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Isolation and Initial Characterization of *Arabidopsis* Mutants That Are Deficient in Phytochrome A[1]

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Phytochrome, a red/far-red-light photoreceptor protein of plants, is encoded by a small gene family. Phytochrome A (PHYA), the product of the *PHYA* gene, is the predominant molecular species of phytochrome in etiolated tissue and has been best characterized biochemically. To define a role for PHYA, we isolated new mutants, designated *fre1* (far-red elongated), in *Arabidopsis thaliana* that were specifically deficient in PHYA spectral activity and protein accumulation. These mutants were identified on the basis of their long hypocotyl phenotype under continuous far-red light. Although the *fre1* mutants lacked the hypocotyl response to continuous far-red light, their responses to continuous white light and to end-of-day far-red-light treatments were normal. Thus, PHYA appears to play only a minor role in the regulation of hypocotyl elongation under natural conditions. In contrast, the *fre1* mutation affected greening; a *fre1* mutant was less able than the wild type to deetiolate after growth in the dark. However, the potentiation effect of a red-light pulse on accumulation of chlorophyll was not changed significantly in the *fre1* mutants. Thus, the function of PHYA might be highly specialized and restricted to certain phases of *Arabidopsis* development.

Incident light modulates both the development and the physiology of plants. Plants have several regulatory photoreceptors that respond to the light environment, the best studied of which is phytochrome (Thomas and Johnson, 1991). Phytochrome is known to regulate a wide variety of responses of plants to light, including induction of seed germination, modulation of elongation growth, promotion of chloroplast development, and induction of flowering (Shropshire and Mohr, 1983). An understanding of the molecular mechanism of phytochrome action is therefore essential to elucidate the complex processes of plant growth and development.

Phytochrome is a chromoprotein existing as a dimer of two 120-kD polypeptides, each with a covalently attached linear tetrapyrrole chromophore (Lagarias, 1985). This pigment undergoes a reversible photo-induced conversion between two distinct forms, a Pr, which is physiologically inactive, and a Pfr, which is active. Recent biochemical (Abe et al., 1989; Somers et al., 1991) and molecular biological (Sharrock and Quail, 1989; Dehesh et al., 1991) analyses have indicated that phytochrome is encoded by a small gene family in angiosperms. In *Arabidopsis*, for which available information is most complete, five distinct phytochrome genes, *PHYA* through *PHYE*, have been reported (Sharrock and Quail, 1989). *PHYA* and *PHYB* genes are also present in rice (Dehesh et al., 1991) and potato (Hayer and Gatz, 1992). Amino acid identities between different members of the family are as low as 50% (Sharrock and Quail, 1989; Dehesh et al., 1991).

Among the members of the phytochrome family, the *PHYA* gene appears to encode the well-characterized light-labile phytochrome, which accumulates at a high level in the dark but is rapidly degraded upon illumination (Sharrock and Quail, 1989; Somers et al., 1991). However, it is noteworthy that PHYA persists at a certain level in the light (Abe et al., 1989; Somers et al., 1991). In contrast to PHYA, PHYB and PHYC accumulate at much lower levels that are unaffected by light (Abe et al., 1989; Somers et al., 1991). The divergence in amino acid sequences among these molecular species and the distinct patterns of their expression imply that each of the species might play distinct roles in development (Furuya, 1989).

To assign a biological function to each phytochrome, mutants that are deficient in one of the molecular species would be of great help (Furuya, 1989; Kendrick and Nagatani, 1991; Reed et al., 1992). In this respect, the *hy3* mutant of *Arabidopsis* (Koornneef et al., 1980) and the *lh* mutant of cucumber (Adamse et al., 1987) are particularly important among a number of phytochrome-related mutants. The *hy3* mutant of *Arabidopsis* has recently been reported to lack PHYB polypeptide (Nagatani et al., 1991; Somers et al., 1991). Furthermore, molecular analysis of mutations at the *HY3* locus show that *HY3* encodes PHYB (Reed et al., 1993). Lack of PHYB was also demonstrated in the *lh* mutant of cucumber (López-Juez et al., 1992) and the *ein* mutant of *Brassica* (Devlin et al., 1992), although the molecular lesions are unknown.

These mutants all exhibit similar deficiencies in phytochrome responses. They lack EOD-FR shoot elongation responses (Adamse et al., 1988a; López-Juez et al., 1990; Nagatani et al., 1991), indicating that PHYB plays a major role

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Abbreviations: EOD-FR, end-of-day far-red light; PHYA, PHYB, and PHYC, phytochrome proteins encoded by the *PHYA*, *PHYB*, and *PHYC* genes, respectively.

in this growth response. This view is consistent with previous physiological observations that the EOD-FR responses are regulated through a "light-stable pool" of Pfr (Jabben and Holmes, 1983). Furthermore, *phyB* null mutations affect elongation responses in various tissues, including hypocotyls, petioles, floral stems, and root hairs (Reed et al., 1993). In addition, these mutants have reduced Chl content and flower earlier than the wild type. Thus, PHYB acts in numerous tissues throughout development to affect growth and physiological processes.

Another class of phytochrome-related mutants includes the *hy1*, *hy2*, and *hy6* mutants of *Arabidopsis* (Koornneef et al., 1980; Chory et al., 1989) and the *au* mutant of tomato (Koornneef et al., 1985). Severe reduction in spectral activity of phytochrome in etiolated tissues is a common feature of these mutants, indicating that they are deficient in PHYA. However, phytochrome apoprotein is present in these mutants (Chory et al., 1989; Parks et al., 1989). Moreover, results of feeding experiments with precursors of phytochrome chromophore in *hy1* and *hy2* (Parks and Quail, 1991) and in *hy6* (J.W. Reed and J. Chory, unpublished data) mutants suggest that these mutants are deficient in the biosynthesis of the chromophore. Therefore, all the molecular species of phytochrome are probably defective in these mutants.

