogenous Phytochromes in Arabidopsis by Three Type-Selective MAb Pools.

Tissue extracts were subjected to immunoblot analysis.
(A) Phytochrome-enriched extracts from 7-day-old dark-grown seedlings probed with *phyA*- (lane 1), *phyB*- (lane 2), and *phyC*- (lane 3) selective MAbs. Immunoblot lanes were loaded with ammonium sulfate-precipitated fractions from 300 (lane 1), 450 (lane 2), and 750 μg (lane 3) of crude extract protein. The dark horizontal lines at the top were used to align strips after development.
(B) Phytochrome-enriched extracts from 7-day-old dark-grown seedlings treated before harvest with 0 (lane 1, D), 6 (lane 2), 12 (lane 3), or 24 (lane 4) hr of continuous red light. Immunoblot lanes were loaded with ammonium sulfate-precipitated fractions from 300 μg (rows phyA and phyB) and 1.2 mg (row phyC) of crude extract protein, and the blots were probed with the appropriate type-selective MAb pool.

respective antigens with approximately the same degree of sensitivity, whereas 073d detected the *phyA*-encoded apoprotein with fivefold lower sensitivity (data not shown). This result may be partly because 073d was originally raised to phytochrome purified from etiolated oat tissue and because the 073d "pool" recognizes only a single epitope. Nonetheless, the *phyA* band is the strongest among the three in extracts from etiolated plants (Figure 2B, lane 1), which suggests a much greater abundance of phytochrome A than of either phytochrome B or C in this tissue. For an approximate quantitation of these differences, electroblots of serial dilutions of phytochrome-enriched extracts from dark-grown tissue were probed with the appropriate MAb pools. By visual assessment, phytochrome A is approximately 25- to 50-fold more abundant than phytochrome B and approximately 500-fold more abundant than phytochrome C (data not shown). In contrast, after 24 hr of red light illumination, phytochrome A levels have dropped approximately 200-fold and phytochromes B and C predominate (Figure 2B, lanes 2, 3, and 4). Under all conditions tested, phytochrome B was consistently 10- to 20-fold more abundant than phytochrome C (data not shown).

*hy*3 Is Severely Deficient in Phytochrome B

The Arabidopsis *hy*3 mutant is characterized by an increased hypocotyl length in light-grown plants, relative to the wild type, a slightly more pale color, and more elongated petioles than wild-type plants (Koornneef et al., 1980; Chory et al., 1989). Based on limited physiological evidence, some have speculated that *hy*3 lacks a light-stable phytochrome (Adamse et al., 1988c; Smith and Whitelam, 1990). Using transcript-specific probes and type-selective MAbs, we have analyzed the levels of the *phyA*, *phyB*, and *phyC* mRNAs and their encoded proteins in wild-type and *hy*3 plants.

Figure 3 (rows a and b) shows that the levels of the *phyA*-encoded protein and mRNA in *hy*3 tissue are similar to those found in the wild type under the conditions tested. Sharrock and Quail (1989) have previously reported that the *phyA* mRNA level remains constant in response to a red light pulse and decreases only slightly under white light. These results are confirmed here because message levels in etiolated seedlings for both wild-type and *hy*3 plants are unchanged after treatment with either 6 or 24 hr of red light (Figure 3, row b). The *phyA*-encoded protein level, in contrast, decreases rapidly to nearly undetectable levels in extracts from both *hy*3 and wild-type plants after 24 hr of red light treatment (Figure 3, row a).

The level of *phyC* mRNA in the tissue of wild-type plants has been shown previously to be low and unaffected by light conditions in early plant development (Sharrock and Quail, 1989). These results are confirmed here for the wild type and extended to the *hy*3 mutation that shows no

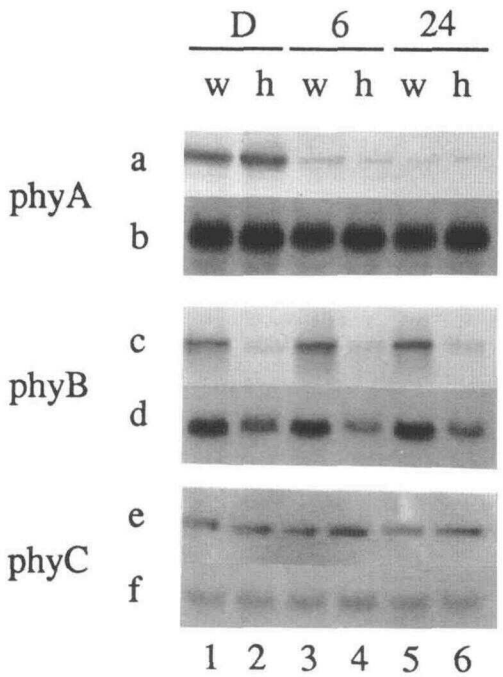

Figure 3. *phyA*, *phyB*, and *phyC* mRNA and Protein Levels in *hy*3 and Wild-Type Arabidopsis.

