Phytochrome A Null Mutants of Arabidopsis Display a Wild-Type Phenotype in White Light

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Phytochrome is a family of photoreceptors that regulates plant photomorphogenesis; the best-characterized member of this family is phytochrome A. Here, we report the identification of novel mutations at three Arabidopsis loci (fhy1, fhy2, and fhy3) that confer an elongated hypocotyl in far-red but not in white light. fhy2 mutants are phytochrome A deficient, have reduced or undetectable levels of PHYA transcripts, and contain structural alterations within the PHYA gene. When grown in white light, fhy2 mutants are morphologically indistinguishable from wild-type plants. Thus, phytochrome A appears to be dispensable in white light-grown Arabidopsis plants. fhy2 alleles confer partially dominant phenotypes in far-red light, suggesting that the relative abundance of phytochrome A can affect the extent of the far-red-mediated hypocotyl growth inhibition response. Plants homozygous for the recessive fhy1 and fhy3 mutations have normal levels of functional phytochrome A. The FHY1 and FHY3 gene products may be responsible for the transduction of the far-red light signal from phytochrome A to downstream processes involved in hypocotyl growth regulation.

INTRODUCTION

Phytochrome is a regulatory photoreceptor that controls plant growth and development in response to signals from the light environment. Phytochrome is composed of a linear tetrapyrrole chromophore attached to an apoprotein moiety and exists in two stable photointerconvertible forms, Pr and Pfr. Photochromicity between inactive, red light (R)-absorbing Pr and active, far-red light (FR)-absorbing Pfr endows phytochrome with the capacity to sense the relative amounts of R and FR (Smith and Whitelam, 1990). It has recently been established that higher plants possess multiple, discrete phytochrome species, the apoprotein components of which are the products of a small family of divergent genes called PHYA, PHYB, PHYC, PHYD, and PHYE (for review see Quail, 1991). Through comparisons of peptide sequence and deduced amino acid sequence, it is now accepted that phytochrome A, the apoprotein of which is the PHYA gene product, is equivalent to the well-characterized light-labile or type I phytochrome (Quail, 1991).

Phytochrome A accumulates to relatively high levels in etiolated plant tissues, but its levels are rapidly depleted upon exposure of such tissue to R or white light (W). This depletion is due to light-induced degradation of Pfr and to light-mediated down regulation of PHYA gene expression (Quail, 1991). Although phytochrome A is still present in fully deetiolated plants, type II or light-stable phytochromes, such as phytochromes B and C, predominate (Smith and Whitelam, 1990; Quail, 1991).

The existence of multiple phytochrome species together with the diversity of phytochrome responses and response modes have led to the suggestion that different phytochromes may have discrete roles or functions (Smith and Whitelam, 1990). Current understanding of phytochrome function has been greatly advanced through the analysis of the long hypocotyl mutants of Arabidopsis (hyl to hy7) that display an elongated hypocotyl when grown in W (Koornneef et al., 1980; Chory et al., 1989; Chory, 1991). These mutants have lost the capacity for the light-mediated inhibition of hypocotyl growth exhibited by wild-type individuals. hyl, hy2, and hy6 mutants appear to be chromophore depleted, thus causing a corresponding depletion in the levels of active phytochrome (both light-labile and light-stable phytochromes; Chory et al., 1989; Parks et al., 1989; Parks and Quail, 1991). hy3 mutations map to the PHYB gene, and hy3 mutants are specifically deficient in phytochrome B activity (Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993). Thus, phytochrome B is the phytochrome species predominantly responsible for W-mediated hypocotyl growth inhibition, and hyl, hy2, and hy6 mutants probably exhibit an elongated hypocotyl in W because of a reduction in phytochrome B activity.

hy3 seedlings display normal inhibition of hypocotyl growth in response to prolonged FR but lack a hypocotyl growth
inhibition response to R (Koornneef et al., 1980; McCormac et al., 1993). This observation is fully consistent with physiological studies indicating that light-labile phytochrome is responsible for the FR-mediated hypocotyl growth inhibition of etiolated seedlings (the so-called FR high-irradiance response) and that a light-stable phytochrome mediates the effect of R (e.g., Beggs et al., 1980). Thus, phytochrome A null mutants might be expected to exhibit an elongated hypocotyl when grown in FR. Two recent papers have described the isolation of ethylmethane sulfonate (EMS)-induced Arabidopsis mutants (hy8 and frel) that exhibit an elongated hypocotyl in FR and are deficient in functional phytochrome A (Nagatani et al., 1993; Parks and Quail, 1993). Here, we describe the isolation and characterization of further mutants that display an elongated hypocotyl in FR (but not in W). Analysis of these mutants has enabled us to extend the conclusions of previous studies (Nagatani et al., 1993; Parks and Quail, 1993). The mutations identified belong to three distinct complementation groups (fhy1, fhy2, and fhy3). Physiological and molecular experiments showed that the fhy2 mutants are phytochrome A deficient and are defective in functional phytochrome A (Nagatani et al., 1993; Parks and Quail, 1993). Here, we describe the isolation and characterization of further mutants that display an elongated hypocotyl in FR (but not in W). Analysis of these mutants has enabled us to extend the conclusions of previous studies (Nagatani et al., 1993; Parks and Quail, 1993). The mutations identified belong to three distinct complementation groups (fhy1, fhy2, and fhy3). Physiological and molecular experiments showed that the fhy2 mutants are phytochrome A deficient and carry mutations in the PHYA gene. fhy1 and fhy3 identify previously undescribed genetic loci that may play a role in the transduction of the signal from phytochrome A to subsequent steps in the FR response pathway.

