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*hy*8, a New Class of Arabidopsis Long Hypocotyl Mutants Deficient in Functional Phytochrome A

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Emerging evidence suggests that individual members of the phytochrome family of photoreceptors may regulate discrete facets of plant photomorphogenesis. We report here the isolation of phytochrome A mutants of Arabidopsis using a novel screening strategy aimed at detecting seedlings with long hypocotyls in prolonged far-red light. Complementation analysis of 10 selected mutant lines showed that each represents an independent, recessive allele at a new locus, designated *hy*8. Immunoblot and spectrophotometric analyses of two of these lines, *hy*8-1 and *hy*8-2, showed that, whereas phytochromes B and C are expressed at wild-type levels, phytochrome A is undetectable, thus indicating that the long hypocotyl phenotype displayed by these mutants is caused by phytochrome A deficiency. A third allele, *hy*8-3, expresses wild-type levels of spectrally normal phytochrome A, suggesting a mutation that has resulted in loss of biological activity in an otherwise photochemically active photoreceptor molecule. Together with physiological experiments, these data provide direct evidence that endogenous phytochrome A is responsible for the "far-red high irradiance response" of etiolated seedlings, but does not play a major role in mediating responses to prolonged red or white light. Because the *hy*8 and the phytochrome B–deficient *hy*3 mutants exhibit reciprocal responsivity toward prolonged red and far-red light, respectively, the evidence indicates that phytochromes A and B have distinct photosensory roles in regulating seedling development.

INTRODUCTION

The regulatory photoreceptor phytochrome is well known to control plant developmental responses to light during all phases of the life cycle (Kendrick and Kronenberg, 1986). This control is exerted by virtue of the photoreceptor's capacity for reversible interconversion between its inactive Pr and active Pfr forms through sequential absorption of red and far-red photons, respectively. However, the diversity of the responses attributed to phytochrome has been difficult to reconcile with the action of a single photoreceptor species (Smith and Whitelam, 1990). Indeed, a variety of accumulated evidence over the years has suggested the presence of at least two pools of phytochrome in plant tissue. In particular, physiological studies have indicated the existence of a photolabile and a photostable pool of biologically active phytochrome (Smith and Whitelam, 1990), and biochemical, immunochemical, and spectroscopic studies have demonstrated two molecular classes of the photoreceptor, termed type 1 and type 2, with distinctive characteristics (Quail, 1991). Type 1 phytochrome is the historically familiar and biochemically well-characterized "etiolated tissue" species that is abundant in dark-grown tissue, but present at low levels in light-grown tissue because of the lability of the Pfr form in vivo. Type 2 is the "green tissue" species that is constitutively present at equal levels in both dark- and light-grown tissue because of the absence of enhanced lability in the Pfr form. As a result, type 1 phytochrome predominates in dark-grown tissue (~100 times higher than type 2), whereas in light-grown tissue, both photoreceptor types are present at roughly comparable levels (Tokuhisa and Quail, 1987). However, these studies established no definitive relationship between the respective physiologically and biochemically defined pools of phytochrome.

Direct molecular evidence that the phytochrome apoprotein is encoded by a family of five divergent genes, designated *phyA*, *phyB*, *phyC*, *phyD*, and *phyE*, has been provided recently from studies with Arabidopsis (Sharrock and Quail, 1989; R. Sharrock, personal communication) and rice (Dehesh et al., 1991; K. Dehesh and P. Quail, unpublished data). This finding raised the questions of whether individual family members have discrete photosensory roles in regulating plant photomorphogenesis, and in which way they relate to the physiologically and biochemically defined species of the photoreceptor.

Molecular and genetic analyses have begun to shed light on these questions. Microsequencing of the purified type 1 phytochrome polypeptide has established unequivocally that this photoreceptor species is encoded by the *phyA* gene (Grimm et al., 1988; Jones and Quail, 1989) and is therefore designated

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as phytochrome A (Quail, 1991; Quail et al., 1991). Phytochromes B and C are of low abundance and photostable and, therefore, have the properties of type 2 phytochrome (Somers et al., 1991; López-Juez et al., 1992). Phytochromes D and E have yet to be analyzed.

Analysis of photomorphogenic mutants has provided the first definitive link between a defined, individual phytochrome species and a specific photoresponse. The *hy*3 mutant of Arabidopsis has recently been shown to be deficient in phytochrome B (Somers et al., 1991). This mutant is defective in responsiveness to continuous red light (Rc), manifested as long hypocotyl growth in Rc (Koornneef et al., 1980; McCormac et al., 1993) and as the absence of "shade-avoidance" and "end-of-day far-red responses" (Nagatani et al., 1991; Whitelam and Smith, 1991). These results establish clearly that phytochrome B is necessary for perception of Rc, and conversely that neither phytochromes A, C, D, nor E can act in Rc perception (at least in the absence of phytochrome B) in eliciting these responses. By contrast, continuous far-red light (FRc) induces normal inhibition of hypocotyl elongation in etiolated seedlings of the *hy*3 mutant (Koornneef et al., 1980; McCormac et al., 1993). This result indicates that phytochrome B is not required for the so-called "far-red high irradiance response" (FR-HIR) (Mancinelli, 1980; Smith and Whitelam, 1990), which could therefore be mediated by phytochromes A, C, D, or E. In support of these conclusions, the long hypocotyl (*lh*) mutant of cucumber, which has a similar photoresponse profile to *hy*3, has also recently been shown to be deficient in phytochrome B (López-Juez et al., 1992) as has the elongated internode (*ein*) mutant of Brassica (Devlin et al., 1992).

The *hy*1 and *hy*2 mutants of Arabidopsis have been shown to be defective in phytochrome chromophore biosynthesis (Parks and Quail, 1991). The data have established that these mutants are at least deficient in photochemically active phytochrome A, and also probably in all phytochromes to a greater or lesser extent. Like *hy*3, the *hy*1 and *hy*2 mutants lack responsiveness to Rc, consistent with a deficiency in functional phytochrome B, but, in contrast to *hy*3, also lack responsiveness to FRc, indicating an additional deficiency in the phytochrome(s) responsible for the FR-HIR (potentially, phytochromes A, C, D, or E).

