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Mutations in the *NPH1* Locus of Arabidopsis Disrupt the Perception of Phototropic Stimuli

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The phototropic response is an important component of seedling establishment in higher plants because it orients the young seedlings for maximal photosynthetic light capture. Despite their obvious importance, little is known about the mechanisms underlying the perception and transduction of the light signals that induce phototropic curvatures. Here, we report the isolation of eight mutants of Arabidopsis that lack or have severely impaired phototropic responses. These *nph* (for nonphototropic hypocotyl) mutants comprise four genetic loci: *nph1*, *nph2*, *nph3*, and *nph4*. Physiological and biochemical characterization of the *nph1* allele series indicated that the *NPH1* locus may encode the apoprotein for a dual-chromophoric or multichromophoric holoprotein photoreceptor capable of absorbing UV-A, blue, and green light and that this photoreceptor regulates all the phototropic responses of Arabidopsis. It appears that the NPH1 protein is most likely a 120-kD plasma membrane-associated phosphoprotein because all of the *nph1* mutations negatively affected the abundance of this protein. In addition, the putative NPH1 photoreceptor protein is genetically and biochemically distinct from the HY4 protein, which most likely acts as a photoreceptor for blue light-mediated hypocotyl growth inhibition. Furthermore, the NPH1 and HY4 proteins are not functionally redundant because mutations in either gene alone affect only one physiological response but not the other, thus providing strong support for the hypothesis that more than one blue light photoreceptor is required for the normal growth and development of a seedling.

INTRODUCTION

Phototropism is an adaptive mechanism by which plants maximize light capture for photosynthesis by detecting lateral differences in irradiance and/or light quality and transducing those stimuli into differential growth patterns that result in directional growth of organs. Blue and UV-A light are the most effective light qualities for inducing the phototropic responses of higher plants (see Iino, 1990); however, green light– (Atkins, 1936; Steinitz et al., 1985) and red light–induced phototropism (Atkins, 1936; Shuttleworth and Black, 1977; Iino et al., 1984; Parker et al., 1989) have also been observed in a limited number of species. Although phototropism was first documented by Darwin (1880) more than a century ago, little is known about the genetic and biochemical components regulating this important response to blue light. All of what is known about the genetic regulation of phototropism comes from studies of mutants of the small crucifer Arabidopsis (for reviews, see Koornneef and Kendrick, 1994; Liscum and Hangarter, 1994).

In contrast to our lack of knowledge about the molecules required for phototropism, detailed photophysiological studies of this response in several monocotyledonous and dicotyledonous species, including Arabidopsis, have resulted in a vast collection of phenomenological observations (for reviews, see Dennison, 1979; Iino, 1990; Firn, 1994). For example, Steinitz et al. (1985) have shown that etiolated, wild-type Arabidopsis seedlings exhibit characteristic blue light–induced first and second positive phototropic responses similar to those observed for other species. Green light, although significantly less effective than blue light, can induce the same phototropic responses observed with blue light irradiation (Steinitz et al., 1985). Compelling physiological evidence that two photosensors are required for phototropic responsiveness to wavelengths greater than 410 nm in etiolated Arabidopsis seedlings was presented by Konjevic et al. (1989). One of these photoreceptors, designated PI, has an apparent absorption maximum at 450 nm; the second, PII, although capable of absorbing at 450 nm, has significantly more absorption at 510 nm. Apparently, only the PII photosensor contributes significantly to green light–induced first positive phototropism (Konjevic et al., 1989). Study of a mutant, strain JK224, isolated by Khurana and Poff (1989), provides genetic evidence for the existence of two photosensory systems for phototropism (Konjevic et al., 1992). The lesion in strain JK224 most likely affects the PI photosensor because this mutant exhibits a 20- to 30-fold shift in the fluence threshold for blue light–induced first positive phototropism but responds normally to first positive fluences of green light (Khurana and Poff, 1989; Konjevic et al., 1992).

Janoudi and colleagues have also utilized Arabidopsis as a model organism to study the complexities of the phototropic fluence responses observed in the laboratory as first and second positive curvatures (Janoudi and Poff, 1990, 1991, 1992,

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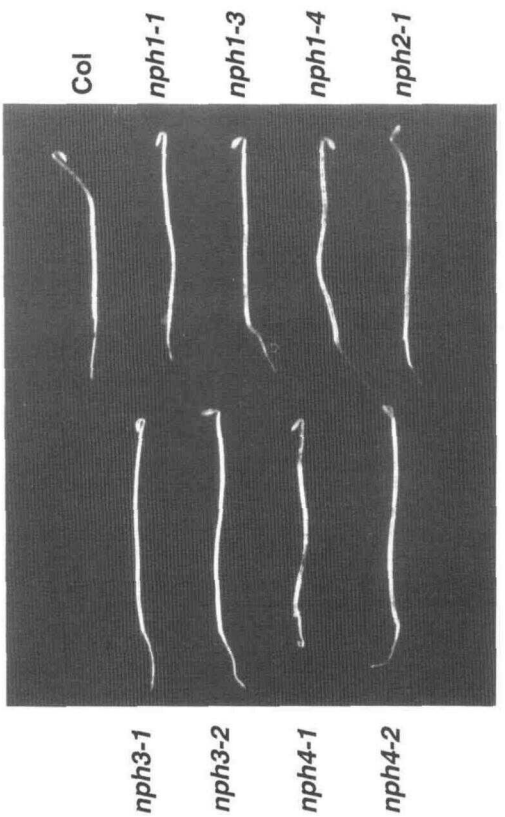