Because phytochrome is an important regulatory molecule, it might be expected that alterations in the abundance of phytochrome would have significant effects on the processes it controls. To investigate this possibility, we have studied the effect of heterozygosity for mutations conferring phytochrome A deficiency (fhy2-1, fhy2-2) on hypocotyl elongation in FR. The results of these experiments show that these alleles display partial dominance and suggest that reduced phytochrome A abundance in fhy2 heterozygotes results in a significant lengthening of the hypocotyl of plants grown in FR.

RESULTS

Isolation and Complementation Analysis of Mutations Conferring an Elongated Hypocotyl in FR

Mutants displaying an elongated hypocotyl in FR were isolated from EMS and γ-irradiation-mutagenized M2 populations of Arabidopsis (for details see Methods). These mutants were then transplanted to soil, grown in W, and allowed to self-pollinate. The resultant (M2) seedlings were then tested for the occurrence of elongated hypocotyls in FR and W. Mutant lines displaying elongated hypocotyls in W were excluded from further analysis. Four independent mutant lines displaying elongated hypocotyls only in FR were studied further. Complementation analysis of these four mutant lines was performed by examining the phenotypes of F1 double heterozygotes obtained in crosses between them (all possible combinations). These experiments showed that the four mutant lines fell into three complementation groups, fhy1 (for far-red elongated hypocotyl), fhy2 (at which two alleles, fhy2-1 and fhy2-2, were identified), and fhy3 (data not shown). fhy1 fhy3 double heterozygotes displayed a hypocotyl of a length that was indistinguishable from that of the wild type when grown in FR. fhy1 and fhy3 are therefore complementing mutations at two separate genetic loci, FHY1 and FHY3, respectively. fhy2-1 fhy2-2 double heterozygotes displayed an elongated hypocotyl of a length that was indistinguishable from that of either mutant homozygote parent when grown in FR. The fhy2-1 and fhy2-2 mutant lines therefore contain noncomplementing, allelic mutations at the FHY2 locus. Analysis of fhy1 fhy2 and fhy2 fhy3 double heterozygotes was more complex due to the partial dominance exhibited by fhy2 alleles (see below). These double heterozygotes displayed a slightly more elongated hypocotyl than the wild type in FR. However, in both cases, the degree of hypocotyl elongation exhibited by the double heterozygote in FR was not as great as that exhibited by either parental homozygote. Thus, the fhy2-1 and fhy2-2 alleles were both assigned to a complementation group distinct from those of fhy1 and fhy3.

The fhy1 and fhy2 mutants were isolated from the γ-ray-treated M2 population, and the fhy3 mutant was isolated from the EMS-treated M2 population. We chose to designate these loci fhy so as to clearly distinguish them from previously described hy loci (hy1 to hy7; Koornneef et al., 1980; Chory et al., 1989; Chory, 1991), at which mutations conferring an elongated hypocotyl in W have been obtained. Because of their profoundly differing photosensitivities, it seems unlikely that any of the fhy1 to fhy3 mutations are allelic to any of the hy1 to hy7 mutations. For reasons that will become apparent, it is very likely that the fhy2 mutations are allelic to mutations at the hy8 and frel loci that confer a deficiency in functional phytochrome A (Nagatani et al., 1993; Parks and Quail, 1993). However, because complementation tests with hy8 and frel alleles have not been performed, it would be premature to reassign the fhy2 mutations to the hy8 or frel complementation groups at present.

Phenotypes of fhy1, fhy2, and fhy3 Mutants

Plants homozygous for the fhy2-1 and fhy2-2 mutations display a hypocotyl phenotype strikingly different from that of the wild type when grown in FR. In these conditions, fhy2 mutant homozygotes are almost identical to dark-grown wild-type plants, exhibiting an extremely elongated hypocotyl, closed hypocotyl hooks, and unexpanded cotyledons. In contrast, wild-type plants have short hypocotyls and expanded cotyledons. When grown in FR, fhy2 mutant homozygotes do not differ significantly from wild-type plants when grown in W or R. These data are presented in Figures 1 and 2. When grown in standard glasshouse conditions, fhy2 mutants are morphologically indistinguishable from the wild type at all stages of the life cycle (data not shown). Plants homozygous for fhy1-1 and fhy3-1...
Phytochrome A Mutants of Arabidopsis

Figure 1. Phenotypes Exhibited by fhy Mutants and Their Corresponding Wild Types.

Seeds of fhy1, fhy2-1, fhy2-2, and fhy3 and their corresponding wild types (wtL, Landsberg erecta for fhy1 and fhy2; wtC, Columbia for fhy3) were sown on agar plates and germinated in the dark for 1 day, as described in Methods. Seedlings were grown for an additional 4 days in the dark (D) or under continuous R, continuous FR, continuous B, or continuous W.

also display an elongated hypocotyl selectively in FR (and not in R or W; Figures 1 and 2). Cotyledon expansion and hypocotyl hook opening are inhibited in FR-grown fhy1-1 and fhy3-1 mutants (Figure 1). Compared with their respective wild types, blue light (B)-treated fhy1, fhy2, and fhy3 mutant hypocotyls are all slightly elongated (Figures 1 and 2). That light-labile phytochrome (phytochrome A) mediates at least some of the effects of B on the inhibition of hypocotyl elongation has been concluded from earlier physiological experiments (Beggs et al., 1980).