Phytochrome-enriched protein extracts and total RNA preparations were made from wild-type (w) and *hy*3 (h) 7-day-old dark-grown seedlings treated before harvest with 0 (D, lanes 1 and 2), 6 (6, lanes 3 and 4), or 24 (24, lanes 5 and 6) hr of continuous red light. Immunoblot lanes were loaded with ammonium sulfate-precipitated fractions from 300 (rows a and c) and 600 (row e) μg of crude extract protein and then probed with type-selective MAbs against phytochromes A (row a), B (row c), and C (row e). RNA blots (rows b, d, and f) containing 5 μg of total RNA per lane were hybridized with the appropriate transcript-specific single-stranded DNA probe.

difference from the wild type in *phyC* mRNA levels (Figure 3, row f). Likewise, wild-type and *hy*3 plants show similarly low levels of *phyC*-encoded protein in extracts of dark-grown and light-treated tissue (Figure 3, row e). We conclude that both wild-type and *hy*3 plants contain constitutively low levels of phytochrome C.

In contrast to the results with *phyA* and *phyC*, the *phyB*-encoded protein and mRNA levels are both depleted in *hy*3 when compared with the wild type (Figure 3, rows c and d). *phyB* mRNA levels in the wild type and *hy*3 remain unchanged by up to 24 hr of red light treatment, but are consistently twofold to threefold lower in *hy*3 under the three conditions tested (Figure 3, row d). More importantly, there is a severe reduction in the *phyB*-encoded protein levels in *hy*3 relative to the wild type (Figure 3, row c). Based on a serial dilution of extracts from wild-type plants, we estimated that phytochrome B in *hy*3 is reduced to a level that is at most 2% to 5% of wild-type levels (data not shown). This reduction occurs consistently to the same degree and is independent of the light conditions (Figure 3, row c).

Koornneef et al. (1980) reported the isolation of 17 independent alleles of *hy*3, but all published characterization of this locus has been done with the Bo64 allele. We examined the phytochrome mRNA and protein levels in three additional members of the *hy*3 complementation group (1053, 548, and M4084) to ensure that the strong reduction of phytochrome B shown above in Bo64 was due solely to a lesion at the *hy*3 locus and to assess the relative severity of the *phyB* decrease among the four different alleles.

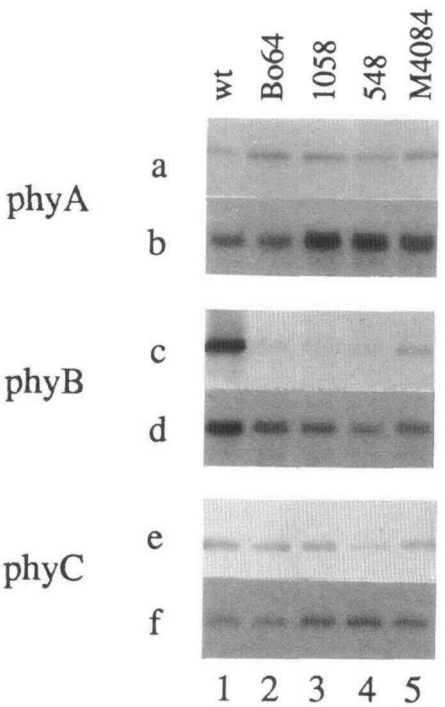

Figure 4. *phyA*, *phyB*, and *phyC* mRNA and Protein Levels in Wild-Type and Four Allelic Mutants of *hy*3 Arabidopsis.