Figure 1. Phototropic Phenotype of Etiolated Arabidopsis Wild-Type and *nph* Seedlings.

Seedlings were grown in darkness for 72 hr and then transferred to unilateral blue light from the left for 10 hr at a fluence rate of 0.2 μmol m^{-2} sec^{-1}. Col, wild-type Columbia ecotype.

1993; Janoudi et al., 1992). These studies clearly indicate that the complex nature of the fluence response curves for phototropism in Arabidopsis is a result of poorly understood adaptation mechanisms occurring at the level of one signal transduction system and not the coactive function of multiple signaling systems. Although all of the aforementioned studies represent significant advances, they have provided few clues regarding the molecular events that occur during the perception and transduction of phototropic stimuli. The notable exceptions are studies using the mutant strain JK224 (Khurana and Poff, 1989; Konjevic et al., 1992). Strain JK224 has provided genetic evidence that blue light–potentiated phosphorylation of a plasma membrane–associated protein is involved early in the signal response pathway for first positive phototropism (Reymond et al., 1992b).

In total, 37 mutants of Arabidopsis with altered phototropic responses have been isolated. To date, only six of these (including strain JK224) have been characterized in any detail (for review, see Liscum and Hangarter, 1994). Furthermore, only two mutants, *rpt1* and *rpt2*, exhibiting altered root phototropism have been assigned to a particular locus (Okada and Shimura, 1992). One of the reasons for the apparent lack of genetic and molecular genetic results, despite the existence of these mutants, is that all but three (strain JK218, *rpt1*, and *rpt2*) exhibit some phototropic responsiveness. Mutants retaining a partial phototropic response, although representing powerful tools for physiological analysis (Khurana and Poff, 1989; Khurana et al., 1989; Konjevic et al., 1992), are difficult to study because the first positive response in Arabidopsis (e.g., ≤10° curvature under simple irradiation conditions) is not easily scored by visual inspection of single seedlings (Steinitz et al., 1985; Steinitz and Poff, 1986). By contrast, mutations that result in phototropic null phenotypes should be readily amenable to molecular genetic analysis. Therefore, we have developed a screen to detect mutants of Arabidopsis that lack phototropism altogether.

In this study, we report the isolation of several mutant lines that lack or have severely impaired phototropic responses and show that these mutants comprise four genetic loci. Results from the genetic and photophysiological characterization of mutants at one locus, *nph1* (for nonphototropic hypocotyl), suggested that the *NPH1* gene encodes the apoprotein for a dual-chromophoric (or multichromophoric) photoreceptor that mediates all the known phototropic responses of Arabidopsis and that the putative NPH1 and HY4 blue light photoreceptor proteins are genetically and biochemically distinct entities. Biochemical characterization of the *nph1* allele series indicated that the NPH1 photoreceptor apoprotein may in fact be a 120-kD plasma membrane–associated phosphoprotein.

RESULTS

Isolation and Genetic Characterization of Phototropic Null Mutants

Unilateral exposure of etiolated wild-type Arabidopsis seedlings to short wavelength blue light for 8 to 10 hr resulted in second positive curvatures (35 to 45°) of the hypocotyl toward actinic light, as shown in Figure 1. We used these conditions to screen seedlings arising from mutagenized seed populations for mutants exhibiting altered second positive phototropism, and isolated eight such mutants. Six mutant lines were isolated by screening nine independent populations of fast neutron–mutagenized M_2 seeds, and two mutant lines were isolated from the T_4 generation of two different parental pools arising from T-DNA insertional mutagenesis. These mutant lines shared an extreme phenotype with respect to the phototropic response to blue light. Figure 1 depicts representative seedlings from each of the mutant lines. Seven of the mutants (*nph1-1*, *nph1-3*, *nph1-4*, *nph3-1*, *nph3-2*, *nph4-1*, and *nph4-2*) exhibited a phototropic null phenotype, and one mutant (*nph2-1*) was weakly phototropic.