FHY2 Maps in the Vicinity of PHYA

To obtain the chromosomal locations of the FHY1 and FHY2 loci, we mapped them with respect to the site of insertion of the T-DNA in transformant line A264. This line carries a T-DNA inserted into the top arm of chromosome 1 (Peng and Harberd, 1993) within DNA spanned by yeast artificial chromosomes EG11A5 and EG12E7 (Grill and Somerville, 1991; C. Recknagel and G. Coupland, unpublished data). EG11A5 and EG12E7 also contain PHYA, the structural gene for the phytochrome A apoprotein (J. Peng and N. P. Harberd, unpublished data). Because EG11A5 and EG12E7 contain Arabidopsis DNA inserts of ~150 kb (Hwang et al., 1991; J. Peng and N. P. Harberd, unpublished data), it follows that the T-DNA insertion site is likely to be within 150 kb of PHYA.

Plants homozygous for fhy1 were crossed with plants homozygous for the T-DNA. The resulting F1 plants were

Figure 2. Photocontrol of Hypocotyl Elongation in Wild-Type and fhy Mutant Arabidopsis Seedlings.

Seeds of fhy1, fhy2-1, fhy2-2, and fhy3 and their corresponding wild types (as given in Figure 1) were sown on agar plates, germinated in the dark for 1 day, and grown for an additional 4 days in the dark (D) or under continuous R, continuous FR, continuous B, or continuous W (as shown in Figure 1). The mean hypocotyl lengths of at least 30 seedlings from each light treatment are plotted. Error bars represent the standard errors of the means.
Table 1. Segregation of Phenotypic Classes Obtained following the Backcrosses of fhy1/fhy1; K/K* × fhy1/FHY1; K/K*

<table>
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<th>Phenotypes</th>
<th>Tall</th>
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<td>Resistant*</td>
<td>Sensitive*</td>
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* K*, kanamycin resistance conferred by the T-DNA (A264), is dominant to kanamycin sensitivity (K*).

As expected, an approximate segregation of 1 plant displaying an elongated hypocotyl length in FR (short; FHY1/fhy1) was observed. In both of the tall and short classes, approximately one-half of the plants were kanamycin resistant and approximately one-half were kanamycin sensitive. Thus, fhy1 segregated independently of the T-DNA, showing that fhy1 is not linked to the T-DNA insertion site and is not in the vicinity of PHYA.

Plants homozygous for fhy1 were crossed with plants homozygous for the T-DNA. The resulting F1 plants were self-pollinated. F2 populations were used to score for kanamycin resistance and for an elongated hypocotyl in FR. As expected, an approximate segregation of 3 plants displaying a wild-type hypocotyl length in FR (short; FHY1/fhy1) to 1 plant displaying an elongated hypocotyl in FR (tall; fhy1/fhy1) was observed. In both of the tall and short classes, approximately one-half of the plants were kanamycin resistant and approximately one-half were kanamycin sensitive. Thus, fhy1 segregated independently of the T-DNA, showing that fhy1 is not linked to the T-DNA insertion site and is not in the vicinity of PHYA.

Plants homozygous for fhy2-1 were crossed with plants homozygous for the T-DNA. The resulting F1 plants were self-pollinated. F2 populations were used to score for kanamycin resistance and for an elongated hypocotyl in FR. As expected, an approximate segregation of 3 plants displaying a relatively short hypocotyl in FR (fhy2-1/FHY2, FHY2/fhy2) to 1 plant displaying an elongated hypocotyl in FR (tall; fhy2-1/fhy2-1) was observed (3136 short, 974 tall). Of the 974 tall plants observed, none was resistant to kanamycin. Thus, fhy1 segregated independently of the T-DNA, showing that fhy1 is not linked to the T-DNA insertion site and is not in the vicinity of PHYA. This latter conclusion is strengthened by the physiological and molecular analyses presented below.

Spectroscopic and Immunochemical Analysis of Phytochrome in fhy1, fhy2, and fhy3 Mutants

In vivo spectrophotometric measurements revealed that etiolated fhy1-1 and fhy3-1 seedlings possess levels of spectrally active phytochrome that do not differ appreciably from those of their respective wild types. On the other hand, as shown in Figure 3, etiolated fhy2-1 and fhy2-2 seedlings contain levels of spectrally active phytochrome that are below the limit of detection of the spectrophotometer. Because the spectral activity of phytochrome from etiolated tissues is very largely determined by the abundance of light-labile phytochrome (e.g., Brockmann and Schäfer, 1982; Somers et al., 1991), it can be concluded that fhy2 seedlings are deficient in spectrally active phytochrome A.

This conclusion is supported by immunoblot analyses of phytochrome A polypeptide levels in wild-type and fhy seedlings. Whereas extracts of etiolated fhy1-1 and fhy3-1 seedlings contain more or less wild-type levels of immunochemically detectable phytochrome A polypeptide, no detectable staining is observed for extracts of either fhy2-1 or fhy2-2 seedlings, as shown in Figure 4. Thus, the fhy2-1 and fhy2-2 mutations appear to reduce phytochrome A abundance to levels below the limit of detection, whereas phytochrome A levels appear normal in fhy1-1 and fhy3-1 seedlings.