Phytochrome-enriched protein extracts and total RNA preparations were made from wild-type (wt, lane 1) Arabidopsis and four allelic mutants of *hy*3 (Bo64, lane 2; 1058, lane 3; 548, lane 4; M4084, lane 5) (Koornneef et al., 1980) after 6 days of growth under continuous white light. Immunoblot lanes were loaded with ammonium sulfate-precipitated fractions from 500 μg (rows a, c, and e) of crude extract protein and then probed with type-selective MAbs against phytochromes A (row a), B (row c), and C (row e). RNA blots (rows b, d, and f) containing 5 μg of total RNA per lane were hybridized with the appropriate transcript-specific single-stranded DNA probe.

Figure 4 shows the results for all three phytochromes after growth for 6 days under continuous white light. The levels of protein encoded by *phyA* and *phyC* are not significantly different in the mutants as compared with the wild type (Figure 4, rows a and e). The variations in band intensity shown in Figure 4 (rows a and e) were not reproduced in all trials and do not correlate with the slightly higher *phyA* and *phyC* mRNA levels observed in alleles 1058, 548, and M4084. All four *hy*3 alleles, however, show the same twofold to threefold reduction of *phyB* mRNA and 20- to 50-fold decrease in *phyB*-encoded protein abundance, relative to the wild type, as described above (Figure 4, rows c and d). The slightly higher phytochrome B level shown by the M4084 allele (Figure 4, row d, lane 5) was not observed in three subsequent trials. These results indicate that four independent mutant alleles of the *HY*3 locus exhibit very similar molecular phenotypes characterized by specific and significant reductions in the *phyB* gene products.

phyA, *phyB*, and *phyC* Expression Is Unaffected in *hy*4 and *hy*5

The *hy*4 and *hy*5 long hypocotyl mutants, like *hy*3, exhibit wild-type levels of photoreversible and immunochemically detectable phytochrome in dark-grown tissue (Koornneef et al., 1980; Chory et al., 1989; Parks et al., 1989). Therefore, we examined *hy*4 and *hy*5 with the type-selective MAbs to determine whether the mutant phenotypes are due to aberrant levels of *phyB*- or *phyC*-encoded protein. Figure 5 shows that in dark-grown tissue, *hy*4 and *hy*5 possess wild-type levels of both the mRNA and phytochrome protein encoded by the three *phyA*, *phyB*, and *phyC* genes. These results indicate that unlike *hy*3, the *hy*4 and *hy*5 phenotypes are unlinked to any alterations in the levels of these phytochromes.

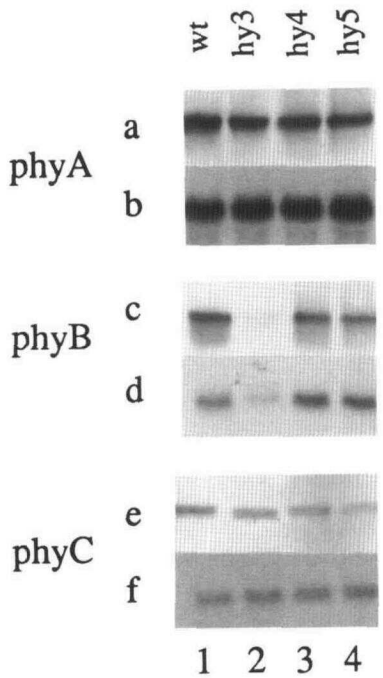

Figure 5. *phyA*, *phyB*, and *phyC* mRNA and Protein Levels in *hy*3, *hy*4, *hy*5, and Wild-Type Arabidopsis.

Phytochrome-enriched protein extracts and total RNA preparations were made from wild-type (wt, lane 1), *hy*3 (lane 2), *hy*4 (lane 3), and *hy*5 (lane 4) Arabidopsis seedlings grown for 7 days in the dark. Immunoblot lanes were loaded with ammonium sulfate-precipitated fractions from 300 (rows a and c) and 600 (row e) μg of crude extract protein and then probed with type-selective MAbs against phytochromes A (row a), B (row c), and C (row e). RNA blots (rows b, d, and f) containing 5 μg of total RNA per lane were hybridized with the appropriate transcript-specific single-stranded DNA probe.

DISCUSSION

Phytochromes in Wild-Type Arabidopsis

From the data in Figures 1 and 2, we conclude that the seven MAbs characterized here clearly discriminate between the *phyA*-, *phyB*-, and *phyC*-encoded phytochromes on immunoblots of plant extracts. We have designated these MAbs as "selective" for their respective antigens rather than "specific" because of the current lack of information on possible cross-reactivity with the remaining two Arabidopsis phytochromes D and E. 073d, a MAb raised to phytochrome purified from dark-grown oat, selectively recognizes the homologous protein in Arabidopsis.