When each of these mutant lines was crossed to its representative wild-type background in a reciprocal manner and the phototropic phenotype was scored in the F_1 and F_2

Table 1. Genetic Analysis of Arabidopsis *nph* Mutants

Crosses[a]	Generation	Curving[b]	Noncurving[b]	χ^2
NPH1-1/NPH1-1 × *nph1-1/nph1-1*[c]	F_1	45	0	
NPH1-1/nph1-1 × *NPH1-1/nph1-1*	F_2	157	50	0.103[d]
NPH2-1/NPH2-1 × *nph2-1/nph2-1*	F_1	58	0	
NPH2-1/nph2-1 × *NPH2-1/nph2-1*	F_2	324	98	0.711[d]
NPH3-1/NPH3-1 × *nph3-1/nph3-1*[c]	F_1	67	0	
NPH3-1/nph3-1 × *NPH3-1/nph3-1*	F_2	206	71	0.059[d]
NPH4-2/NPH4-2 × *nph4-2/nph4-2*[c]	F_1	64	0	
NPH4-2/nph4-2 × *NPH4-2/nph4-2*	F_2	310	163	22.579[e]

[a] Data represent wild-type (male) to mutant (female) crosses; similar results were obtained with reciprocal crosses.
[b] Wild-type seedlings have curving hypocotyls (>10°); mutants have noncurving hypocotyls (≤10°).
[c] Similar results were obtained with other alleles of this locus.
[d] χ^2 expected ratio, 3 wild type:1 mutant; $P > 0.05$.
[e] χ^2 expected ratio, 3 wild type:1 mutant; $P < 0.005$.

generations, we observed that the *nph1*, *nph2*, and *nph3* loci all segregated as single, recessive nuclear mutations, as shown in Table 1. The *nph4* locus, although segregating as a single nuclear mutation, appeared incompletely recessive (Table 1). Table 2 shows the results from complementation tests among all of the mutant lines. The eight mutants comprise four genetic loci, represented by three alleles of *nph1*, a single *nph2* allele, two alleles of *nph3*, and two alleles of *nph4*. In addition to complementation tests among the eight mutant lines isolated in this study, each line was crossed to the only previously isolated mutant line null for hypocotyl phototropism, strain JK218 (Khurana and Poff, 1989), and the presumptive PI photoreceptor mutant line, strain JK224 (Khurana and Poff, 1989; Konjevic et al., 1992). We found that strain JK218 represents a third mutant *NPH3* allele, *nph3-3*; strain JK224 is an allele of the *nph1* mutant series and has been designated *nph1-2* (Table 2). Because the *NPH1* locus may encode a photoreceptor for the phototropic response to blue light (Khurana and Poff, 1989; Konjevic et al., 1992), we focused our initial efforts on the *nph1* allele series.

F_2 segregation analysis of crosses between *nph1/nph1* M_2 plants, carrying the homozygous *glabrous1* (*gl1*) mutation, and wild-type plants (*NPH1/NPH1 GL1/GL1*) showed linkage between the *NPH1* and *GL1* loci on chromosome 3. It was observed that the mutant trichome phenotype cosegregated with the phototropic null phenotype in 162 of 240 *nph1/nph1* individuals, indicating that these loci are within 26 centimorgans of each other.

Characterization of the Tropic Responses of the *nph1* Allele Series

As mentioned previously, studies of blue light– and green light–induced phototropism in etiolated Arabidopsis seedlings

Table 2. Complementation Analysis of Arabidopsis *nph* Mutants

Crosses[a]	Curving[b]	Noncurving[b]
nph1-1/nph1-1 × *nph1-3/nph1-3*	0	17
nph1-1/nph1-1 × *nph1-4/nph1-4*	0	130
nph1-2/nph1-2[c] × *nph1-1/nph1-1*[d]	0	28
nph1-2/nph1-2 × *nph1-4/nph1-4*[d]	0	12
nph1-3/nph1-3 × *nph1-2/nph1-2*[d]	0	38
nph1-4/nph1-4 × *nph1-3/nph1-3*	0	64
nph3-1/nph3-1 × *nph3-2/nph3-2*	0	53
nph3-1/nph3-1 × *nph3-3/nph3-3*[e]	0	57
nph3-2/nph3-2 × *nph3-3/nph3-3*[e]	0	48
nph4-1/nph4-1 × *nph4-2/nph4-2*	0	58
nph1-1/nph1-1 × *nph3-1/nph3-1*[f]	50	0
nph2-1/nph2-1 × *nph1-1/nph1-1*[f]	12	0
nph2-1/nph2-1 × *nph3-2/nph3-2*[f]	61	0
nph3-2/nph3-2 × *nph4-1/nph4-1*[f]	56	0
nph4-2/nph4-2 × *nph1-1/nph1-1*[f]	79	0
nph4-2/nph4-2 × *nph2-1/nph2-1*[f]	84	0

[a] Crosses were made with the first mutants listed (male) to second mutant listed (female).
[b] Wild-type seedlings have curving hypocotyls (>10°); mutants have noncurving hypocotyls (≤10°).
[c] This mutant was previously designated as strain JK224 (Khurana and Poff, 1989).
[d] The progeny of this cross were analyzed for first positive phototropism, as described in Methods.
[e] This mutant was previously designated as strain JK218 (Khurana and Poff, 1989).
[f] Similar results were obtained with all other allele combinations of these loci.