Isolation and Characterization of Arabidopsis Genomic DNA Clones Containing the PHYA Gene

To obtain hybridization probes for use in RNA and DNA gel blot analyses of the fhy mutants, Arabidopsis genomic DNA copies of spectrally active phytochrome that are below the limit of detection of the spectrophotometer. Because the spectral activity of phytochrome from etiolated tissues is very largely determined by the abundance of light-labile phytochrome (e.g., Brockmann and Schäfer, 1982; Somers et al., 1991), it can be concluded that fhy2 seedlings are deficient in spectrally active phytochrome A.

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Isolation and Characterization of Arabidopsis Genomic DNA Clones Containing the PHYA Gene

To obtain hybridization probes for use in RNA and DNA gel blot analyses of the fhy mutants, Arabidopsis genomic DNA
clones containing PHYA were isolated from a genomic DNA clone library (see Methods). Two such clones, λPHYA81 and λPHYA91, were selected for further analysis. Restriction fragment maps of λPHYA81 and λPHYA91 are shown in Figure 5. Hybridization of oligonucleotide probes (phyA5' and phyA3'; see Methods) containing sequence close to the 5' and 3' ends of the phytochrome A cDNA (see Methods; Sharrock and Quail, 1989) to digests of λPHYA81 and λPHYA91 DNA permitted the identification of approximate 5' and 3' ends of the PHYA gene, as shown in Figure 5. The entire PHYA gene together with 5' and 3' flanking genomic DNA is contained within the overlapping λPHYA81 and λPHYA91 clones. DNA from λPHYA81 and λPHYA91 was used as hybridization probes in the RNA and DNA gel blot experiments described below.

**Analysis of PHYA mRNA Transcripts in fhy2 Mutants**

The abundance of PHYA mRNA in fhy2-1 and fhy2-2 mutant homozygotes was analyzed in RNA gel blot experiments. Etiolated plant material was used in these experiments because PHYA transcripts are known to accumulate to relatively high levels in these conditions (Sharrock and Quail, 1989). Total RNA was isolated from etiolated wild-type and mutant seedlings.

**Figure 4. Immunological Detection of Phytochrome A in Wild-Type and fhy Mutant Seedlings.**

Protein extracts, enriched for phytochrome by ammonium sulfate precipitation, were prepared from 6-day-old etiolated wild-type (wt, Landsberg erecta; wtC, Columbia) and fhy mutant seedlings. Each lane was loaded with 25 μg of protein.

**Figure 5. Restriction Fragment Maps of Genomic DNA Clones Containing PHYA.**

The inserts of λPHYA81 and λPHYA91 are Arabidopsis genomic DNA fragments containing the PHYA gene. Restriction fragment maps of these clones are compared as shown. The position of the PHYA gene is based on approximate 5' and 3' transcript end positions determined using oligonucleotides phyA5' and phyA3'(see Methods). The Sall site at the ends of both clone inserts is from the cloning site of the λFIX II cloning vector. The HindIII site shown very close to, and 3' of, the phyA5' position may represent a sequence polymorphism between Landsberg erecta and Columbia DNA because this site is not present in the phytochrome A cDNA sequence (Sharrock and Quail, 1989).
Irradiation mutagenesis of Arabidopsis often generates deletion (Wilkinson and Crawford, 1991) or rearrangement (Shirley et al., 1992) mutations. To determine if the PHYA gene in the irradiation-induced fhy2 mutants had sustained any such alterations, its structure was investigated in DNA gel blot experiments. The data are presented in Figures 7 and 8. A hybridization probe containing the entire Arabidopsis PHYA gene (λPHYA91) detected novel EcoRI fragments (9.1 and 1.3 kb) in DNA from fhy2-1. These novel fragments replace a 6.2-kb EcoRI fragment seen in DNA from the wild type (FHY2) and fhy2-2 (Figure 7). The 6.2-kb fragment seen in FHY2 contains most of the PHYA gene (see Figure 5). This restriction fragment alteration cannot be due to the occurrence of a mutation creating a novel EcoRI site within the PHYA gene in fhy2-1 because the sum of the sizes of the new fragments observed (9.1 + 1.3 = 10.4 kb) would then be expected to be equal to 6.2 kb. Furthermore, λPHYA91 also detects novel fragments in HindIII digests of genomic DNA from fhy2-1 (data not shown). Thus, the fhy2-1 mutant line contains a rearrangement mutation of PHYA. As expected, restriction fragment alterations were not observed when EcoRI digests of DNA from fhy1-1 and fhy3-1 were probed with the PHYA gene (data not shown).

Experiments to define more precisely the site of the rearrangement mutation in the PHYA gene of the fhy2-1 mutant are shown in Figure 8. Probes 1, 2, and 3 are internal DNA fragments purified from an Arabidopsis phytochrome A cDNA (Sharrock and Quail, 1989; for locations of probes 1, 2, and 3, see Figure 8A). Probe 1 hybridizes with the 9.1-kb, probe 2 with both the 9.1- and 1.3-kb, and probe 3 with the 1.3-kb (see Methods). A radiolabeled hybridization probe containing the PHYA gene (a subfragment of λPHYA81) identified a 4-kb transcript that was present in the wild type, present at greatly reduced levels in fhy2-2, and not detectable in fhy2-1, as shown in Figure 6. The levels of PHYA transcript in fhy1-1 and fhy3-1 were not significantly different from that of the wild type (data not shown).