Transgenic Arabidopsis seedlings overexpressing phytochrome B exhibit enhanced sensitivity to Rc but wild-type responsiveness to FRc (Wagner et al., 1991; McCormac et al., 1993). This result is consistent with the behavior of endogenous phytochrome B deduced from the *hy*3 mutant studies and underscores the lack of involvement of this photoreceptor species in the FR-HIR. By contrast, transgenic Arabidopsis overexpressing phytochrome A exhibits enhanced sensitivity to FRc (Whitelam et al., 1992; McCormac et al., 1993). Together, these results are consistent with the possibility, although do not prove, that the capacity to mediate the FR-HIR may be an intrinsic property of phytochrome A.

Accumulated biochemical and physiological data also provide circumstantial evidence that phytochrome A may be responsible for the FR-HIR. Action spectroscopy has shown that the responsiveness of etiolated seedlings to FRc declines rapidly upon greening, indicating that this response is under the control of a physiologically defined photolabile phytochrome pool (Beggs et al., 1980; Holmes and Schäfer, 1981). Because the time course of this decline was found to be similar to the decline in levels of the abundant, spectrophotometrically detectable type 1 phytochrome, the data are consistent with the possibility that this photolabile phytochrome pool may be responsible for the FR-HIR.

Regardless of the validity of this specific proposal, however, because phytochrome B does not appear to be involved in the FR-HIR, we reasoned that mutants defective in the activity of the phytochrome mediating this response would retain phytochrome B, and, therefore, retain responsiveness to Rc. If correct, this prediction might explain why previous exhaustive screens have not detected mutants in phytochromes other than phytochrome B. These screens were performed in white light, presumably precluding detection of mutants in the FR-HIR. Based on this prediction, we have devised a photobiological screen involving sequential screening of mutagenized populations of Arabidopsis seedlings in FRc and Rc. We provide evidence that mutants selected for lack of responsiveness to FRc, but which retain wild-type responsiveness to Rc, are selectively deficient in functional phytochrome A.

RESULTS

A Two-Step Screen Identifies a New *HY* Locus

A search for long hypocotyl mutants in an m_2 population of Arabidopsis was initiated by growing seedlings under FRc. Figure 1 outlines the screening strategy. For the wild type, FRc causes the seedlings to grow very short due to severe inhibition of hypocotyl elongation and to display fully opened and expanded cotyledons. These wild-type seedlings also appear yellow because far-red light is not sufficient to stimulate photochemical chlorophyll production or the development of a fully functional photosynthetic apparatus. A screen of m_2 seedlings allowed facile identification of plants that did not respond to FRc but rather continued to develop as etiolated seedlings. Because at least one class of mutants exhibiting this phenotype has been shown to result from a deficiency in chromophore biosynthesis (e.g., *hy*1, Figure 1), thereby producing seedlings potentially deficient in all functional phytochromes, a screen utilizing FRc is not sufficient alone to identify a new *HY* locus.

Long hypocotyl mutants identified under FRc were therefore selected and carried to the next generation (m_3), where they were then tested for their responsiveness to Rc. Under these conditions, we predicted that seedlings would respond through the action of phytochrome B, and, thus, allow discrimination between chromophore or signal transduction mutants and mutants specifically affected in the FR-HIR photoreceptor species. The particular Rc growing conditions used here caused wild-type seedlings and any putative new mutants to

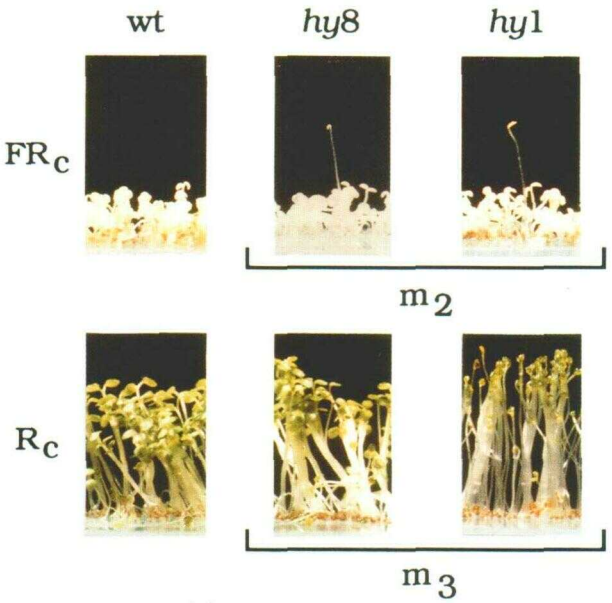

Figure 1. Phytochrome A Mutant Selection Strategy.

Populations of wild-type and m_2 seed were sown onto agar plates and germinated as described in Methods. Seedlings were then grown for 3 days in continuous far-red light (FRc). Under these conditions, wild-type seedlings (wt) are short with open and expanded cotyledons, whereas both newly arising mutants (*hy*8) and previously described phytochrome chromophore biosynthesis mutants, such as *hy*1, (Parks and Quail, 1991), are readily identifiable as tall individuals with closed, unexpanded cotyledons rising above the bulk population exhibiting wild-type behavior. However, these different mutant classes are indistinguishable from each other under these growth conditions. To distinguish between these two classes, the putative long hypocotyl mutants selected under FRc were grown to maturity, and the m_3 seeds produced were plated and grown for 3 days in continuous red light (Rc). Wild-type and putative FR-HIR photoreceptor mutants (*hy*8) have partially elongated hypocotyls but fully opened and expanded cotyledons under these conditions. By contrast, phytochrome chromophore biosynthesis mutants, such as *hy*1, are taller and display partially closed, poorly expanded cotyledons.

grow only slightly shorter than dark-grown plants, but to display fully opened, well-developed green cotyledons (Figure 1). In contrast, putative signal transduction or chromophore-biosynthetic mutants did not respond to Rc, as demonstrated by their longer hypocotyls and poorly developed, underexpanded cotyledons (Figure 1). Individuals displaying this lack of normal response to Rc were eliminated from our selected pool. Conversely, FRc-unresponsive mutants that responded normally to Rc in the subsequent m_3 generation were selected as potential FR-HIR–specific mutants for further characterization.