The rapid loss of *phyA*-encoded protein in red light (Figure 2B, row phyA) is similar to the well-characterized lability of phytochrome A in other species (Colbert et al., 1983; Quail, 1991). In contrast, we conclude from our present data that the *phyB* and *phyC* genes both encode light-stable phytochromes (Figures 2 and 4). From immunoblots of serial dilutions of phytochrome-enriched plant extracts, we estimate that in both etiolated and green tissue, phytochrome B abundance is approximately 10- to 20-fold greater than phytochrome C (data not shown; Figures 2 and 4). The low phytochrome C abundance is, in general, congruent with the observed low level of *phyC* mRNA (Figures 2 and 4). Therefore, we conclude that phytochrome B is the predominant species of the two light-stable types of phytochrome identified here.

On the other hand, phytochrome C may be the least abundant of all three phytochrome proteins examined here. Despite the rapid and marked decrease (approximately 200-fold) in phytochrome A abundance upon illumination

(Figure 2B, row phyA), a significant steady-state level of this protein is still detectable in light-grown plants (Figure 4, row a). When the fivefold lower sensitivity of 073d for phytochrome A (see Results) is considered, the content of the light-labile *phyA*-encoded protein in extracts from green plants may still be fivefold to 10-fold greater than phytochrome C. Therefore, based strictly on abundance, a role for phytochrome A in green plants is just as likely as one for phytochromes B and C. Conversely, the presence of both light-stable forms in etiolated plants allows for the possibility that they also function very early in light-mediated plant development. One approach to assignment of specific functions to individual phytochromes is to identify mutants deficient in only one of the photoreceptor family members.

Phytochrome in the *hy* Mutants

Some of the six long hypocotyl mutants of Arabidopsis (*hy*1, *hy*2, *hy*3, *hy*4, *hy*5, and *hy*6) have long been candidates for being deficient in photoactive phytochrome because of experiments indicating a lack of responsiveness to red and far-red light in the control of hypocotyl length (Koornneef et al., 1980; Chory et al., 1989). Recent evidence indicates that *hy*1 and *hy*2 (Parks et al., 1989; Parks and Quail, 1991) and perhaps *hy*6 (Chory et al., 1989) are deficient in chromophore biosynthesis. These mutants, therefore, are likely to be deficient in all functional phytochromes and not useful in determining the actions of individual phytochromes. The remaining *hy*3, *hy*4, and *hy*5 mutants show normal levels of spectrally detectable phytochrome in etiolated plants, but the lack of a minor species, such as phytochrome B or C, still could go undetected. We applied our type-selective MAbs to extracts of these plants to address this possibility.

We found no evidence in *hy*4 or *hy*5 for a deficiency in any of the three phytochrome proteins assayed here (Figure 5). The results with *hy*4 remain consistent with the long-standing hypothesis that it lacks some component of the blue light receptor transduction pathway (Koornneef et al., 1980; Liscum and Hangarter, 1991). The primary lesion in *hy*5 remains unknown.

*hy*3 has been the best candidate for a mutant deficient in a single type of phytochrome in Arabidopsis. Whereas previous studies with this mutant have shown that dark-grown tissue contains apparently normal levels of spectrally and immunochemically detectable phytochrome (Koornneef et al., 1980; Chory et al., 1989; Parks et al., 1989), *hy*3 seed has been reported to be reduced in phytochrome content (Cone, 1985). In addition, although both red and far-red light strongly inhibit hypocotyl growth in wild-type plants, red light is ineffective in *hy*3 (Koornneef et al., 1980). The *lh* cucumber mutant shows a similar response (Adamse et al., 1987) and, based on more extensive photophysiological studies, has been inferred to lack a light-stable phytochrome (Adamse et al., 1988a).