In the tomato *au* mutant, PHYB appears to be functional, because this mutant responds to the EOD-FR treatments in a normal way (Adamse et al., 1988b). Nonetheless, the *au* mutation appears not to be a mutation in the *PHYA* gene, because the apparent homolog of the *PHYA* gene in tomato maps to a chromosome location distinct from the *au* locus (R. A. Sharrock, personal communication). It is still possible that the *au* mutation affects not only PHYA but also the other molecular species of phytochrome (Reed et al., 1992). Thus, although PHYA is the predominant species in etiolated tissue and has been best characterized biochemically, definition of the biological roles for PHYA still awaits isolation of mutations in the *PHYA* gene itself.

In this paper we present the isolation and the initial characterization of a new class of *Arabidopsis* mutants that are deficient specifically in PHYA. Our screening strategy was based on the assumption that responses of the etiolated seedlings to continuous far-red light (often referred to as far-red high-irradiance responses) are mediated by PHYA (Smith and Whitelam, 1990). Accordingly, we screened for long hypocotyl mutants under continuous far-red light to obtain mutants specifically deficient in PHYA spectral activity and protein accumulation. The two lines described here define mutations at a single locus, *FRE1* (far-red elongated). The *fre1* mutants exhibit normal responses for hypocotyl growth inhibition by EOD-FR irradiation and continuous white light treatments and appear normal after growth in continuous white light. However, the deetiolation process is affected to some extent. Thus, PHYA appears to play a highly specialized role in *Arabidopsis* development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The alleles used in the present study were *hy1* (21.84N), *hy3* (Bo64) (Koornneef et al., 1980), *hy3* (8–36) (Reed et al., 1993), and *hy6-1* (Chory et al., 1989). These mutants were either isolated in the Landsberg *erecta* background (*hy1*, *hy3*) or backcrossed into the Landsberg *erecta* background (*hy6*). The wild type used in the present study was Landsberg *erecta*. New mutants were isolated from M2 seeds of *Arabidopsis thaliana* (ecotype Landsberg *erecta*) mutagenized with ethyl methanesulfonate (Lehle Seeds, Tucson, AZ). Seeds of the M_3 generation of the putative mutants were used to test for rescue with biliverdin, a precursor of phytochrome chromophore (Elich and Lagarias, 1987; Elich et al., 1989) and for the immunochemical detection of PHYA. Further analysis was performed in the M_4 and M_5 generations of the putative mutants.

For most of the experiments, seeds were sown on 0.5% agar plates containing one-tenth strength Murashige-Skoog salt mixture (Murashige and Skoog, 1962). For the biliverdin test, seeds were grown on the agar plates containing 0.125 mM biliverdin (Sigma) and 7.5 mM Hepes-KOH (pH 7.4) in addition to the Murashige-Skoog salt mixture (Parks and Quail, 1991). For the deetiolation experiments, plates containing Murashige-Skoog medium with or without Suc were used.

In most experiments, seeds were placed in white light for 1 d before the far-red-light treatments to induce germination. For the deetiolation experiments, the seeds were incubated at 4°C for 43 h and then in constant white light for 16 h before transfer to the dark. For the screening of mutants in the M_2 seeds, plates were placed under continuous far-red light for 5 d. For the determination of phytochrome, seedlings were grown for 6 d in the dark. For the Chl experiments, seedlings were grown in the dark for 4 d instead of 6 d. For the detection of PHYB, plants were grown under continuous white light for 3 to 5 weeks in pots. All the growth and light treatments were done at 23°C.

Isolation of Mutants and Genetic Analysis

Approximately 4000 and 2000 M_2 seeds were screened for the long hypocotyl phenotype under continuous far-red light from each of 9 and 10 independent parental groups. Each parental group was derived from 1000 M_1 parents (Lehle Seeds). The putative mutants that showed more than 4-fold longer hypocotyls than the other plants were then grown in pots to obtain the M_3 seeds. The M_3 seeds were then tested for the dependence of the long hypocotyl phenotype on biliverdin. Among the putative mutants in which the long hypocotyl phenotype was biliverdin independent, three were further characterized in the M_4 generation. Because two of the three showed substantially reduced levels of PHYA polypeptide, these two variants were further analyzed genetically.

Light Sources

White light of about 6.0 W m^{-2} from white fluorescent tubes (FL20S.W.SDL.NU; National, Tokyo) was used for the growth of plants except that "white" light of 4.0 and 8.5 W m^{-2} from red/blue fluorescent tubes (FL20S.BRF; Toshiba, Tokyo) was used for the EOD-FR and the Chl accumulation experiments, respectively. Continuous far-red light of 3.5 W m^{-2} for the screening of mutants and hypocotyl elongation

assays was obtained from special far-red fluorescent tubes (FL20S.FR-74; Toshiba) filtered through 3-mm red acrylic (Shinkolite A102; Mitsubishi Rayon, Tokyo). For the EOD-FR experiments, far-red light of 1.8 W m^{-2} from the far-red tubes wrapped with red and blue film (red No. 22, blue No. 72; Tokyo Butai Shomei, Tokyo) was used. Red light of 4.5 W m^{-2} for the Chl accumulation experiments was obtained from the red/blue fluorescent tubes filtered through the red acrylic. Spectral energy distributions of the light sources for red and far-red irradiation were calculated from the percent transmission spectra of the filters and energy distributions of the fluorescent tubes supplied from the company (Fig. 1). Phytochrome extraction and measurement were done under a dim green safelight (Nagatani et al., 1989).