To determine whether this two-step screen had identified a new *HY* locus, we performed genetic complementation analyses. Our m_2 population consisted of 2.5×10^5 seed divided equally among 32 separate families represented by 1000 m_1 parents per family. From this seed, we initially identified 58 long hypocotyl mutants under FRc. This number was reduced to 18 prospective new mutants after the second screen of the m_3 generation under Rc. Test crosses of 10 of these putative mutant lines to each other yielded no instance of complementation of the long hypocotyl phenotype displayed under FRc (data not shown). Because these 10 mutant lines were each derived from a separate m_2 family, we concluded that the mutants represent independent alleles at the same locus.

Additional test crosses of selected mutant lines to the wild-type RLD parent and the *hy*1, *hy*2, *hy*3, *hy*5, and *hy*6 mutants of Arabidopsis showed full complementation of the long hypocotyl response seen under FRc in all cases (data not shown). F_2 progeny of selfed F_1 plants from the backcross between the new mutant lines and the wild-type parent segregated in a 3:1 ratio of wild type to mutant for hypocotyl length under FRc (data not shown). We did not perform a test cross to the *hy*4 mutant because this mutant is known to be specifically defective in blue light responsiveness (Koornneef et al., 1980). The *hy*7 mutant represents a recent, incompletely characterized addition to the long hypocotyl mutant group (Chory, 1991) and was not tested here. However, because *hy*7 was selected under white light, it has a photosensory phenotype distinct from the newly isolated mutants, and, therefore, most likely represents a separate locus. Together, the data indicate that all of the mutants identified by the two-step screen represent independent, recessive mutations in a single, newly identified locus that will be referred to hereafter as *hy*8.

Phytochrome A Is Selectively Affected in *hy*8 Mutants

Our screening strategy was designed to target the action of the FR-HIR photoreceptor, which, based on indirect evidence, seemed most likely to be phytochrome A. If our prediction were valid, then the *hy*8 locus would either represent the *phyA* gene itself or another gene whose product is closely linked to the synthesis or action of phytochrome A. To test directly whether the phytochrome A molecule was selectively affected in the *hy*8 mutants, we examined the levels of immunochemically detectable phytochromes A, B, and C in three different *hy*8 lines that had been selected for further characterization from three separate m_1 families. Figure 2 shows that two distinct classes of mutants were found. Phytochromes B and C each appear equal in abundance and molecular mass in each *hy*8 mutant relative to the wild-type parent. In contrast, phytochrome A is undetectable in the *hy*8-1 and *hy*8-2 mutants. This result provides support for the proposal that the *hy*8 mutation has a selective effect on phytochrome A, as opposed to a general effect on all phytochromes. In contrast to the *hy*8-1 and *hy*8-2 alleles, phytochrome A polypeptide levels appear normal for the *hy*8-3 line compared to the wild type (Figure 2).

Spectrophotometric analysis further indicates that phytochrome A is affected in the *hy*8 mutants. Etiolated wild-type seedling tissue contains high levels of phytochrome A and relatively low levels of the other phytochrome types (Quail, 1991;

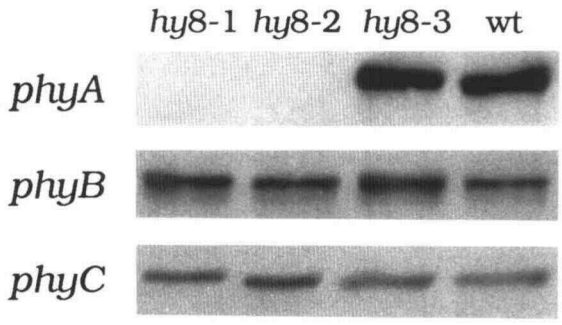

Figure 2. *phyA*-, *phyB*-, and *phyC*-Encoded Protein Levels in the Wild Type and Three *hy*8 Mutants of Arabidopsis.

Phytochrome-enriched protein extracts were made from etiolated Arabidopsis seedlings carrying wild-type (wt) or one of three *hy*8 mutant alleles (*hy*8-1, *hy*8-2, and *hy*8-3). Immunoblot lanes were loaded with ammonium sulfate–precipitated protein (17.5 μg per lane for *phyA* and *phyB* immunoblots, and 35 μg per lane for the *phyC* immunoblot) and then probed with type-selective monoclonal antibodies against phytochromes A, B, or C.

Somers et al., 1991). As a result, it can be estimated that ≥90% of the phytochrome spectrophotometric signal measured in etiolated tissue results from phytochrome A.

Figure 3 shows the levels of spectrophotometrically detectable phytochrome present in crude protein extracts of etiolated Arabidopsis tissue from the wild type and each of the three *hy*8 mutants. The *hy*8-1 and *hy*8-2 mutants contain no spectrophotometrically detectable phytochrome significantly above background, whereas the *hy*8-3 mutant contains levels similar to the wild-type parent. These data parallel those of the immunoblot analysis (Figure 2) and indicate that the lack of immunochemically detectable polypeptide in the *hy*8-1 and *hy*8-2 lines resulted from the absence of phytochrome A polypeptide rather than an alteration of the immunochemical properties of the polypeptide. Together, these results provide support for the proposal that a severe deficiency of phytochrome A is responsible for the phenotype observed in these two *hy*8 mutants. Conversely, the presence of wild-type levels of spectrophotometrically measurable phytochrome A in the *hy*8-3 line (Figure 3) indicates that this allele leads to production of an apparently fully photochemically active photoreceptor molecule that is defective in its regulatory activity.