Our results demonstrate that the phytochrome B levels in *hy*3 are reduced to an amount that is at most 2% to 5% of wild-type levels (Figure 3; data not shown). The deficiency is present in both dark-grown and green tissue, and the level of reduction is independent of growth conditions (Figures 3 and 4). In addition, *phyB*-encoded protein levels in the four *hy*3 alleles tested here were all reduced to approximately the same extent (Figure 4). Currently, it cannot be excluded that the detection of a residual, very low abundance band in the four *hy*3 alleles at the same molecular mass as phytochrome B results from a weak cross-recognition of *phyD*- or *phyE*-encoded protein by the *phyB* MAb pool. If so, phytochrome B levels are even lower than estimated here. The testing of this possibility awaits the completion of the cloning of *phyD* and *phyE* cDNAs. A similar but less marked decrease (twofold to threefold) in *phyB* mRNA abundance was seen in all the treatments and *hy*3 alleles (Figures 3 and 4). These reductions are unique to *phyB* among the phytochromes studied here because *phyA* and *phyC* gene products in *hy*3 are at wild-type levels (Figures 3 and 4).

We suggest that a deficiency in phytochrome B is responsible for the phenotype conferred by *hy*3. The evidence for this conclusion is threefold. First, as outlined above, the *hy*1 and *hy*2 mutants appear very similar to *hy*3 and, having now been shown to be chromophore deficient (Parks and Quail, 1991), are probably depleted in all photoactive phytochromes. Although a reduction in phytochrome B alone could be largely responsible for the long hypocotyl phenotypes of *hy*1, *hy*2, and *hy*3, a more complex interaction between phytochromes is more likely (see below). Second, the initial demonstration in *hy*3 of aberrant hypocotyl growth in red light (see above; Koornneef et al., 1980) has been extended by further studies (Whitelam and Smith, 1991) that tie the *hy*3 morphology and physiology even more closely to that of the *lh* cucumber mutant. In both *hy*3 and *lh*, white light-grown plants supplemented with continuous far-red light exhibit only a very small increase in elongation growth relative to the wild type (Whitelam and Smith, 1991). This "shade avoiding" response is considered to be, in principle, the same as the end-of-day far-red effect (Kasperbauer, 1971; Gaba and Black, 1985) that is also lacking in the *lh* line, a putative light-stable phytochrome mutant (Adamse et al., 1988a; Lopez-Juez et al., 1990). Third, the overexpression of Arabidopsis phytochrome B in Arabidopsis has been shown recently to result in an extremely short hypocotyl in light-grown plants (Wagner et al., 1991). Together with observations on wild-type and *hy*3 plants (Koornneef et al., 1980), these three phenotypes present a graded series of hypocotyl lengths in which increased extension is inversely correlated with the phytochrome B level. In combination, the above three arguments strongly implicate the reduction in phytochrome B as the cause of the *hy*3 phenotype.

The associated but nonproportional reduction of both *phyB* mRNA and protein in *hy*3 suggests a mutation that affects both the translatability and stability of the *phyB* transcript. It has been well established in eukaryotes that premature termination of translation can result in reduced message stability (Urlaub et al., 1989; Cheng et al., 1990). The deliberate introduction of nonsense and frameshift mutations within exons of genes from both plants and animals has been shown to reduce effectively the final levels of both gene products (Daar and Maquat, 1988; Vancanneyt et al., 1990). This precedent, together with the recent mapping of *phyB* to the same chromosome (2) as *hy*3 (S. Kempin and E.M. Meyerowitz, personal communication; Koornneef et al., 1983), is consistent with the possibility that the mutation lies within the *phyB* transcript. Successful complementation of the *hy*3 phenotype with the *phyB* gene would demonstrate the genetic equivalence of the *HY*3 and *phyB* loci.

Phytochrome and the Control of Hypocotyl Extension

The inhibition of hypocotyl extension in etiolated seedlings by continuous irradiation has been well established as an example of a phytochrome-mediated HIR (Mancinelli, 1980; Kendrick and Kronenberg, 1986). The exact mechanism of this response is unknown, but probably involves the coaction or interaction of both phytochrome and a separate blue light receptor to obtain the full inhibition response (Thomas and Dickinson, 1979; Liscum and Hangarter, 1991).