**fhy2-1 Mutants Show a Structural Rearrangement of the PHYA Gene**

A radiolabeled genomic λ DNA clone (λPHYA81; insert size 14.5 kb) containing the entire Arabidopsis PHYA gene plus flanking DNA (see Figure 5) detected restriction fragment alterations in EcoRI-digested genomic DNA from fhy2-1 with respect to EcoRI-digested DNA from fhy2-2 and the wild type (FHY2). The differences between fhy2-1 and FHY2 are due to partial digestion of DNA because a control genomic DNA fragment detected identical restriction fragment patterns in all lanes when hybridized with the same filter (data not shown). The 6.2- and 3.5-kb EcoRI fragments detected by λPHYA91 in FHY2 DNA are equivalent to the internal 6.2- and 3.5-kb EcoRI fragments of λPHYA91 (see Figure 5). The remaining unlabelled fragment (in all samples) is detected by DNA flanking the 3.5-kb EcoRI fragment at the 3' extremity of λPHYA91. The DNA flanking the 6.2-kb EcoRI fragment at the 5' extremity of λPHYA91 may be of insufficient length to give a detectable signal (see Figure 5).
Figure 8. Location of the Rearrangement Junction in the PHYA Gene in fhy2-1.

(A) Restriction fragment map of the Arabidopsis phytochrome A cDNA (calculated from sequence data; Sharrock and Quail, 1989), identifying fragments 1, 2, and 3 used in the DNA blot hybridization experiments shown in (B). The positions of the translation start (ATG) and stop (TAG) codons are as shown. The likely positions of introns in the genomic DNA sequence of PHYA (based on the fact that intron positions are conserved in the PHYA genes of oat, maize, rice, and pea [Hershey et al., 1987; Sato, 1988; Cristensen and Quail, 1989; Kay et al., 1989] and in the PHYB gene of Arabidopsis [Reed et al., 1993]) are as indicated. The highlighted region shows the EcoRV-KpnI fragment within which the rearrangement junction in fhy2-1 lies.

(B) Radiolabeled subfragments of the Arabidopsis phytochrome A cDNA (probes 1, 2, and 3; see above) also detected restriction fragment differences between EcoRI digests of DNA from fhy2-1 and FY2. In addition to the 6.2-kb EcoRI fragment in FY2 (1.3 kb in fhy2-1), probe 3 also detects the 3.5-kb EcoRI fragment (see Figure 5) because it overlaps the EcoRI site in the cDNA sequence. Faint hybridization to a low molecular weight fragment (seen in both FY2 and fhy2-1) may be due to cross-hybridization with additional phytochrome or phytochrome-related sequences in the genome.
EcoRI fragments originally seen in DNA from fhy2-1 with the λPHYA91 probe (Figure 8B). Thus, probe 1 lies 5' of the rearrangement, probe 2 spans the rearrangement junction, and probe 3 is 3' of it. The rearrangement junction in fhy2-1 therefore lies within the EcoRV-KpnI fragment highlighted in Figure 8A and is close to the center of the transcribed region of PHYA.

The rearrangement in fhy2-1 can occur in one of two ways. First, insertion of DNA into PHYA at the rearrangement junction site identified above may have occurred. To create the novel 9.1- and 1.3-kb EcoRI fragments described above, the minimum size of this possible insertion is 4.2 kb (such that this insertion itself carries a single EcoRI site). Second, an interstitial inversion (with one breakpoint junction located in the center of PHYA, the other at an unknown site) may have occurred. Both of these types of rearrangement would be expected to create a null allele.

**fhy2 Mutations Are Partially Dominant**

To determine whether the fhy2-1 and fhy2-2 mutations are dominant or recessive, plants homozygous for these mutations were backcrossed to the Landsberg erecta progenitor strain (FHY2/FHY2). Hypocotyl elongation in FR of the resulting heterozygotes (fhy2-1/FHY2; fhy2-2/FHY2) was compared with that of mutant (fhy2-1/fhy2-1; fhy2-2/fhy2-2) and wild-type (FHY2/FHY2) homozygotes. The data are presented in Figure 9. Although they are nowhere near as elongated as mutant homozygote hypocotyls, the hypocotyls of heterozygotes (fhy2-1/FHY2 and fhy2-2/FHY2) are significantly longer than those of wild-type homozygotes (FHY2/FHY2) when grown in FR. Thus, the fhy2-1 and fhy2-2 mutations confer partially dominant phenotypes in FR. The phytochrome A−deficient hy8 and fre1 mutants were found to confer recessive phenotypes in FR (Nagatani et al., 1993; Parks and Quail, 1993). This apparent discrepancy with our results may be explained by differences in duration or fluence rate of the FR exposures used. In addition, the effects of heterozygosity for mutations conferring phytochrome A deficiency are subtle and require the observation of a number of heterozygous individuals for a convincing demonstration of their existence.

Similar experiments showed that fhy1-1/FHY1 and fhy3-1/FHY3 heterozygotes display hypocotyl lengths in FR that are indistinguishable from that of wild type (data not shown). Thus, the fhy1-1 and fhy3-1 mutations confer recessive phenotypes in FR.