Hypocotyl Elongation Experiments

For the initial hypocotyl test, 10 to 20 seedlings of the putative mutants in the M_3 generation were treated with continuous far-red light for 6 d on the agar plates with or without biliverdin, and the hypocotyl lengths were determined. To examine the hypocotyl responses in the M_5 generation, about 60 seedlings were subjected to the treatments. To examine the hypocotyl responses to the EOD-FR treatments in the M_5 generation, about 60 seedlings sown on the agar plates were treated with 8-h white light/16-h dark cycles for 6 d, and the hypocotyl lengths were determined. At the end of each light period, the seedlings were treated with or without far-red light for 30 min. As controls, we examined the *hy3* and *hy6* mutants and the wild-type plants in all of the experiments described above. In addition to these, the *hy1* mutant was tested in the biliverdin experiments.

Immunochemical and Spectral Detection of Phytochrome

For the immunochemical detection of PHYA in the M_4 generation, about 500 seeds were grown in the dark. The whole etiolated seedlings were then glass homogenized in the presence of 1 mL of phytochrome extraction buffer (100 mM Tris-HCl, 28 mM 2-mercaptoethanol, 5 mM EDTA, and 0.5 mM PMSF [pH 8.3]) per g of tissue (Nakazawa et al., 1991). Immunochemical detection of PHYA in the extracts was performed as described by Nagatani et al. (1991). For the immunochemical detection of PHYB in the M_5 generation, about 0.4 g of young leaves of the light-grown plants were processed in the same way. For the SDS-PAGE, a 7.5% acrylamide gel was used. The monoclonal anti-PHYA and anti-PHYB antibodies used in the present study were mAP5 (Nagatani et al., 1985) and mAT1 (López-Juez et al., 1992), respectively.

For the in vivo spectrophotometry of phytochrome, about 0.5 g of etiolated seedlings were packed into a stainless steel cuvette with glass windows (10 mm in diameter and about 4-mm light path), and the difference spectrum for phytochrome phototransformation was determined at 4°C (Suzuki et al., 1980) with a spectrophotometer (model 3410; Hitachi, Tokyo) equipped with an actinic irradiation unit (Nagatani et al., 1989). M_5 seedlings were used for the in vivo measurements.

Deetiolation Experiments

The M_5 seeds sown on plates with or without Suc were subjected to a cold treatment and then placed in the dark for various numbers of days before transfer to 16-h light/8-h dark cycles in a growth chamber (3×10^{16} quanta cm^{-2} s^{-1}). Three days after the transfer to light, the numbers of seedlings with unfolded cotyledons were counted as deetiolated.

Potentiation of Chl Accumulation

The procedures were based on those of Lifshitz et al. (1990) with slight modifications. About 80 etiolated seedlings were irradiated with a 3-min pulse of red light, kept in the dark for 4 h, then transferred to continuous white light, and harvested at different times for determination of the level of total Chl. Chl was extracted from the upper half of the 20 to 30 seedlings with no seed coat attached to the cotyledons into *N,N*-dimethylformamide. The level of Chl was determined by fluorescence. Seeds from the M_4 generation of the *fre1* mutants were used.

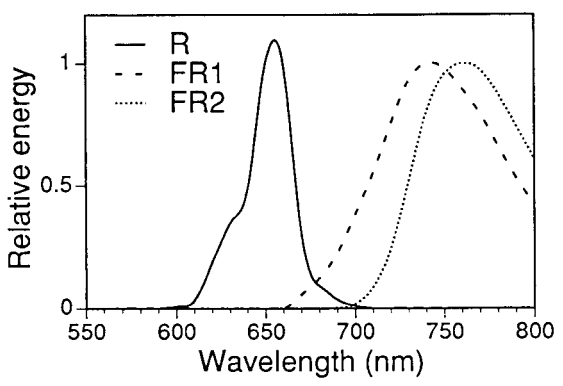

Figure 1. Calculated spectral energy distributions of light sources. R, Red light from white fluorescent tubes filtered through red acrylic; FR1, far-red light from far-red fluorescent tubes filtered through red acrylic; FR2, far-red light from far-red-light fluorescent tubes filtered through red and blue film.

RESULTS

Isolation of New Long Hypocotyl Mutants in Far-Red Light

We screened 56,000 M_2 progeny of a population of ethyl methanesulfonate-mutagenized seeds for variants having a longer hypocotyl than the wild type under continuous far-red light. We picked 16 putative mutants that exhibited a 4- to 8-fold increase in hypocotyl length compared to the wild type. Subsequently, we harvested M_3 seeds from 14 of the 16 original lines. To identify chromophore-deficient mutants in loci such as *hy1* or *hy2*, we examined whether or not the wild-type phenotype was restored by the addition of biliverdin, a precursor of phytochrome chromophore (Elich and Lagarias, 1987; Elich et al., 1989), in these lines. Although the short hypocotyl phenotype was restored with biliverdin to some extent in seven of the 14 lines, the phenotype was biliverdin independent in the seven other lines (data not shown). Thus, the latter seven lines did not appear to be chromophore-related mutants.