Table 3. Second Positive Hypocotyl Phototropism in Etiolated Arabidopsis Wild-Type and *nph1* Seedlings

Genotype	Curvature (°)		
	UV-A Light[a]	Blue Light[b]	Green Light[a]
Col	59.3 ± 3.4 (26)	43.9 ± 2.5 (25)	42.2 ± 1.8 (44)
nph1-1	−1.9 ± 1.9 (31)	−0.2 ± 0.9 (26)	−0.2 ± 1.6 (27)
nph1-3	0.0 ± 1.4 (24)	−1.2 ± 1.1 (31)	0.1 ± 0.7 (31)
nph1-4	0.0 ± 0.6 (18)	0.4 ± 1.1 (25)	0.9 ± 1.4 (27)
Est	55.0 ± 2.7 (32)	49.1 ± 2.5 (36)	39.0 ± 2.0 (45)
nph1-2	54.4 ± 3.0 (31)	48.9 ± 2.5 (36)	33.0 ± 2.0 (45)

Seedlings were handled as described in Figure 1, except for varied light conditions. The phototropic response of seedlings was measured after 10 hr of exposure to light of the indicated quality. Data represent the mean ± SE from a minimum of two replicate experiments. Numbers of seedlings are given within parentheses. Col, wild-type Columbia ecotype; Est, wild-type Estland ecotype.

[a] UV-A and green light were given at 0.2 μmol m^{-2} sec^{-1}.

[b] Blue light was given at a fluence rate of 0.1 μmol m^{-2} sec^{-1}.

(Steinitz et al., 1985) have shown that these responses are mediated via two photosensory systems, PI and PII (Konjevic et al., 1989), and that the *nph1-2* mutant (previously strain JK224) most likely affects the PI photoreceptor specifically (Khurana and Poff, 1989; Konjevic et al., 1992). Although the *nph1-2* mutant has provided genetic evidence for a second photosensory system in the blue/green light–mediated phototropic response of Arabidopsis, its leaky nature precludes any conclusion regarding the relationship of the PI and PII photosensory systems beyond the existence of two independent chromophores. Thus, it is unclear whether the activities of the PI and PII systems reside in a single, dual-chromophoric holoprotein or in separate and unique photoreceptor holoproteins. Because the *nph1-2* mutant was generated by ethyl methanesulfonate (EMS) mutagenesis and hence most likely contains a point mutation and because we do not yet know the molecular nature of the lesion, neither of the aforementioned scenarios can be ruled out. However, having multiple mutant alleles at the *NPH1* locus that result in a phototropic null phenotype in blue light allowed us to determine the relationship between PI and PII.

Table 3 shows that *nph1-2* exhibits a blue light–induced second positive curvature similar to the wild type, as has been shown previously with high fluences of blue light (Khurana and Poff, 1989). Furthermore, we also observed wild-type responsiveness of *nph1-2* to green light (Table 3), demonstrating that the normal green light responsiveness of this mutant includes second as well as first positive phototropism (Konjevic et al., 1992). However, the nonphototropic response of the strong *nph1* alleles (*nph1-1*, *nph1-3*, and *nph1-4*) observed after extended irradiation with blue light was also evident with green light (Table 3). Similar phototropic null responses of the strong *nph1* alleles were observed at peak first positive fluences of blue light (data not shown).

Phototropism in higher plants can be induced by UV-A light (Shropshire and Withrow, 1958; Thimann and Curry, 1960; Everett and Thimann, 1968; Baskin and Iino, 1987) in addition to blue/green light. The UV-A response has generally been thought to act through a single photoreceptor that absorbs both UV-A and blue light, such as a flavoprotein (see Briggs and Iino, 1983). However, given evidence for the function of two genetically separable photosensory systems in blue light– and green light–induced phototropism in etiolated Arabidopsis (Konjevic et al., 1992), the hypothesis for multiple photoreceptors for phototropism in oat seedlings (Zimmerman and Briggs, 1963), and the genetic separation of blue from UV-A light–induced hypocotyl growth inhibition in Arabidopsis (Young et al., 1992), it remains formally possible that blue and UV-A light sensitivities for phototropism may require unique gene products. Therefore, we investigated the UV-A light–induced phototropic responsiveness of the *nph1* allele series because they are putative blue-light photoreceptor mutants.

As shown in Table 3, the phototropic responses of etiolated *nph1* mutants to UV-A light paralleled those observed in blue and green light: the strong alleles of *nph1* (*nph1-1*, *nph1-3*, and *nph1-4*) exhibited no phototropic curvature in UV-A, blue, or green light, whereas the response of the weak allele, *nph1-2*, was similar to the wild type for all three light qualities. Together, the results from experiments with different light qualities indicated that if the *NPH1* gene product encodes a photoreceptor for phototropism, as suggested by photophysiological studies of *nph1-2* (Khurana and Poff, 1989; Konjevic et al., 1992), it must encode the apoprotein for a single photoreceptor holoprotein containing both the PI and PII chromophores because severe alleles of *nph1* result in a phototropic null phenotype in all light qualities tested.