However, even apart from the role of blue light, it is unlikely that phytochrome B is the sole phytochrome photoreceptor involved in the control of hypocotyl growth, as might be first concluded from this study. Biochemical and spectral studies of *aurea*, a long hypocotyl tomato mutant (Koornneef et al., 1985), have shown there to be an extreme reduction in the level of the light-labile phytochrome A (Parks et al., 1987; Adamse et al., 1988b). As well, spectral evidence for the presence of functional phytochrome in petals and Norflurazon-treated leaves and further photophysiological results have led to the conclusion that *aurea* probably possesses normal levels of light-stable phytochrome and is deficient only in the light-labile type (Adamse et al., 1988b). Confirmation of this conclusion awaits testing of the mutant with type-specific antibodies. If true, it then appears likely that the long hypocotyl phenotype can result either from the reduction of a light-stable phytochrome, as in *hy*3 and *lh*, or from a decrease in the light-labile phytochrome A, as in *aurea*. At the other extreme, the overexpression of phytochrome A in tomato (Boylan and Quail, 1989), tobacco (Nagatani et al., 1991), and Arabidopsis (Boylan and Quail, 1991) causes a shortened hypocotyl similar to the results obtained with the overexpression of phytochrome B in Arabidopsis (Wagner et al., 1991).

Hence, the mutant studies indicate that neither phytochrome A nor phytochrome B is sufficient alone to direct normal hypocotyl extension, whereas the overexpression of either results in similarly hypershortened hypocotyls. These results suggest that the two phytochromes initiate either the same or separate transduction chains in which the degree of hypocotyl extension in wild-type plants is dependent on the levels of both *phyA* and *phyB* Pfr established in the light. However, it is not clear whether the two phytochromes act additively or cooperatively or are quantitatively interchangeable.

Previous studies of the HIR inhibition of hypocotyl growth in etiolated and deetiolated seedlings, together with our results, now suggest that phytochromes A and B may mediate different components of the HIR response. Beggs et al. (1980) showed that the inhibitory effect of continuous far-red light in *Sinapis alba* was severely reduced after a brief red light pulse had decreased phytochrome A levels. Inhibition by continuous red light remained unchanged by this pretreatment. This result implies that a light-stable phytochrome (e.g., phytochrome B) could mediate the red light component of hypocotyl inhibition and is consistent with the absence of such a response in the phytochrome B-deficient *hy*3 (Koornneef et al., 1980). In contrast, inhibition of hypocotyl elongation by far-red light is normal in *hy*3 (Koornneef et al., 1980), which correlates with the normal levels of phytochrome A that we observed (Figure 3). Other reports showing a loss of the far-red component of the HIR in light-treated plants (Jose and Vince-Prue, 1977; Thomas and Dickinson, 1979) similarly suggest that this aspect of hypocotyl extension is normally mediated through the light-labile phytochrome A.

The above interpretation that, in the control of hypocotyl growth, phytochromes A and B separately mediate the far-red and red light components of the HIR, respectively, remains tentative. However, it illustrates that with the application of phytochrome type-specific molecular probes to the analysis of photomorphogenic mutants, we can begin to develop testable hypotheses to elucidate further the functions of the different phytochromes in plant development.

METHODS

Plant Material and Growth Conditions

The initial isolation and characterization of the long hypocotyl mutants of *Arabidopsis thaliana* cv Landsberg used in this study are found in Koornneef et al. (1980). Seeds of the wild type, *hy*3 (Bo64, 1053, 548, M4043), *hy*4 (2.23 N), and *hy*5 (Ci 88) were surface-sterilized in 1.5% sodium hypochlorite (v/v), 0.02% Triton X-100 and sown onto germination media (Valvekens et al., 1988). Seeds were stratified at 4°C for 2 days in the dark, given a 10-min white light treatment, and grown at 22°C. All dark-treated plants were grown for 7 days, with some material receiving either

6 or 24 hr of continuous red light (0.20 W/m^2) just before harvest. The red light source was fluorescent light filtered through red plastic (600-nm cutoff). Plants grown in white light (cool-white fluorescent plus incandescent) received 6 days of continuous irradiation (8.8 W/m^2). All tissue was weighed, frozen in liquid nitrogen, and stored at −80°C.

Plant Protein Extraction and Analyses

Preparation of extracts enriched in phytochrome was according to Tokuhisa and Quail (1987) with the following modifications. Frozen tissue was thawed and ground for 3 to 5 min under red light at a 1:1 ratio (grams to milliliters) in undiluted extraction buffer containing protease inhibitors. The final extract volume was adjusted with ice water to double that of the original buffer volume that was used for extraction. The final protease concentrations were 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 1 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 30 mM sodium bisulfite. Polyethyleneimine was added to 0.1%, and the final pellet was resuspended in approximately 5% of the original volume in resuspension buffer (Tokuhisa and Quail, 1987) containing protease inhibitors at twice the above concentrations.