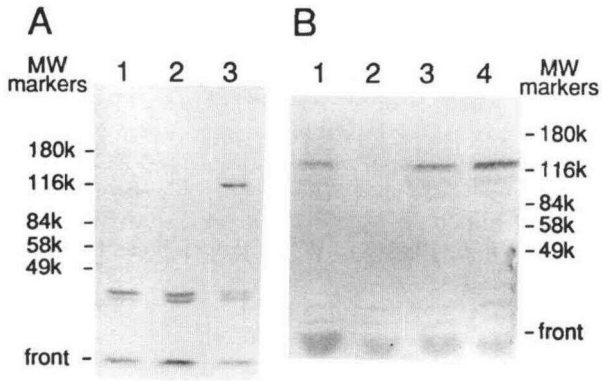

Figure 2. Immunoblot detection of PHYA and PHYB. A, Detection of PHYA in extracts from etiolated tissues with monoclonal antibody mAP5. Lane 1, *fre1–1*; lane 2, *fre1–2*; lane 3, wild type. Each lane contained 25 μg of total protein. Mol wt (MW, expressed in thousands) markers were from Sigma (Prestained Kit). B, Detection of PHYB in extracts from light-grown tissues with monoclonal antibody mAT1. Lane 1, Wild type; lane 2, *hy3* mutant; lane 3, *fre1–1* mutant; lane 4, *fre1–2* mutant. Each lane contained 120 μg of total protein. Mol wt markers were the same as above.

Three of the seven biliverdin-independent variants were analyzed for accumulation of PHYA polypeptide in the M_4 generation. Preliminary immunoblot analysis for PHYA with a monoclonal antibody demonstrated that the levels of PHYA polypeptide in the etiolated seedlings were substantially reduced in two of the three lines tested (see below). When these two lines were crossed with the wild-type strain, the F_1 heterozygotes resembled the wild-type parent under far-red light, showing that the mutations were recessive. Moreover, F_1 double heterozygotes arising from a cross between the two mutants appeared etiolated in far-red light, indicating that the two mutations failed to complement each other. They both complemented a *phyB* mutant, indicating that they did not fall in the *PHYB* gene. Thus, these two lines appeared to define mutations at a new locus. We tentatively designated this locus *fre1*, for far-red elongated. The two lines described above are now referred to as *fre1–1* and *fre1–2*. We assessed whether the *fre1* mutations were linked to the *PHYA* gene by examining the F_2 progeny of crosses of *fre1–1* or *fre1–2* to a *dis1* (distorted trichomes) line. The *PHYA* gene maps on chromosome 1 very close to *dis1* (Cherry et al., 1992). Of 238 F_2 progeny having long hypocotyls under far-red light, just three had distorted trichomes. These data indicate that the *fre1* mutations are closely linked to the *dis1* locus and, therefore, to the *PHYA* gene.

Phytochrome Analysis in the *fre1* Mutants

We analyzed phytochrome protein in the *fre1* mutants. Immunoblot analysis for PHYA with a monoclonal antibody mAP5 (Fig. 2A) revealed no PHYA signal in the etiolated tissue of the *fre1–1* mutant (lane 1), whereas the protein was detected clearly in the wild type at approximately 116 kD (lane 3). The other allele, *fre1–2*, showed only a faint signal (lane 2). In contrast, immunoblot analysis of the extracts from light-grown plants with a PHYB-specific monoclonal antibody (Fig. 2B) indicated the presence of PHYB at normal levels in both *fre1* mutants (lanes 3 and 4). As previously reported (Nagatani et al., 1991), the *hy3* mutant showed no signal on the blot (lane 2).

Because PHYA is the predominant molecular species of phytochrome in etiolated tissue (Somers et al., 1991), we examined whether spectral activity of phytochrome was detected by difference spectroscopy in etiolated *fre1* mutants. As expected, no significant signals of phytochrome were detected in plants with either of the two alleles of *fre1*, whereas a typical difference spectrum for phytochrome photoconversion was observed in the wild type (Fig. 3).

Hypocotyl Elongation Responses in the *fre1* Mutants

We examined the effect of continuous irradiation with far-red or white light on hypocotyl elongation in the mutants. Both white and far-red light had inhibitory effects in the wild type (Fig. 4) as previously reported (Koornneef et al., 1980). The *fre1* mutants totally lacked the response to continuous far-red light, whereas they retained a normal response to white light (Fig. 4). Thus, the hypocotyl lengths of the *fre1* mutants were the same in far-red light as in the dark (Fig. 4). In contrast, the *hy3* mutant responded to the far-red light quite normally, but its response to white light was reduced (Fig. 4) as previously reported (Koornneef et al., 1980). Under the same conditions, the *hy6* mutant showed deficiencies in both responses (Fig. 4).

We examined the effect of exogenously added biliverdin, a precursor of the phytochrome chromophore phytochromobilin, on the long hypocotyl phenotype of the *fre1* mutants under continuous far-red light. As described previously (Parks and Quail, 1991), biliverdin restored the wild-type length of hypocotyls to both the *hy1* and *hy6* mutants under

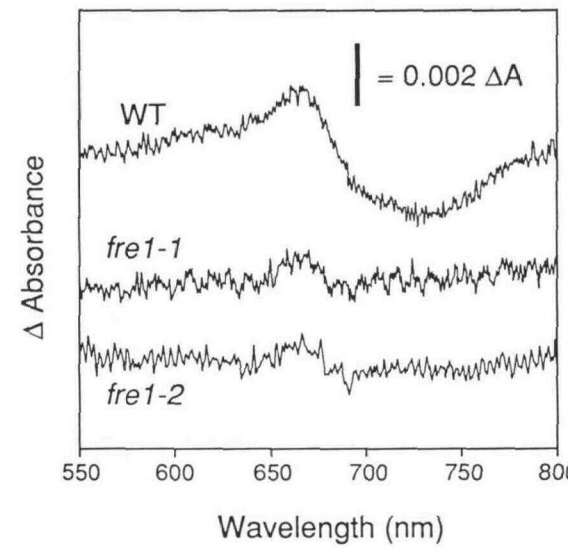

Figure 3. In vivo measurement of spectral activity of phytochrome in etiolated seedlings of the *fre1* mutants. Difference spectra (red minus far-red) of the etiolated tissue of the *fre1* mutants (*fre1–1* and *fre1–2*) and wild-type plants (WT). Bars indicate *A* differences.