Table 4. Second Positive Hypocotyl and Root Phototropism in De-Etiolated Arabidopsis Wild-Type and *nph1* Seedlings

Genotype	Curvature (°)	
	Hypocotyl	Root
Col	51.6 ± 3.9 (48)	−15.0 ± 1.1
nph1-1	−1.0 ± 2.0 (48)	−1.1 ± 1.2
nph1-3	2.7 ± 2.1 (36)	2.1 ± 2.2
nph1-4	0.8 ± 0.9 (47)	−0.5 ± 1.3
Est	58.6 ± 3.5 (46)	−21.1 ± 1.6
nph1-2	26.3 ± 3.8 (40)	−8.5 ± 1.9

Seedlings were grown on vertical plates. After 23.5 hr in darkness, plates were transferred to continuous white light given from above for 48 hr and then subjected to continuous unilateral blue light (0.1 μmol m^{-2} sec^{-1}) for 10 hr. Plates were projected so that curvatures could be determined, as described in Methods. Data represent the mean ± SE from a minimum of three replicate experiments. Because hypocotyl and root curvature measurements were made for each seedling, the number of seedlings analyzed are given within parentheses to the right of the hypocotyl data only. Col, wild-type Columbia ecotype; Est, wild-type Estland ecotype.

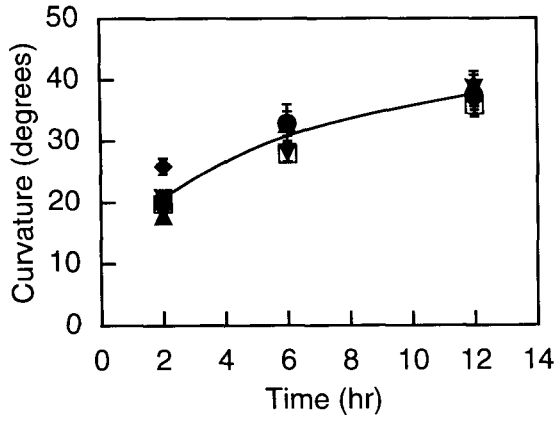

Figure 2. Time Course for Hypocotyl Gravitropism in Etiolated Arabidopsis Wild-Type and *nph1* Seedlings.

Seedlings were grown on vertical plates. After 71.5 hr in darkness, plates were rotated 90°, on edge, under a dim green safelight and returned to darkness. At the indicated times, plates were removed from darkness and projected so that curvatures could be determined as described in Methods. Each data point represents the mean response of a minimum of 25 seedlings from at least three replicate experiments. Vertical error bars represent the standard error of the mean. Because the symbols often overlap, some individual symbols and error bars are not readily visible. Similar results were obtained with the wild-type Estland ecotype (the genetic background of *nph1-2*). □, wild-type Columbia ecotype; ●, *nph1-1*; ▲, *nph1-2*; ♦, *nph1-3*; ▼, *nph1-4*.

Although experiments designed to pose questions about the phototropic response of higher plants most often employ etiolated seedlings, plants in the natural environment need to respond to changes in light direction after they have become de-etiolated and photosynthetically competent (Iino, 1990). Thus, we examined the blue light–dependent phototropic response of hypocotyls of de-etiolated wild-type seedlings and *nph1* mutants. As with etiolated seedlings, strong *nph1* mutants lacked blue light–induced phototropic curvature of de-etiolated hypocotyls, as shown in Table 4. The weak *nph1-2* allele responded ~40 to 45% as well as the wild type did under the same conditions (Table 4). In addition to hypocotyls, roots of de-etiolated seedlings of Arabidopsis are also phototropic, exhibiting a negative response (Okada and Shimura, 1992). Like de-etiolated hypocotyls, strong alleles of *nph1* showed a lack of response in the roots, whereas the weak allele (*nph1-2*) exhibited a partial response (Table 4).

Another tropistic response of higher plants is the response to gravity, specifically the negative tropic response of stems and the positive tropic response of roots (Firn and Digby, 1980). Although many of the signal perception–transduction steps involved in phototropism and gravitropism are likely to be unique to each of the responses, components directly involved in changes in growth rates on opposite flanks of stem or root tissues during both phototropic and gravitropic response could be shared. Genetic support for this hypothesis comes from previous studies of Arabidopsis phototropism mutants, showing that although phototropism and gravitropism are genetically separable, they indeed have shared gene products (Khurana and Poff, 1989; Khurana et al., 1989; Okada and Shimura, 1992). As would be expected if the *NPH1* gene product functioned as a photoreceptor, hypocotyls of all of the *nph1* mutants exhibited a wild-type gravitropic response, as shown in Figure 2. Root gravitropism is also apparently not affected by the *nph1* mutations (data not shown).