Another approach to assessing the photochemical activity of phytochrome A is to determine whether the levels of the photoreceptor decline rapidly in etiolated tissue exposed to red light as a result of conversion to the labile Pfr form (Parks and Quail, 1991; Somers et al., 1991). Figure 4 shows that the immunochemically detectable polypeptide in the *hy*8-3 line declines in irradiated seedlings in a manner parallel to that of the wild type. This result indicates that the phytochrome A molecule in this mutant is not only photoconvertible to the Pfr form in apparently normal fashion, but is also recognized by the cellular machinery that preferentially degrades this form of the photoreceptor in vivo. This result thus confirms and extends the direct spectrophotometric measurements for *hy*8-3 (Figure 3).

*hy*8 Mutants of Arabidopsis Demonstrate Separate Photosensory Roles for Phytochromes A and B

The availability of the *hy*8 mutants described here provides the opportunity to assess directly the role of phytochrome A in plant photomorphogenesis. Figures 5 and 6 compare the light-controlled phenotypic responses of the three *hy*8 mutants with those of other previously described photomorphogenic mutants of Arabidopsis. Dark-grown seedlings of all lines, whether wild-type or mutant, are essentially indistinguishable from one another, with long hypocotyls, closed hooks, and yellow, unseparated, and unexpanded cotyledons (Figures 5, 6A, and 6B). This result demonstrates that the phenotypic aberrations in the mutants are light-dependent and, therefore, specifically involve defects in the plants' photosensory response system. White light–grown seedlings of all *hy*8 mutant lines are essentially indistinguishable from the wild type in regard to all morphological parameters considered here, including inhibition of hypocotyl elongation (Figures 5, 6A, and 6B) and cotyledon development (Figure 6A; data not shown). This result indicates that phytochrome A does not have a discernible

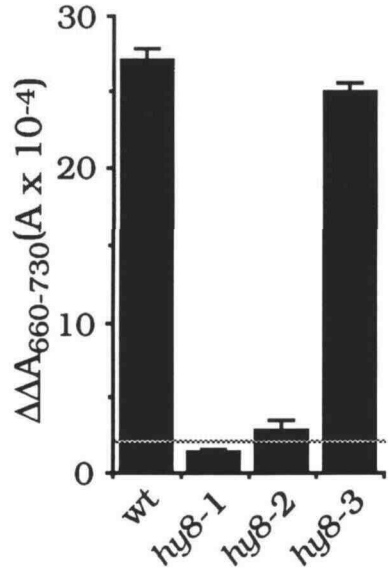

Figure 3. Spectrally Detectable Phytochrome in Crude Extracts of Etiolated Arabidopsis Seedlings.

Crude extracts were prepared from etiolated Arabidopsis seedlings carrying wild-type (wt) or one of three *hy*8 mutant alleles (*hy*8-1, *hy*8-2, and *hy*8-3), and phytochrome was measured spectrophotometrically as described in Methods. The dashed horizontal line denotes the detection limit for the instrument.

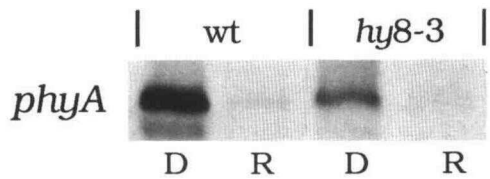

Figure 4. Light-Induced Phytochrome A Turnover in the Wild Type and *hy*8-3 Mutant of Arabidopsis.

Direct protein extracts were made from etiolated and red light–treated *hy*8-3 and wild-type (wt) Arabidopsis seedlings as described in Methods. Lanes were loaded with 50 μL of each sample extract, and after electrophoresis and transfer, the immunoblot was probed with a type-selective monoclonal antibody against phytochrome A. Lanes D, 4-day-old dark-grown tissue; lanes R, 4-day-old dark-grown tissue that had received 6 hr of continuous red light just prior to harvest. The somewhat lower initial level of phytochrome A in dark-grown *hy*8-3 than in wild-type seedlings in this experiment was due to partial loss during sample preparation (cf. Figure 2).

role in Arabidopsis seedling development under these white light irradiation conditions. This observation therefore verifies our suspicion that previous screening protocols performed in white light may have failed to detect phytochrome A mutants because no abnormal phenotype was apparent. By contrast, the well-established long hypocotyl phenotype of the phytochrome B–deficient *hy*3 (Somers et al., 1991) and chromophore-deficient *hy*1 (Parks and Quail, 1991) mutants is readily apparent in white light (Figures 6A and 6B).

The phenotypic differences developed in Rc are qualitatively parallel to those in white light but are less pronounced. Again, all three *hy*8 mutants are indistinguishable from the wild type with respect to hypocotyl elongation and cotyledon development (Figures 5, 6A, 6B, and 6C). Inhibition of hypocotyl elongation in Rc is, in general, substantially less than in white light, but is nevertheless significantly greater for the wild-type and *hy*8 seedlings than for the *hy*3 and *hy*1 mutants (Figures 6A and 6B). Note in Figures 6A and 6B that the Landsberg wild type is somewhat more inhibited by Rc than the RLD wild type. As a result, the differential in hypocotyl length that we observed here between the *hy*1 and *hy*3 mutants and their Landsberg parental line is consistent with the original data of Koornneef et al. (1980). The relatively small differential observed between the *hy*8 and the *hy*1 and *hy*3 mutants would thus be anticipated to be somewhat greater if the mutants were compared in the same parental genetic background. This suggestion is supported by the clear differences in cotyledon development between these mutant lines in response to Rc (Figure 6C). Whereas the *hy*8 mutants develop open hooks and open, expanded cotyledons, *hy*1 and *hy*3 retain partially closed hooks, and small, unseparated cotyledons similar to those of completely dark-grown seedlings (Figures 6A and 6C). We have found this cotyledon phenotype to be a more reliable and pronounced diagnostic index of lack of Rc perception than hypocotyl length. Together, the data provide quantitative support for a central premise on which the genetic screen was initially based; namely, that whereas phytochrome B is necessary for Rc perception, phytochrome A is not.