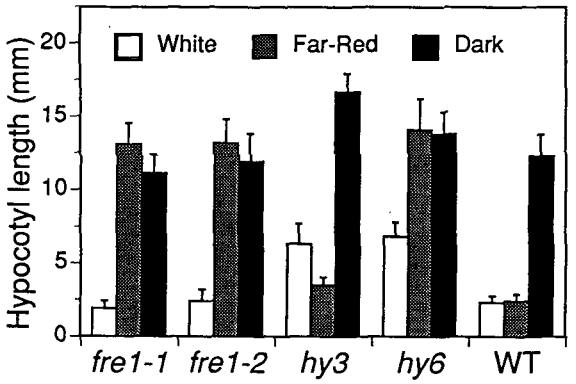

Figure 4. Hypocotyl elongation under continuous light. Hypocotyl lengths of the plants grown under continuous white light, continuous far-red light, or total darkness were determined. Bars indicate SD. WT, Wild type.

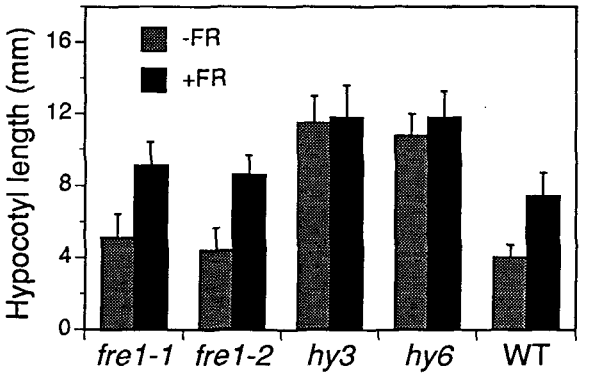

Figure 6. Responses of hypocotyls to EOD-FR treatments. Hypocotyl lengths of plants treated without far-red (−FR) or with far-red (+FR) light were determined. Bars indicate SD. WT, Wild type.

continuous far-red light (Fig. 5). In contrast, biliverdin had no effect on the phenotype of the *fre1* mutants.

We also analyzed the promotion of hypocotyl elongation in the *fre1* mutants by EOD-FR treatments. As shown in Figure 6, hypocotyls of the *hy3* and *hy6* mutants were significantly longer than those of the wild type, even when the plants were not treated with far-red light, and far-red light did not have any additional effects. In contrast, the plants with the two *fre1* alleles exhibited normal elongation responses to the treatments (Fig. 6). This observation is in striking contrast to the observation made in the continuous far-red-light experiments (Fig. 4), in which the *fre1* mutant totally lacked the response to far-red light, whereas the *hy3* mutant retained the response.

Greening in the *fre1* Mutants

Although severe retardation of greening has been reported in phytochrome-deficient mutants such as the *hy1*, *hy2*, and *hy6* mutants (Chory et al., 1989; Lifshitz et al., 1990), the *fre1* mutants greened normally when grown under continuous white light (data not shown). We examined the greening process in the *fre1-1* mutant in greater detail by observing its ability to recover from etiolation. We found that dark-grown seedlings of this mutant lost the ability to deetiolate after shorter periods in the dark than the wild type (Fig. 7A). When dark-grown seedlings were transferred to white light after various times in the dark, a smaller proportion of mutant seedlings than wild-type seedlings deetiolated. Whereas the wild type lost its ability to deetiolate after 4 to 6 d of growth in the dark, the *fre1-1* mutant lost its ability to deetiolate after between 3 and 5 d of growth in the dark (Fig. 7A).

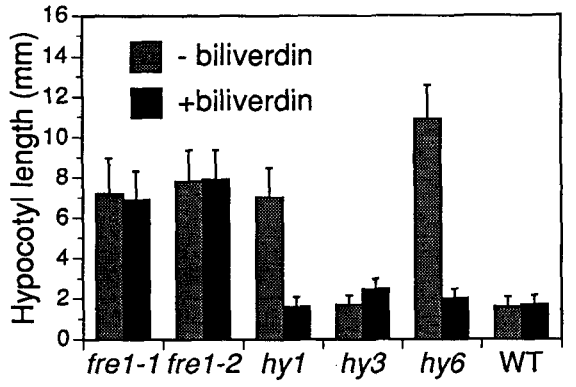

Figure 5. Hypocotyl elongation under continuous far-red light in the presence and absence of biliverdin. Bars indicate SD. WT, Wild type.