Light-Induced Phosphorylation of a 120-kD Plasma Membrane-Associated Protein in Wild-Type and *nph1* Seedlings

Blue light has been shown to potentiate the rapid phosphorylation of a high molecular mass plasma membrane–associated protein, both in vivo and in vitro, in all monocotyledonous and dicotyledonous species examined, including Arabidopsis (Reymond et al., 1992a, 1992b). Several photophysiological studies provide correlative evidence that this rapid biochemical response to blue light represents an early step in the signal transduction of phototropic stimuli (for review, see Short and Briggs, 1994). Reymond et al. (1992b) have shown that crude microsomal membranes isolated from 3- to 4-day-old etiolated *nph1-2* mutants exhibit severely impaired phosphorylation of a 120-kD protein relative to wild-type Arabidopsis membranes, providing strong genetic support for the hypothesis that the phosphorylation event is involved in the phototropic signaling pathway.

When we examined microsomal membranes from all of the *nph1* mutants, we observed a complete lack of blue light–potentiated phosphorylation of the 120-kD protein for each of the mutant alleles, as shown in Figure 3A. The results presented here for *nph1-2* are in contrast with the observations of Reymond et al. (1992b), who reported a slight light induction in the phosphorylation of the 120-kD protein in microsomal membranes from *nph1-2* seedlings at fluences of blue light greater than 3300 μmol m^{-2}. However, we did not observe any reproducible light-induced phosphorylation of the 120-kD protein in *nph1-2* membranes, even at fluences two orders of magnitude above those required to saturate the wild-type response (data not shown). The reason for this discrepancy is not known.

Close examination of silver-stained gels after SDS-PAGE showed that wild-type membranes contained a protein band at 120 kD (Figure 3B) that comigrated with the radioactive band on the autoradiograph (Figure 3A). Furthermore, the 120-kD protein present in wild-type membranes exhibited a lower mobility during SDS-PAGE after it had been phosphorylated in vitro (Figure 3B). A similar change in mobility of the phosphorylation target protein has been reported for pea plasma membranes isolated from in vivo–irradiated third internode tissue (Short et al., 1993). The lowered mobility of the 120-kD protein observed with in vitro–irradiated wild-type Arabidopsis membranes was dependent upon three factors: blue light, ATP, and Mg^{2+} (data not shown). These results suggested that

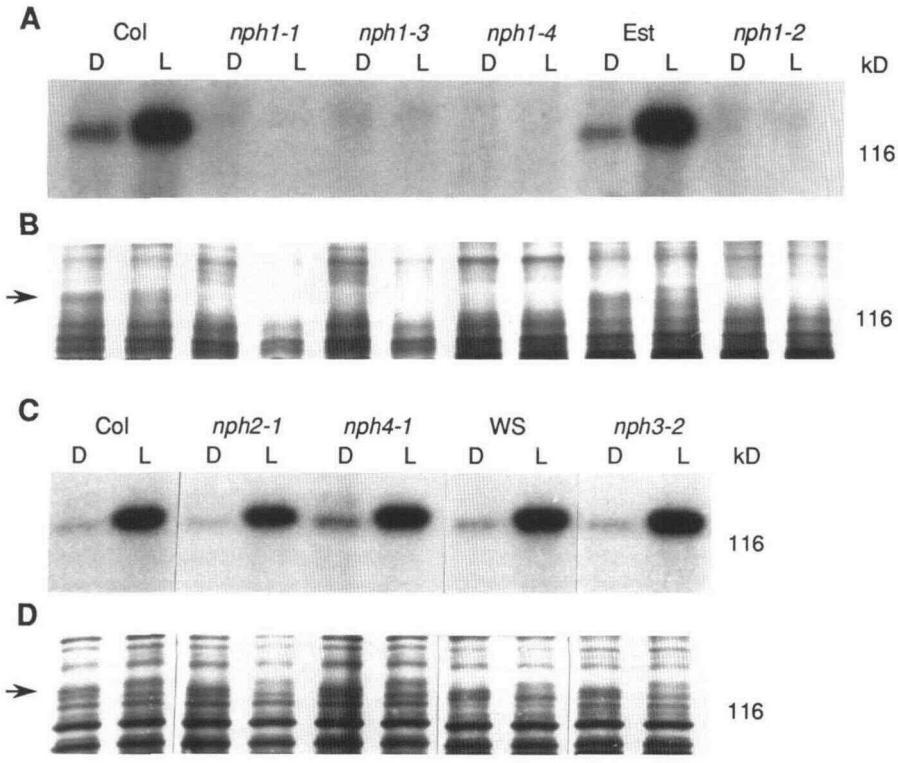

Figure 3. In Vitro Blue Light–Induced Phosphorylation of a 120-kD Protein in Microsomal Membranes from Arabidopsis Wild-Type Seedlings and *nph* Mutant Seedlings.