**DISCUSSION**

This paper describes the isolation and characterization of Arabidopsis mutants that display an elongated hypocotyl phenotype in FR but not in W. These mutants fall into three complementation groups, identifying the FHY1, FHY2, and FHY3 loci. Previous publications on this subject have described the identification of mutants defining single complementation groups (hy8 and fre1; Nagatani et al., 1993; Parks and Quail, 1993) that are probably equivalent to FHY2. A possible reason for our success in identifying the fhy1 mutant is that the agar medium used in our mutant screening experiments lacked sucrose. Exogenous sucrose affects hypocotyl growth and partially suppresses fhy1 phenotype (G. Whitelam, unpublished data) and so may have masked the presence of mutants equivalent to fhy1 in the previous mutant screens (Nagatani et al., 1993; Parks and Quail, 1993). In addition, the hypocotyl elongation displayed by fhy1 in FR, although significantly greater than that of the wild type, is considerably less dramatic than that of the fhy2 mutants. It is possible that, because of their less severe phenotypes, such mutants were simply overlooked in previous experiments. The fhy3 phenotype is not suppressed by exogenous sucrose (G. Whitleam, unpublished data). At present, we cannot explain why previous experiments failed to identify mutants equivalent to fhy3.

The results of our experiments showed that the fhy2-1 and fhy2-2 mutant lines contain mutant alleles of PHYA. Phytochrome A is undetectable in fhy2-2, and this mutant accumulates PHYA transcripts at levels that are greatly reduced with respect to that of the wild type. The fhy2-1 mutant line lacks detectable phytochrome A, lacks detectable PHYA transcripts, and contains a structurally altered PHYA gene.
interrupts the PHYA transcriptional unit, suggesting that fhy2-1 is a phytochrome A null mutant. These observations conclusively demonstrate that seedlings homozygous for mutations at PHYA exhibit an elongated hypocotyl when grown in FR.

When grown in FR, fhy2 mutants are almost indistinguishable from wild-type plants grown in the dark. This characteristic distinguishes these mutants from previously characterized phytochrome-related hy mutants (hy1, hy2, hy3, and hy6). These mutants display long hypocotyls when grown in W. However, the length of these hypocotyls is not as great as that observed in dark-grown (etiolated) seedlings, possibly because of the action in W of photoreceptors (B, UV-A and UV-B receptors) other than phytochrome. The fact that FR-grown fhy2 mutants mimic etiolated wild-type plants implies that phytochrome A is the principal active photoreceptor in FR.

With the possible exception of hy5, none of the hy mutants appears to be a phytochrome-related signal transduction chain mutant (Chory, 1992). We have identified two novel mutations (fhy1-1 and fhy3-1) that confer an elongated hypocotyl in FR (but not in W) and do not belong to the fhy2 (PHYA) complementation group. Unlike the fhy2 mutants, fhy1-1 and fhy3-1 confer recessive phenotypes in FR. The FHY1 and FHY3 loci appear not to be extragenic regulators of PHYA because levels of spectrophotometrically and immunochromatically detectable phytochrome A appear to be normal in both fhy1-1 and fhy3-1 mutants. Because fhy1-1 and fhy3-1 display an elongated hypocotyl in FR, but a hypocotyl indistinguishable from the wild type in R or W, it seems likely that these mutations specifically affect components of a phytochrome A signal transduction pathway. Thus, the FHY1 and FHY3 gene products do not appear to be involved in the transduction of phytochrome B signals. In addition, it is clear that the fhy1 and fhy3 mutants differ from hy5 mutants, which display a long hypocotyl in all light regimes (Koornneef et al., 1980; Chory, 1992). Hypocotyl elongation and inhibition of cotyledon expansion of the fhy1-1 and fhy3-1 mutants in FR are not as pronounced as in the fhy2 (phytochrome A-deficient) mutants. The fhy1-1 and fhy3-1 mutations may be reduced-function rather than loss-of-function mutations, thus reducing but not completely abolishing FR-mediated hypocotyl growth inhibition. Alternatively, the FHY1 and FHY3 gene products may not have sole responsibility for the signal transduction steps that they identify. Further analyses of the functions of FHY1 and FHY3 are in progress.

The analysis of plants heterozygous for fhy2 alleles shows that FR-grown hypocotyls of fhy2/FHY2 plants are longer than those of FR-grown FHY2/FHY2 plants. Thus, the fhy2 alleles are dominant over the wild-type allele (FHY2). Because FR-grown fhy2/FHY2 hypocotyls are much closer in length to FR-grown FHY2/FHY2 hypocotyls than to FR-grown fhy2/fhy2 hypocotyls, the fhy2 alleles are partially dominant. The molecular analyses presented above indicate that the fhy2 mutations are loss-of-function or reduced-function alleles of PHYA. Dominant loss-of-function alleles exert their effects because the wild-type allele is needed in two copies to confer the wild-type phenotype. This usually reflects a close relationship between the level of wild-type gene product and phenotype. Thus, the fhy2 alleles probably display partial dominance because phytochrome A is less abundant in fhy2/FHY2 heterozygotes than in FHY2/FHY2 homozygotes, and because the FR-mediated hypocotyl growth inhibition response is sensitive to this difference in abundance.