The time at which seedlings lost their ability to deetiolate depended on the experimental conditions. For example, the presence of Suc in the medium delayed the loss of ability to deetiolate in both the wild type and the mutants (Fig. 7B). Similarly, both wild-type and mutant seedlings grown in far-red-rich light retained their ability to deetiolate upon transfer to white light for several days longer than if transferred from the dark (data not shown). In these experiments, the *hy3* mutant was better able to deetiolate than the *fre1-1* mutant (Fig. 7, data not shown), although in the absence of Suc the *hy3* mutant appeared to have a deetiolation phenotype intermediate between those of the wild type and the *fre1-1* mutant (Fig. 7A).

We examined the potentiation of Chl accumulation by a red-light pulse in the two *fre1* mutants, the *hy3* mutant and the wild type (Fig. 8). This response is known to be regulated by phytochrome (Raven and Shropshire, 1975). The rate of Chl accumulation after transfer from dark to continuous white light varied slightly among the lines tested. The increases in Chl content over 5 h were 21-fold (*fre1-1*), 30-fold (*fre1-2*), 28-fold (*hy3*), and 33-fold (wild type). This experiment was performed twice with similar results (data not shown). Although we cannot exclude the possibility that the lower Chl accumulation of the *fre1-1* mutant was a property of the particular seed batch used in this experiment, these results appear consistent with the results of the deetiolation experiment described above, in which the *fre1-1* mutant was deficient in deetiolation (Fig. 7A).

However, despite these possible differences in rates of Chl accumulation upon deetiolation, the various lines responded similarly to pretreatment with a potentiating red-light pulse.

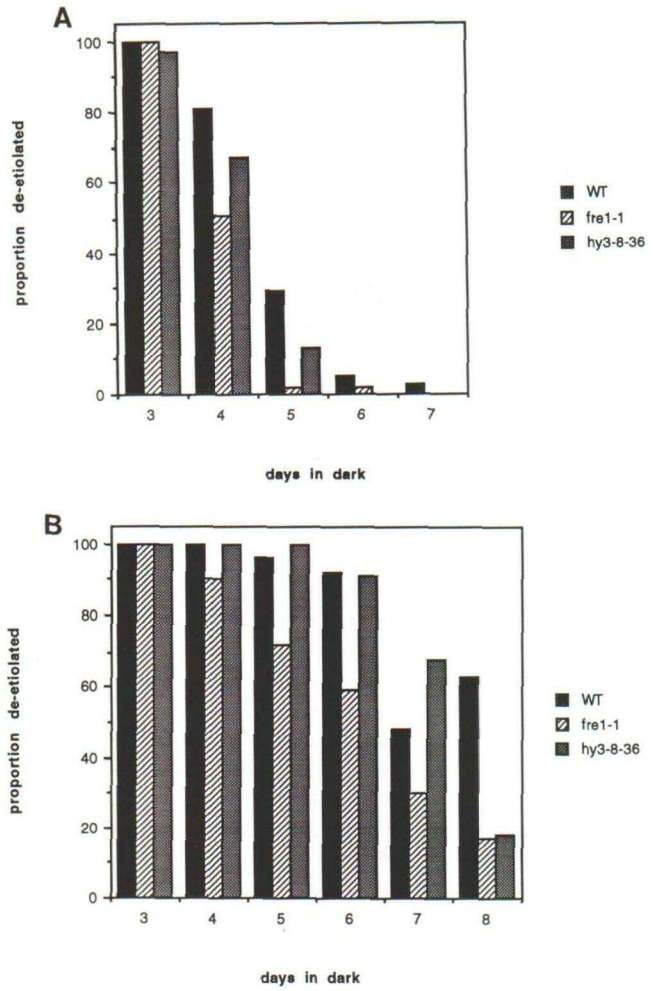

Figure 7. Recovery of etiolated seedlings upon transfer to white light. Wild-type (WT) (Ler), *fre1–1*, and *hy3–8–36* seedlings were grown in the dark for different lengths of time and then transferred to white light. Shown is the proportion of seedlings that appeared deetiolated 3 d after transfer to white light. Between 30 and 65 seedlings were assessed for each data point.

In all four lines, the rate of Chl accumulation was increased (Fig. 8). Furthermore, the effectiveness of the red-light pulse appeared to be similar among all of the lines tested. From Figure 8, we estimated the accelerations of Chl accumulation by the red-light pulse for the different strains to be 1.3 (*fre1–1*), 1.3 (*fre1–2*), 1.1 (*hy3*), and 1.2 h (wild type).

Phenotypes of Mature *fre1* Plants

Although the *fre1* mutants were severely deficient in both PHYA protein (Figs. 2 and 3) and in the response to continuous far-red light (Fig. 4) at the seedling stage, mature plants grown under continuous white light looked quite normal (Fig. 9). The shape and color of the leaves were not distinguishable from those of the wild type, and the two *fre1* mutants flowered at about the same time as the wild type. In contrast, the *hy3* mutant grown under the same conditions exhibited

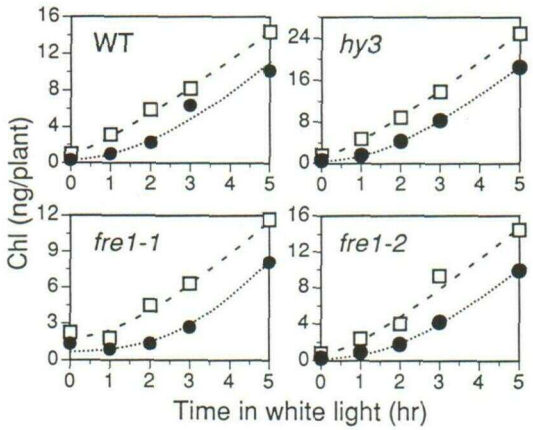

Figure 8. Potentiation of rapid greening by a pulse of red light. Etiolated seedlings were treated with a pulse of red light before transfer to continuous white light. Both pretreated plants (□) and dark controls (●) were harvested for Chl assay at different times following transfer to white light. WT, Wild type.

significantly longer petioles and paler color and flowered early (Koornneef et al., 1980; Goto et al., 1991; Nagatani et al., 1991; Reed et al., 1993; data not shown), and the *hy1*, *hy2*, and *hy6* mutants are very pale and also flower early (Koornneef et al., 1980; Goto et al., 1991; Chory, 1992; Reed et al., 1993).