The arrows to the left of **(B)** and **(D)** indicate the position of the 120-kD protein in an unphosphorylated form. Molecular mass is indicated to the right as determined by the migration of *Escherichia coli* β-galactosidase (116 kD). Col, wild-type Columbia ecotype; Est, wild-type Estland ecotype; WS, wild-type Wassilewskija ecotype; D, mock irradiated control; L, blue light irradiated.

(A) Autoradiograph of in vitro–phosphorylated (see Methods) crude microsomal membranes from 3-day-old etiolated wild-type Columbia and Estland seedlings and *nph1* (*nph1-1*, *nph1-2*, *nph1-3*, and *nph1-4*) mutant seedlings following SDS-PAGE.

(B) Silver stain of the SDS–polyacrylamide gel from which the autoradiograph in **(A)** was generated.

(C) As shown in **(A)**, except that membranes from wild-type Columbia and Wassilewskija seedlings and *nph2-1*, *nph3-2*, and *nph4-1* mutant seedlings were used. Similar results were obtained with the other alleles of *nph3* and *nph4*.

(D) Silver stain of the SDS–polyacrylamide gel corresponding to the autoradiograph in **(C)**.

the lower mobility of the target protein results from the addition of multiple phosphate molecules to the 120-kD protein by a Mg^{2+}-dependent, light-activated kinase. Such multiple phosphorylations presumably result in an anomalous mobility of a phosphorylated protein relative to its nonphosphorylated counterpart when subjected to SDS-PAGE because they alter the ability of SDS to bind to a given phosphoprotein by charge exclusion (Beebe and Corbin, 1986; Guilfoyle et al., 1990). Silver-stained gels also showed that the lack of phosphorylation in the *nph1* mutants (Figure 3A) is correlated with severely reduced levels of the 120-kD target protein (Figure 3B). Given our levels of resolution, we estimated that each of the *nph1* mutants had a maximum of 10% of the 120-kD protein of the wild type (data not shown). When microsomal membranes from the *nph2*, *nph3*, and *nph4* mutants were phosphorylated in vitro, we observed that the abundance of the 120-kD protein (Figure 3D) and the light-dependent phosphorylation (Figure 3C) and change in electrophoretic mobility of that protein (Figure 3D) were indistinguishable from those observed for wild-type membranes. Together, these data indicate that a blue light–responsive 120-kD plasma membrane–associated protein may be the putative NPH1 photoreceptor and that the *NPH2*, *NPH3*, and *NPH4* loci are not required for the light-potentiated phosphorylation of the 120-kD protein.

We also examined the blue light–potentiated phosphorylation response in the *hy4* long hypocotyl mutant (Figures 4A and 4B). The *hy4* mutants exhibit altered blue light–dependent hypocotyl growth inhibition (Koornneef et al., 1980; Liscum and Hangarter, 1991). Recently, the *HY4* gene was cloned (Ahmad and Cashmore, 1993), and its sequence is similar to that of DNA photolyases, a class of flavoproteins that catalyze the blue light–dependent excision of pyrimidine dimers in UV-damaged DNA (Sancar, 1994). Although the *HY4* gene product does not appear to be a functional photolyase (Ahmad and Cashmore,

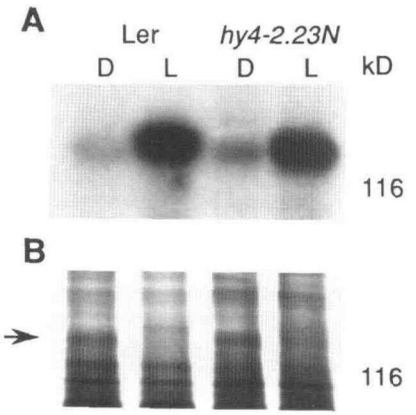

Figure 4. In Vitro Blue Light–Induced Phosphorylation of a 120-kD Protein in Microsomal Membranes from Arabidopsis Wild-Type Seedlings and *hy4-2.23N* Mutant Seedlings.

The arrow at left indicates the position of the 120-kD protein in an unphosphorylated form. Molecular mass is indicated to the right as determined by the migration of *Escherichia coli* β-galactosidase (116 kD). Ler, wild-type Landsberg *erecta* ecotype; D, mock irradiated control; L, blue light irradiated.

(A) Autoradiograph of in vitro–phosphorylated (see Methods) crude microsomal membranes from 3-day-old etiolated wild-type Landsberg *erecta* seedlings and *hy4-2.23N* mutant seedlings following SDS-PAGE.

(B) Silver stain of the SDS–polyacrylamide gel from which the autoradiograph in **(A)** was generated.