We have shown that the fhy2 mutants contain mutations at PHYA. We will therefore be referring to fhy2-1 and fhy2-2 as phyA-1 and phyA-2, respectively, in subsequent publications. Perhaps the most dramatic implication of our findings is that, when grown in W, phytochrome A-deficient mutants have a phenotype that is indistinguishable from that of the wild type (see also Nagatani et al., 1993; Parks and Quail, 1993). Because fhy2-1 is likely to be a phytochrome A null mutation, our observations suggest that this normal phenotype cannot be attributed to the presence of low levels of phytochrome A conferred by an incompletely null mutation. Of all of the phytochrome species, phytochrome A is the best characterized in terms of its spectral, physiological, and molecular properties. Our experiments show that phytochrome A is dispensable and plays a relatively minor role in the photomorphogenesis of Arabidopsis plants grown in W.

METHODS

Arabidopsis Lines and Plant Maintenance

The Arabidopsis thaliana Landsberg erecta laboratory strain was obtained from Maarten Koornneef (Wageningen Agricultural University, The Netherlands). The T-DNA transformant line A264 was obtained from George Coupland (Cambridge Laboratory, John Innes Centre, Norwich, UK). The genetic nomenclature used in this paper follows the conventions established at the Third International Arabidopsis Meeting (East Lansing, MI, April 1987; Koornneef and Stam, 1992). Genotypes are italicized, the wild-type genotype is capitalized (e.g., FHY2), the mutant genotype is represented in lowercase letters, and independent alleles are given identifying numbers (e.g., fhy2-1 and fhy2-2).

For routine plant maintenance, seeds were imbibed on moistened filter paper at 4°C for 4 days (to break dormancy) and then planted on "Arabidopsis mix" (2 parts Levington's M3 potting compost to 1 part grit/sand). Plants were then grown in standard glasshouse conditions at 15 to 20°C. Transgenic plants were grown according to United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF) regulations (License Number PHF 1418/8/22).

γ-Irradiation Mutagenesis

γ-Irradiation mutagenesis was performed using the cesium-137 source at the University of Nottingham, United Kingdom. Approximately 10,000 dry Landsberg erecta seeds were exposed to 90-kR γ rays. The seeds were then chilled and planted (as described above) in 10 batches of ~1000 seeds each. The plants were allowed to self-pollinate, and M2 seeds were then bulk harvested from each batch.
Isolation of Mutants Displaying an Elongated Hypocotyl in Far-Red Light

The γ-ray-treated M₂ material and the additional ethylmethane sulfonate (EMS)-treated M₂ material (Columbia [embossed] Leile Seed Co., Tucson, AZ) were screened for mutants displaying an elongated hypocotyl in far-red light (FR). Seeds, in batches of ~10,000, were evenly sown onto the surface of 0.8% agar in mineral salts (Whitehead et al., 1992) in clear Perspex boxes that were 12 × 22 × 7 cm deep. The seeds were allowed to imbibe for 4 days at 4°C in the dark and induced to germinate by exposure to white fluorescent light (W; photon flux rate, 400 to 700 nm; 150 μmol m⁻²sec⁻¹) at 20°C (for 30 min). The seeds were allowed to germinate for 1 day in the dark at 22°C and then transferred to continuous, broad band FR for 4 days. The FR source consisted of the output of water-cooled 100 W incandescent lamps filtered through black Plexiglass (type FRF 700; Westlake Plastics, Lenex Mills, PA) providing a photon flux rate, 700 to 800 nm, of 11 μmol m⁻² sec⁻¹ and the same spectral photon flux rate diurnal cycles as the FR source described by Rich et al. (1985).

Putative long hypocotyl mutants were transplanted to soil and grown in a controlled environment room at 20°C under continuous white fluorescent light (photon flux rate, 400 to 700 nm, 170 μmol m⁻² sec⁻¹) to promote rapid flowering, and allowed to self-pollinate. M₃ seed were germinated as described above, and the resultant seedlings were grown under the following light regimes: continuous FR (as described above); continuous W (cool-white fluorescent tubes, photon flux rate, 400 to 700 nm, 100 μmol m⁻² sec⁻¹); continuous red light (R; output of warm-white fluorescent tubes filtered through 1-cm-deep solution of copper sulfate [1.5% w/v] and one layer of No. 406 primary red cinnemoid [Rank Strand, Isleworth, UK], photon flux rate, 600 to 700 nm, 6 μmol m⁻² sec⁻¹); continuous blue light (B; output of cool-white fluorescent tubes filtered through one layer of No. 419 dark blue cinnemoid [Rank Strand], photon flux rate, 400 to 500 nm, 6 μmol m⁻² sec⁻¹).

The mutants isolated were designated fhy1-1, fhy2-1, fhy3-1, and fhy3-1; fhy is for far-red elongated hypocotyl. The fhy2-1 and fhy2-2 mutations were isolated from different batches of γ-ray-treated M₃ seed. They are, therefore, the products of independent mutagenic events at the FHY2 locus.

Linkage Mapping

In the genetic mapping experiments, F₁ and F₂ material was screened for individuals displaying an elongated hypocotyl in FR as described above, except that kanamycin (50 mg/L) was incorporated into the agar medium. Following 3 days of continuous FR, mutant individuals displaying an elongated hypocotyl were clearly visible (the kanamycin does not affect hypocotyl elongation in FR). The plates were then placed in W for 3 days during which time the kanamycin-resistant plants developed expanded green cotyledons, whereas the kanamycin-sensitive plants did not. The numbers of individuals displaying an elongated hypocotyl in FR and/or kanamycin resistance were recorded; genetic linkage distances were calculated as described by Koornneef and Stam (1992).