DISCUSSION

The mutant screen described here was designed to identify seedlings deficient specifically in PHYA. One-half of the long hypocotyl lines scored under continuous far-red light were probably alleles of the previously described *hy1*, *hy2*, or *hy6*

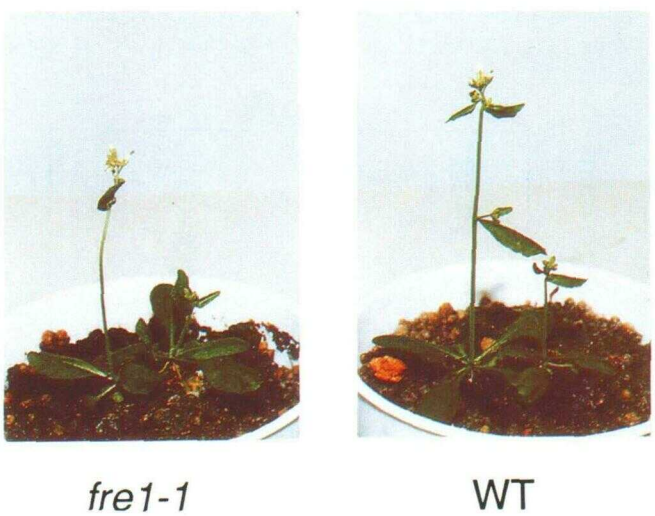

Figure 9. Mature plants of the *fre1–1* mutant and the wild type. Photographs of plants grown for 35 d under continuous white light.

loci, because their long hypocotyl phenotype was rescued with biliverdin, a precursor of phytochrome chromophore, as previously reported (Parks and Quail, 1991). This class of mutants is known to be deficient in PHYA activity, among others (Chory et al., 1989; Parks et al., 1989). The other mutants had phenotypes distinct from previously described mutants (see below) and are putative *phyA* mutants. We examined three of these lines and found that two of the three exhibited severe deficiencies in the accumulation of PHYA (Fig. 2A) and in PHYA spectral activity (Fig. 3). The mutations in these lines fall in a single complementation group we have called *FRE1*. *FRE1* maps very close to *PHYA*, suggesting that the mutations might actually lie in the *PHYA* gene.

Based on the western blot experiment (Fig. 2A), we estimate that the level of PHYA in the *fre1-1* mutant is less than 10% of the wild-type level. On the other hand, we detected a faint PHYA signal at a level close to the detection limit in the *fre1-2* mutant. Thus, *fre1-2* might be a "leaky" allele of the *fre1* locus. No significant phytochrome signals were detected in the etiolated tissues of the *fre1* mutants by in vivo difference spectroscopy (Fig. 3). Thus, the lack of PHYA polypeptide in the *fre1* mutants was confirmed in vivo. We estimate that the levels of spectrally active PHYA were less than 10% of the normal levels in both mutants. Although small increases in *A* in the red region were detected after the irradiation with far-red light in the *fre1* samples, these signals appear not to be due to phytochrome photoconversion, because the increases were not accompanied by reduction in the far-red region (Fig. 3).

The results presented here demonstrate clearly that the *fre1* mutants define a new class of phytochrome-related mutants. Although quite a number of phytochrome-related mutants have been described, the *fre1* mutants are unique in various ways. First, elongation growth under white light was not affected by the *fre1* mutations either at seedling (Fig. 4) or mature (Fig. 9) stages. In contrast, all of the known phytochrome-related mutants including the *hy1, 2, 3,* and *6* mutants of *Arabidopsis* (Koornneef et al., 1980; Chory et al., 1989), the *au* mutant of tomato (Koornneef et al., 1985), the *lh* mutant of cucumber (Adamse et al., 1987), the $ma3^R$ mutant of sorghum (Pao and Morgan, 1986; Childs et al., 1991), and the *ein* mutant of *Brassica* (Devlin et al., 1992) exhibit elongated morphology under white light. Second, unlike the *fre1* mutants, all of the mutants described previously except for the *au* mutant of tomato (Parks et al., 1987) contain normal levels of PHYA polypeptide. Although the *hy1, hy2,* and *hy6* mutants are known to be deficient in spectral activity of PHYA (Koornneef et al., 1980; Chory et al., 1989), PHYA polypeptide is present in these mutants (Chory et al., 1989; Parks et al., 1989).

Although they are deficient in PHYA, several lines of evidence indicate that the *fre1* mutants are not affected in PHYB. First, the PHYB polypeptide accumulated to a normal level in the light-grown tissues of the *fre1* mutants (Fig. 2B). Consistent with a previous report (Somers et al., 1991), the apparent mol wt of PHYB was slightly greater than that of PHYA. Second, the *fre1* mutations map to a position on chromosome 1 distinct from *PHYB*, which maps on chromosome 2. Third, visible phenotypes of *hy3* (*phyB*) mutants such as paler color, long petioles, and accelerated flowering (Chory et al., 1989; Nagatani et al., 1991; Chory, 1992) were not seen in the *fre1* mutants (Fig. 9). Finally, the *fre1* mutants exhibited no deficiency in EOD-FR elongation. Because *hy3* (*phyB*) mutations are known to affect these phenotypes (Adamse et al., 1988a; Nagatani et al., 1991; Whitelam and Smith, 1991; Reed et al., 1993), we conclude that PHYB is functional in the *fre1* mutants.