The most striking phenotypic differences between lines are those displayed under FRc. As described above, in strict contrast to the wild type, all *hy*8 mutant lines exhibit essentially uninhibited hypocotyl elongation (Figures 5, 6A, and 6B) and retain apical hooks and unseparated, unexpanded cotyledons (Figure 6C), similar to completely dark-grown seedlings. The *hy*3 mutant, on the other hand, is indistinguishable from the wild type under these conditions (Figures 6A, 6B, and 6C), as shown originally by Koornneef et al. (1980). The data thus provide quantitative support for the second central premise on which the genetic screen was based; namely, that whereas phytochrome B is not necessary for FRc perception, the postulated FR-HIR photoreceptor, phytochrome A, is necessary.

The reciprocal photosensory roles of phytochromes A and B in mediating the effects of far-red and red light, respectively, are strikingly illustrated by direct comparison of the hypocotyl and cotyledon development of the *hy*8 and *hy*3 mutants under Rc and FRc conditions (Figures 6A and 6C). Together, these data imply further that the phenotype displayed by the chromophore-deficient mutant *hy*1 under FRc is likely to be due primarily to a deficiency of functional phytochrome A, and under Rc to a deficiency of functional phytochrome B (Figures 6A, 6B, and 6C).

Previous studies utilizing photomorphogenic mutants have presented evidence that both the shade-avoidance and the end-of-day far-red light responses are mediated principally, if not exclusively, through the action of phytochrome B (Nagatani et al., 1991; Somers et al., 1991; Whitelam and Smith, 1991;

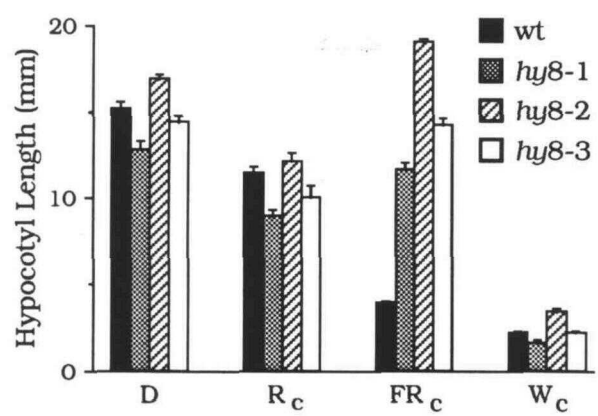

Figure 5. Light-Regulated Hypocotyl Elongation in the Wild Type and Three *hy*8 Mutants of Arabidopsis.

The wild type and three *hy*8 mutants (*hy*8-1, *hy*8-2, and *hy*8-3) were sown onto agar plates and germinated in darkness as described in Methods. Germinated seedlings were then grown for an additional 3 days in darkness (D) or under continuous red (Rc), continuous far-red (FRc), or continuous white (Wc) light. The mean hypocotyl length is plotted for 10 seedlings from each growth regime. Bars represent one standard error of the mean.

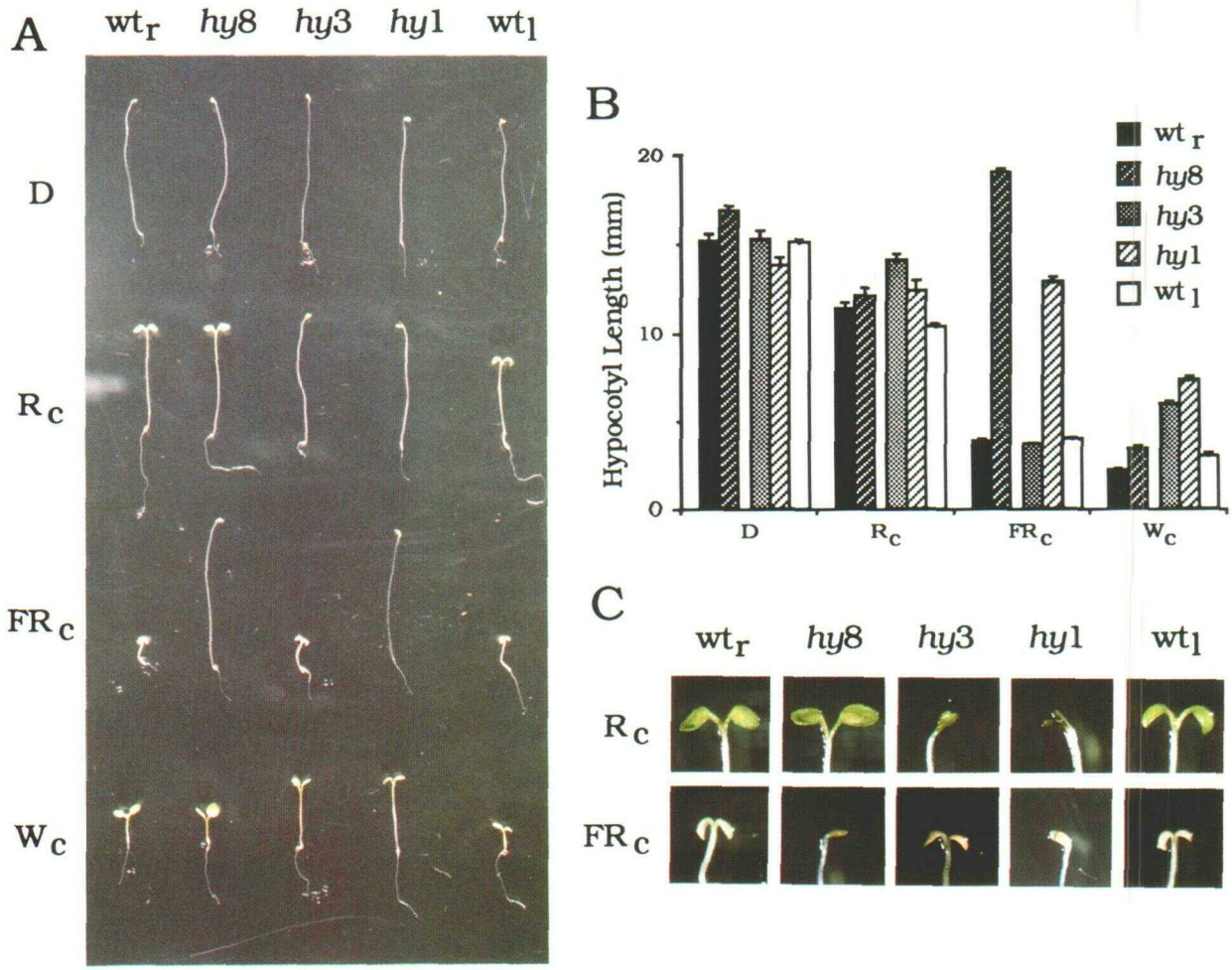

Figure 6. Photomorphogenesis in *hy*1, *hy*3, and *hy*8 Mutants and Corresponding Parental Wild Types of *Arabidopsis*.