In Vivo Phytochrome Spectrophotometry

Seeds were germinated as described above, and seedlings were grown for 6 days in absolute darkness. Phytochrome photoreversibility was measured using a dual-wavelength spectrophotometer (DW 2-A; Aminco, Silver Spring, MD) with the measuring beams set at 660 and 730 nm. Samples consisted of 0.8 g of whole seedlings gently packed into a 2.3-mm light path cuvette.

Phytochrome Extraction and Immunoblot Analysis

Phytochrome-enriched extracts of 6-day-old etiolated seedlings were prepared using the procedure described by Somers et al. (1991). Protein extracts were separated on denaturing 8% polyacrylamide gels using the buffer system of Laemmli (1970) and electroblotted onto nitrocellulose. Immunostaining of phytochrome A was performed as described by McCormac et al. (1992) using spent culture medium from the monoclonal hybridoma LAS32 as primary antibody.

Isolation of Genomic DNA Clones Containing PHYA

Genomic DNA was isolated from frozen Arabidopsis tissue (homogenous for the gibberellin-insensitive [ga] mutation, Landsberg erecta background) and purified by CsCl banding (Hauge and Goodman, 1992). This DNA was partially digested with Sau3A, and a subsequent partial fill-in reaction was performed with dGTP and dATP, resulting in 5'-GA overhangs. This DNA was ligated into a λFIX II vector (Stratagene), which had been digested with XhoI, followed by partial fill-in with dTTP and dCTP to create 5'-TC overhangs. The resulting phage library was screened using a degenerate 44-mer oligonucleotide (NH2) containing a region of sequence strongly conserved between PHYA, PHYB, and HYC (encoding amino acids 301 to 315 in the sequences shown in Sharrock and Quail, 1989). The oligonucleotide was end labeled using Tα polynucleotide kinase and γ³2P-ATP (Sambrook et al., 1989). Hybridizing phages were plaque purified and sorted into clones containing PHYA, PHYB, or PHYC by restriction fragment mapping. Clones λPHYA81 and λPHYA91 were shown to contain the PHYA gene by the following criteria. First, EcoRI digestion of these clones produced a 6.2-kb fragment that hybridized with the NH2 oligonucleotide, as expected for PHYA (Sharrock and Quail, 1989). Second, oligonucleotide probes derived from the 5′ and 3′ ends of regions of the PHYA sequence (Sharrock and Quail, 1989) hybridized to λPHYA91 and λPHYA91 DNA. These oligonucleotides were phyA5' (SAGGTGGTGATCGAGGCCAAG-3: the final G is 111 bp upstream of the starting ATG codon in the phytochrome A cDNA sequence; Sharrock and Quail, 1989; Reed et al., 1993), thus confirming that λPHYA81 and λPHYA91 contain PHYA and not PHYB or PHYC. DNA from phages λPHYA81 and λPHYA91 was used in the hybridization experiments described below.

Analysis of the PHYA Gene Transcripts

Seeds were imbibed for 7 days at 4°C in the dark. The seeds were then given a 10-min pulse of W to induce germination and were then grown in the dark for an additional 5 days. The etiolated seedlings were harvested in green light and immediately frozen in liquid nitrogen. Up to 0.2 g of frozen tissue was ground to a fine powder and resuspended in 2 mL of extraction buffer (150 mM LiCl, 5 mM EDTA, 5% SDS, 80 mM Tris-HCl, pH 9.0). Following two phenol/chloroform
extractions, nucleic acids were precipitated with one-tenth volume of 3 M sodium acetate and one volume of isopropanol. The pellet was resuspended in 3 M LiCl on ice for 10 min. The RNA was pelleted again by centrifugation, washed with 75% ethanol, dried, and resuspended in H2O. Approximately 10 μg of RNA was fractionated on a 1.3% agarose gel (containing 0.66 M formaldehyde) and transferred onto Hybond-N membrane (Amersham International) via capillary action. The RNA was hybridized with an 11.5-kb SalI genomic DNA fragment (from λPHYA81) containing the Arabidopsis PHYA gene (labeled with phosphorus-32 by random primer extension; Feinberg and Vogelstein, 1983). Hybridization conditions were as described previously (Peng and Harberd, 1993).

Identification of Restriction Fragment Alterations in PHYA

Arabidopsis genomic DNA was prepared using a miniprep method (modified from Tai and Tanksley, 1991) and digested with excess EcoRI. The DNA digests were separated on an 0.8% agarose gel and transferred onto a Hybond-N membrane via capillary action. The Arabidopsis phytochrome A cDNA (Sharrock and Quail, 1989) was obtained from Peter Quail (Plant Gene Expression Center, Albany, CA). Subfragments of the phytochrome A cDNA obtained following restriction endonuclease cleavage were fractionated through 0.8% low melting point agarose in TBE (Sambrook et al., 1989), 1 ng mL⁻¹ ethidium bromide. The fragments required were purified from the gel. λPHYA91 DNA and phytochrome A cDNA subfragments were labeled via random primer extension (Feinberg and Vogelstein, 1983) and used in hybridization experiments as described previously (Peng and Harberd, 1993).

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