The samples were subjected to SDS-PAGE (Laemmli, 1970) in a 4.5%/6% stacking/resolving gel (acrylamide:bis 30:0.8). Gel-fractionated proteins were electroblotted to nitrocellulose (Millipore Corporation, Bedford, MA) at 90 V for 5 hr or overnight at 100 mA. Blocking of unoccupied sites on the blot was in 0.4% milk buffer according to Parks et al. (1987).

Culture supernatant from the MAb cell line 073d (Shanklin, 1988; Parks et al., 1989) was used at a 1:200 dilution in 0.4% milk buffer to detect the *phyA* gene product on electroblots. The cell culture supernatants of six hybridoma lines producing the MAbs B1, B7, B8 and C1, C11, C13 raised against the bacterially overexpressed *phyB* and *phyC* gene products, respectively, were each used at a 1:100 dilution in two type-selective pools to detect their respective proteins in plant extracts. Primary antibody incubations were for 2 hr at room temperature, followed by three 10-min washes in 0.4% milk buffer. Goat anti-mouse Ig antibody conjugated to alkaline phosphatase (Promega) was used in a 1:5000 dilution for the final 1-hr incubation. Immunochemically detectable bands were visualized in a solution of 100 mM Tris, pH 8.9, 100 mM NaCl, 5 mM $MgCl_2$ to which 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were added to final concentrations of 75 and 150 μg/mL, respectively.

Bacterial Overexpression of Phytochromes and Antibody Production

The polymerase chain reaction was used to obtain the entire protein coding regions of the *phyA*, *phyB*, and *phyC* genes (Sharrock and Quail, 1989). Appropriate restriction sites were appended to the polymerase chain reaction primers before amplification to facilitate the cloning of the products into the vector pET-3c (Rosenberg et al., 1987) in frame with the initiator ATG of the plasmid. The host strain BL21 (DE3) of *Escherichia coli* was transformed with each of the three pET-3c constructs by standard techniques (Sambrook et al., 1989). Growth was in 2% Tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM KPO_4, pH 7.2, and induction of the transformants was with isopropyl β-D-thiogalactopyranoside as described by Studier and Moffat (1986). The insoluble inclusion body fractions were purified according to Nagai and Thøgersen (1987) (DNase added to 1 mg/mL), and the final pellets were solubilized in 1% SDS, 6 M urea, 50 mM Tris, pH 7.8, 0.5 mM DTT, and 1 mM EDTA.

The solubilized inclusion bodies were subjected to SDS-PAGE, and the highest molecular mass band was excised and electro-eluted into 0.01% SDS, 190 mM glycine, and 25 mM Tris. The *phyB* and *phyC* gel-purified apoproteins were diluted 1:1 into 2 × reconstituted adjuvant (RIBI Adjuvant System, RIBI Immunochem Research, Inc., Hamilton, MT) to a final volume for injection of no greater than 100 μL per mouse. Two Swiss-Webster and two BALB/c mice were injected subcutaneously with 50 μg of the appropriate antigen every 10 to 12 days until an adequate titer was raised, as assayed by dot immunoblot analyses. Mice were prepared 5 days before fusion by an intravenous hyperimmunization. The subsequent electrofusion protocol and postfusion hybridoma maintenance were as described by Karu (1991). Hybridoma supernatants were transferred to immunological assay plates using a robotic sampling system (Karu et al., 1985). The cell lines were assayed by ELISA and immunoblots, and select hybridomas were subcloned and stored in liquid nitrogen.

RNA Preparation and Blot Hybridization

RNA isolation and blot methods were as described in Sharrock and Quail (1989). RNA was isolated from 1 to 2 g of whole plant Arabidopsis tissue by the sarcosine-phenol-LiCl method. Samples of 5 μg of total RNA were separated on formaldehyde-containing agarose gels and transferred to GeneScreen hybridization membranes (Du Pont). Membranes were prehybridized and hybridized at 42°C in buffers containing 50% formamide. Transcript-specific hybridization probes for the Arabidopsis *phyA*, *phyB*, and *phyC* mRNAs were ^{32}P-labeled single-stranded antisense DNAs derived from the 3′ ends of the corresponding cDNA clones (Sharrock and Quail, 1989). Blots were washed for 30 min in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 65°C, and autoradiography was done at −80°C with an intensifying screen. Because of variability in both the specific activity of the hybridization probes and in the time of exposure, direct comparisons between different RNA blots are not meaningful.

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