Three long hypocotyl mutants (*hy*1, *hy*3, and *hy*8-2) and their corresponding parental wild types (wt_l, Landsberg for *hy*1 and *hy*3; wt_r, RLD for *hy*8-2) were sown onto agar plates and germinated in darkness (D) as described in Methods. Germinated seedlings were then grown for an additional 3 days in darkness or under continuous red (Rc), continuous far-red (FRc), or continuous white (Wc) light.

(A) Representative seedlings from each mutant line and the wild type grown under the indicated light regime.

(B) Mean hypocotyl length for 10 seedlings of each mutant and the wild type grown under each light regime. Each mutant is grouped with its own wild-type parent. Bars represent one standard error of the mean.

(C) Morphology of cotyledons of mutant and wild-type seedlings grown under continuous red or continuous far-red light.

López-Juez et al., 1992). This observation predicts that plants specifically deficient in any other phytochrome type would be unaffected in either of these responses. Figure 7 shows the response of the *hy*8, *hy*3, and *hy*1 mutants and their corresponding wild-type parents to an end-of-day far-red light treatment. Wild-type seedlings responded to a brief end-of-day far-red light treatment by growing taller than plants that had not received this treatment (Figure 7). It has been proposed that this response results from the photoconversion of light-stable phytochrome B into the inactive Pr form at the light-to-dark transition, thus precluding the photoreceptor from maintaining its inhibitory activity during the subsequent dark period. According to this proposal, hypocotyl growth is released from inhibition by Pfr and becomes maximal throughout the dark period, in contrast to control plants where Pfr formed during the white light period remains and is inhibitory during the dark period.

Neither the *hy*1 nor the *hy*3 mutants displayed this elongation response to end-of-day far-red light treatment when compared to controls where no terminal far-red light was given (Figure 7). In fact, these seedlings elongated more under both light regimes than the wild-type parent, consistent with the fact that white light–regulated growth inhibition is hampered in these lines which lack functional phytochrome B (Figure 6).

In contrast, the *hy*8 mutant lines all showed an end-of-day far-red light response similar to the wild-type parent (Figure 7). This result suggests that the action of phytochrome A does not contribute to this photoresponse, and, thus, provides further strong evidence for the proposal that phytochromes A and B exercise distinct roles in controlling plant growth and development.

The developmental phenotype displayed by the *hy*8 mutants from the young seedling stage to mature adults (including floral initiation and development) was indistinguishable from the wild type under our standard continuous white light regime (data not shown). This behavior is in contrast to that of the *hy*3 mutant which displays a more spindly adult phenotype than does the wild type. Therefore, the data suggest that phytochrome A may not have a major role in regulating juvenile or adult Arabidopsis development, at least under these conditions.

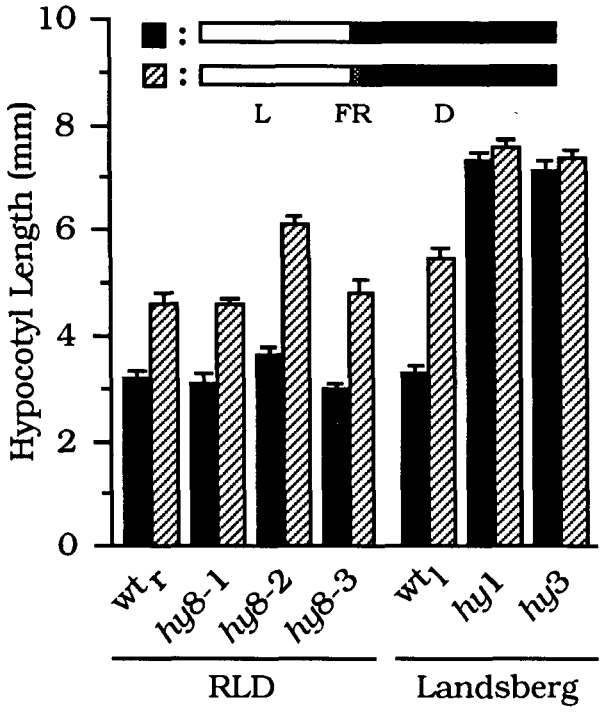

Figure 7. Effect of End-Of-Day Far-Red Light Treatment on *hy*1, *hy*3, and *hy*8 Mutants and Corresponding Parental Wild Types of Arabidopsis.

Long hypocotyl mutants (*hy*1, *hy*3, *hy*8-1, *hy*8-2, and *hy*8-3) and their corresponding parental wild types (wt_l, Landsberg for *hy*1 and *hy*3; wt_r, RLD for the three *hy*8 mutants) were sown onto agar plates and cold treated as described in Methods. Mutant and wild-type seeds were germinated for 2 days under continuous white light. Germinated seedlings were then transferred to a growth cycle consisting of 10 hr of white light (L) followed by 14 hr of darkness (D, black columns), or 10 hr of white light followed by 15 min of far-red light (FR) followed by 13.75 hr of darkness (hatched columns). Hypocotyls were measured after the end of the fourth light cycle. The mean hypocotyl length is plotted for 10 mutant or wild-type seedlings from each growth regime. Bars represent one standard error of the mean. The diagram at the top of the figure schematically illustrates the two different growth regimes.