The simplest and most likely explanation for our data is that the *FRE1* locus encodes PHYA itself. The close linkage of the mutations to the *dis1* locus is consistent with this model. However, we cannot exclude the possibility that the *FRE1* gene stabilizes phytochromes or is required for the expression of phytochrome genes specifically at the etiolated stage. It is noteworthy that the *au* mutant of tomato, which is also deficient in PHYA polypeptide at the etiolated stage (Parks et al., 1987), might be a chromophore-related mutation (Reed et al., 1992). Molecular characterization of the *fre1* mutations will answer the question definitively.

The *fre1* mutants fail completely to inhibit hypocotyl elongation in response to continuous far-red light (Fig. 4). In this respect, they behave similarly to the *hy1* and *hy2* mutants, which are probably deficient in all molecular species of phytochrome (Chory et al., 1989; Parks et al., 1989; Parks and Quail, 1991). In contrast, the response is not affected in the *hy3* mutants (Koornneef et al., 1980), which have mutations in the *PHYB* gene (Reed et al., 1993). These results suggest that this response is controlled almost solely by PHYA. As reviewed by Smith and Whitelam (1990), this type of response has been postulated to be mediated by a light-labile pool of phytochrome on the basis of physiological experiments. This view is in good accordance with the present results because PHYA is the only phytochrome species known to be light labile (Somers et al., 1991).

The *fre1* mutants appear quite normal at later stages of development. When grown under continuous white light, the color of the leaves of the *fre1-1* mutant was indistinguishable from that of the wild type (Fig. 9), whereas the *hy1, hy2, hy3,* and *hy6* plants are substantially paler than the wild type (Chory et al., 1989; Chory, 1992). Furthermore, whereas PHYB-deficient mutants grown under white light exhibit a typical shade-avoiding appearance (López-Juez et al., 1990; Whitelam and Smith, 1991; Chory, 1992) characterized by elongation of hypocotyl and other tissues, increased apical dominance, and a low Chl level (Smith, 1982); the *fre1* mutants exhibited no such phenotypes (Figs. 4 and 9). Thus, PHYA appears to play only a minor role in maintaining the level of Chl under continuous light illumination and in growth regulation under natural conditions. However, it is possible that the lines isolated in the present study carry leaky alleles. Further molecular characterization of these alleles is in progress.

We observed some deficiency in the greening process during deetiolation in the new mutants. The *fre1-1* mutant lost the ability to deetiolate after less time in the dark than the wild type (Fig. 7). However, this phenomenon was statistical and did not apply to every seedling examined. Further work will be required to determine whether these data reflect a requirement for PHYA for deetiolation or whether, for example, the mutants have a defect in some other process such as seed maturation.

The slight deficiency of Chl accumulation in the *fre1-1* mutant after transfer of the seedlings from the dark to light (Fig. 8) is consistent with the hypothesis that this mutant has an impaired ability to deetiolate. However, the *fre1-2* mutant accumulated Chl normally (Fig. 8). Moreover, the deficiency observed in the *fre1-1* mutant was much smaller than those reported for the *hy1* mutants (Lifshitz et al., 1990) despite the severe deficiency of PHYA activity in both the *fre1* (Figs. 2A and 3) and the *hy1* (Koornneef et al., 1980; Chory et al., 1989) mutants. In addition, the *hy3* (*phyB*) mutant behaved like the wild type in these experiments. Hence, the greening process triggered by the transfer of the plants from the dark to light might be controlled mainly by a molecular species other than PHYA or PHYB, or blue light might compensate for the absence of PHYA or PHYB.

The potentiation effect of a red-light pulse on accumulation of Chl is known to be regulated by a phytochrome on the basis of physiological experiments (Raven and Shropshire, 1975). However, the acceleration by a red-light pulse of the accumulation curves relative to the dark control is not altered in the *hy1* and *hy2* mutants, although the rate of Chl accumulation is substantially reduced in these mutants (Lifshitz et al., 1990). The acceleration of Chl accumulation was also observed for the *fre1* mutants (Fig. 8). Thus, this response might be under control of a special molecular species of phytochrome that is not affected even by a mutation such as *hy1*. Alternatively, an extremely low level of PHYA may be enough to mediate this response.

CONCLUDING REMARKS

In the present paper we describe a novel class of phytochrome-related photomorphogenic mutants that are most likely deficient specifically in PHYA. Comparison of the growth responses between the new *fre1* mutants and the *hy3* mutant, which has a mutation at the *PHYB* locus (Reed et al., 1993), reveals that the *hy3* (*phyB*) and *fre1* mutants have nonoverlaping phenotypic deficiencies. Thus, we conclude that PHYB but not PHYA plays a major role in growth responses under natural conditions. Similarly, our results suggest that PHYA plays only a limited role in the greening process. However, these conclusions are tentative, because we do not know the molecular lesions of the mutations. Nonetheless, the *fre1* mutants are potential tools for elucidation of functions of PHYA.

NOTE ADDED IN PROOF

While this manuscript was in press, a report of very similar mutants appeared: **Parks BM, Quail PH** (1993) *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5:** 39–48.

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