DISCUSSION

The data presented here show that the *hy*8 class of Arabidopsis mutants defective in the capacity to respond to FRc is selectively deficient in functional phytochrome A. Moreover, the evidence strongly indicates that the *hy*8 locus is the *phyA* gene itself. This conclusion is based on the fact that we have isolated two contrasting classes of *hy*8 alleles (Figure 2): the *hy*8-1 and *hy*8-2 alleles, which produce no detectable levels of phytochrome A, and the *hy*8-3 allele, which produces normal levels of a phytochrome A molecule that is indistinguishable from the wild type in regard to photochemical activity and competence for Pfr-specific in vivo degradation but that lacks regulatory activity. Although definitive support for this conclusion must await sequencing of the *phyA* gene in the *hy*8 mutants, the alternative, that the mutations are at a second locus whose alternate alleles could either specifically eliminate the *phyA* gene product or simply inhibit its activity, seems less probable. The *hy*8-3 mutant allele thus has the potential to provide valuable information on the molecular mechanism of action of phytochrome A. Because the mutant photoreceptor molecule has retained normal capacity to perceive red and far-red light signals but has lost the capacity to regulate photomorphogenesis, sequencing of this ethylmethane sulfonate–induced mutant *phyA* gene should identify a residue that is specifically critical to the regulatory action, as opposed to the photoperception function, of the phytochrome A molecule. In addition, mutagenesis of the *hy*8-3 line will permit screening for second-site suppressors of the phytochrome A lesion and, therefore, potential identification of a signal transduction partner for the photoreceptor.

Because the evidence indicates that phytochrome A deficiency is responsible for the long hypocotyl phenotype observed in the *hy*8 mutants under FRc irradiation conditions, it follows that phytochrome A is necessary for the FR-HIR. Conversely, it would appear that phytochromes B, C, D, or E, either alone or together, are not capable of mediating the FR-HIR. Moreover, because the phytochrome B–deficient *hy*3 mutant exhibits an FR-HIR indistinguishable from wild type (Figure 6; McCormac et al., 1993), phytochrome A activity is not dependent on phytochrome B in this photosensory mode. Thus, the evidence strongly favors the conclusion that phytochrome A is the primary, if not exclusive, FR-HIR photoreceptor, consistent with previous physiological studies (Beggs et al., 1980; Holmes and Schäfer, 1981; Smith and Whitelam, 1990). On the other hand, the possible involvement of phytochromes C, D, or E in an interactive or synergistic manner with phytochrome A cannot presently be ruled out.

Because the *hy*8 mutants display essentially wild-type responsiveness to continuous white light (Wc) or Rc (Figures 1, 5, and 6) and to end-of-day far-red light treatments (Figure 7), it appears that phytochrome A plays little, if any, role in mediating photoregulation of seedling development under these irradiation conditions. This observation thus verifies the initial prediction on which the genetic screening strategy was based and provides a likely explanation for why phytochrome A mutants have not been isolated in previous screens. Conversely, the behavior of the *hy*3 mutants has shown that phytochrome B is necessary for responsiveness to Rc and Wc as well as to end-of-day far-red light treatments (Figures 6 and 7; Nagatani et al., 1991; Somers et al., 1991; Whitelam and Smith, 1991; McCormac et al., 1993). The wild-type phenotype of the phytochrome A–deficient *hy*8 mutants in Rc and Wc indicates, therefore, that the activity of phytochrome B is fully exerted independently of phytochrome A in this photosensory mode. As was the case for phytochrome A, however, the data do not conclusively demonstrate that phytochrome B operates independently of phytochromes C, D, or E.

Taken together, the above data establish that phytochromes A and B have reciprocal and independent photosensory roles in mediating responsiveness to FRc and Rc, respectively, as summarized in Table 1. The unresponsiveness of the chromophore-deficient *hy*1 and *hy*2 mutants (Parks and Quail, 1991) to both Rc and FRc (Figure 6; Koornneef et al., 1980) is consistent with this conclusion (Table 1) because deficiencies in functional photoreceptors of both types are known (phytochrome A; Parks et al., 1989) or expected (phytochrome B). Data from transgenic Arabidopsis seedlings overexpressing phytochrome B also support this conclusion (Table 1) because these seedlings exhibit enhanced responsiveness to Rc but not to FRc (McCormac et al., 1993). Similarly, the enhanced responsiveness to FRc of transgenic seedlings overexpressing phytochrome A (Whitelam et al., 1992) is consistent with the activity of this photoreceptor species in the FR-HIR (Table 1).

Table 1. Summary of the Responsiveness of Mutant and Transgenic Overexpresser Lines of Arabidopsis to Continuous Red or Far-Red Irradiation

Arabidopsis Line[a]	Phytochrome Levels[b,c]	Responsiveness[c]	
		Rc	FRc
WT	A^+B^+	+	+
*hy*8	A^-B^+	+	–
*hy*3	A^+B^-	–	+
*hy*1, *hy*2	A^-B^-	–	–
AOX	$A^{++}B^+$	+ +	+ +
BOX	A^+B^{++}	+ +	+

[a] WT, wild type; *hy*, long hypocotyl mutants; AOX, phytochrome A overexpresser; BOX, phytochrome B overexpresser.
[b] A, phytochrome A; B, phytochrome B.
[c] Rc, continuous red irradiation; FRc, continuous far-red irradiation; +, WT; + +, enhanced; –, deficient.

The single apparent inconsistency in this general picture at present is the hypersensitivity of phytochrome A overexpressers to Rc (Table 1; Boylan and Quail, 1991; Whitelam et al., 1992). These data indicate that phytochrome A possesses the intrinsic capacity to mediate responses to Rc even though endogenous phytochrome A does not appear to exercise this capacity in the wild-type seedling. The reason for this apparent aberrant activity is unknown but could involve high levels of introduced phytochrome A sustained even in light-grown transgenic seedlings and/or ectopic expression of the photoreceptor driven by the constitutive cauliflower mosaic virus 35S promoter used in these experiments (Boylan and Quail, 1991). Regardless of the ultimate explanation of this phenomenon, however, the assembled evidence indicates that the separate photosensory roles played by endogenous phytochromes A and B in seedling development are intrinsic properties of the individual photoreceptor molecules.

The most intriguing and perhaps unexpected question to emerge from this study is: What is the biological function of phytochrome A? Whereas the aberrant phenotype of *hy*3 had indicated previously that phytochrome A is not sufficient for normal development (Koornneef et al., 1980; Nagatani et al., 1991; Somers et al., 1991; Whitelam and Smith, 1991), the normal development of *hy*8 in Wc from the seedling stage (Figures 1, 5, and 6) to mature adult (data not shown) implies that phytochrome A is also not necessary for normal photomorphogenesis, at least under these irradiation conditions.

Is the sole function of phytochrome A to mediate the FR-HIR and, if so, under what circumstances in the natural spectral environment would this be important? Insufficient data are currently available to definitively assess these questions. Several well-known phytochrome-regulated phenomena, such as photoperiodic control of flowering, seed dormancy, and the so-called "low fluence" and "very low fluence" responses of etiolated seedlings (Kendrick and Kronenberg, 1986), are yet to be examined in the *hy*8 mutants. Of particular interest in this regard will be the "classic" or low fluence phytochrome responses that can be induced by a short pulse of red light and reversed by a subsequent pulse of far-red light. One situation in the natural environment in which the FR-HIR might have a physiologically important role may be the far-red light–enriched environment that would be experienced by an etiolated seedling emerging from the soil under a leafy canopy (Smith and Whitelam, 1990). The inhibition of hypocotyl elongation, opening and expansion of the cotyledons (Figures 1 and 6), and induction of incipient chloroplast development (Mohr, 1977) experienced under FR-HIR conditions might represent initiation of the necessary transition from heterotrophic to autotrophic development, even in the absence of maximum photosynthetically active radiation. It might be expected, however, that the inhibitory effect on elongation growth would be transient, and as phytochrome A levels dropped, the shade-avoidance regulatory role of phytochrome B would assume

dominance and elongation growth would again accelerate in response to the enhanced levels of far-red light. Thus, if this scenario is correct, phytochromes A and B would appear to have transiently antagonistic roles in regulating early seedling photomorphogenesis. The roles of phytochromes C, D, and E in this and other developmental responses await elucidation.

METHODS

Plant Material

Four-day-old m_2 and wild-type *Arabidopsis thaliana* ecotype RLD seedlings (Lehle Seed Co., Tucson, AZ) were used for the initial mutant screen. Photomorphogenic mutant controls used were the *hy*1 and *hy*3 long hypocotyl mutants and their wild-type parent ecotype Landsberg (Koornneef et al., 1980). All seeds used for screening and subsequent experiments were routinely soaked and sterilized for 30 min in white light in a solution containing 1% hypochlorite and 0.2% SDS, washed with six changes of sterile water, and then sown onto 0.8% agar (Bio-Rad) containing 2.16 g/L of Murashige-Skoog salts (Gibco) in Petri dishes. Plates were then stored in darkness at 4°C for 2 days before germination. Germination was induced by placing the plated seeds in white light for 2 hr at 21°C. Germination and initial growth proceeded in darkness at 21°C for 1.5 days (shoots were just beginning to emerge from the seed coats), except for the end-of-day far-red light experiments, in which seeds were germinated in continuous white light (Wc) for 2 days at 21°C. These seedlings were then transferred to different growing conditions depending on the screen or experiment performed.

Mutant Selection

The m_2 population consisted of $\sim 2.5 \times 10^5$ ethylmethane sulfonate–mutagenized seed representing 32 independent parental groups consisting of 1000 m_1 parents per group. Each group contained ~8000 m_2 seed that were evenly sown among 10 Petri dishes, as described above. Dark-grown seedlings (1.5 days old) were transferred to continuous far-red light (FRc) at 3.5 W/m^2 (special red fluorescent tube F48T12/232/VHO, Sylvania, Danvers, MA; with one layer of far-red transmitting Plexiglas, cutoff at 690 nm, FRS700, Westlake Plastics, Lemmi Mills, PA) and grown for an additional 3 days at 21°C.

Putative mutants were identified as very tall seedlings with closed, unexpanded cotyledons (an etiolated appearance; Figure 1). Each putative mutant was transplanted to soil and grown in a greenhouse under constant white light illumination to promote rapid flowering and seed production. m_3 seed collected from each putative mutant were sown and germinated as described above. Multiple plates of 1.5-day-old dark-grown seedlings were transferred to different light regimes consisting of continuous red light (Rc) at 3.5 W/m^2 (special red fluorescent tube F48T12/236/VHO, with one layer of Roscolene No. 823; Musson Theatrical, Santa Clara, CA), FRc, or Wc at 4.5 W/m^2 (F40CW cool-white fluorescent tubes), and grown for 3 days at 21°C. Putative phytochrome A mutants were selected from these individual groups of m_3 seed by their ability to respond normally to Rc (Figure 1). These putative mutants were then transferred to soil and grown in a greenhouse to generate seed for subsequent analysis.

Protein Extraction and Immunochemical Analysis

Direct extracts and phytochrome-enriched extracts were prepared from 4-day-old dark-grown tissue according to the methods of Parks and Quail (1991) and Somers et al. (1991), respectively. Extracted proteins were separated in 6% polyacrylamide gels using SDS-PAGE (Laemmli, 1970), followed by electroblotting, and subsequent immunochemical detection of separated proteins, as described previously (Parks et al., 1987, 1989). Hybridoma supernatants and their working concentrations used to selectively detect the *phyA*-, *phyB*-, and *phyC*-encoded polypeptides are described by Somers et al. (1991).

Spectrophotometry

Phytochrome in crude extracts of 4-day-old etiolated Arabidopsis was measured spectrophotometrically, as described previously (Tokuhisa et al., 1985). To increase light scatter, $CaCO_3$ was added at a ratio of 0.4 g to 0.6 mL of extract just prior to measurement. Crude extracts used for spectrophotometric measurements consisted of the first protein extraction supernatant generated according to Somers et al. (1991).

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