1993), its homology to a class of known flavoprotein photoreceptors suggests that it is a photoreceptor for the blue light–dependent hypocotyl growth inhibition response. However, as shown in Figures 4A and 4B, HY4 is not required for the blue light–mediated phosphorylation of the 120-kD protein because microsomal membranes from a deletion mutant allele (*2.23N*) of *HY4* (Koornneef et al., 1980; Ahmad and Cashmore, 1993) exhibited wild-type levels of light-potentiated phosphorylation of that protein. Furthermore, the levels of the 120-kD protein and its change in electrophoretic mobility when phosphorylated in vitro were identical for both *hy4* and its corresponding wild type.

In addition to the separation of biochemical phenotypes in the *hy4* and *nph1* mutants, the functions of the putative HY4 and NPH1 photoreceptors can be unambiguously separated photophysiologically, as shown in Figure 5. When wild-type, *hy4*, and *nph1* seedlings were grown for 3 days in continuous blue light from above, followed by 24 hr of unilateral blue light, wild-type seedlings exhibited both hypocotyl growth inhibition and phototropism, whereas *hy4* and *nph1* seedlings exhibited only the mutant phenotype for which they were selected. Furthermore, heterozygous F_1 progeny from a cross between *hy4-105* and *nph1-1* plants exhibited a wild-type phototropic response and a blue light–dependent hypocotyl growth inhibition response intermediate between the *nph1* and *hy4* parents (Figure 5). The intermediate hypocotyl length of the heterozygote was expected, even if the two mutants were complementary, because *hy4* is semidominant (Koornneef et al., 1980). Liscum et al. (1992) demonstrated that blue light–dependent hypocotyl growth inhibition and phototropism are genetically separable processes and suggested that the photoreceptors mediating those responses may also be separable. Although the molecular relationship, if any, between *NPH1* and *HY4* is not currently known, the data presented here indicate that the putative NPH1 and HY4 photoreceptor proteins are not the same molecule and do not represent functionally redundant perception systems.

DISCUSSION

Because Arabidopsis has gained acceptance as a model system for studying a variety of problems in plant biology, many groups have taken advantage of its attributes to study the genetic and molecular genetic regulation of responses to the environment. Responses to the light environment represent just one area of plant biology enriched by the study of Arabidopsis mutants (for reviews, see Chory, 1993; Deng, 1994; Koornneef and Kendrick, 1994; Liscum and Hangarter, 1994; Whitelam and Harberd, 1994). With such a genetic approach, several classes of mutants can be isolated for a given physiological response to the environment. These include perception,

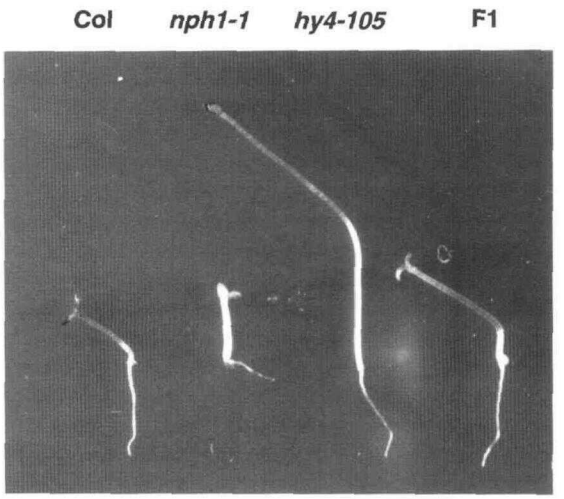

Figure 5. Comparison of Blue Light–Induced Phototropism and Hypocotyl Growth Inhibition in Arabidopsis Wild-Type, *nph1-1*, *hy4-105*, and Heterozygous *nph1 hy4* Seedlings.

Seedlings were grown for 3 days in continuous blue light (3.5 μmol m^{-2} sec^{-1}) given from above and then transferred to continuous unilateral blue light (0.2 μmol m^{-2} sec^{-1}) given from the left for 10 hr. To eliminate potential problems with the use of mutants in different ecotypical backgrounds, the *hy4-105* allele (see Methods) was used in these experiments. Col, wild-type Columbia ecotype; F1, *nph1-1 hy4-105* heterozygote.

transduction, and response mutants. The *nph* mutants reported here span these classes. Of particular interest are the *nph1* mutants that appear altered with respect to perception of light signals.

Analysis of the *nph1* allele series has demonstrated that, although isolated for altered blue light–dependent phototropic response of hypocotyls of etiolated seedlings, three of the *nph1* alleles (*nph1-1*, *nph1-3*, and *nph1-4*) can be considered null mutants. These mutants lack not only the blue light–dependent first and second positive hypocotyl phototropism of etiolated seedlings, but also the response of etiolated seedlings to UV-A and green light (see following discussion) as well as hypocotyl and root phototropism of de-etiolated seedlings. These results clearly demonstrate that the *NPH1* locus is an essential component of the signaling pathways for all the phototropic responses previously described for Arabidopsis (Steinitz and Poff, 1986; Okada and Shimura, 1992). No such universal phototropism mutant has been previously described for higher plants. In fact, analysis of two root phototropism mutants, *rpt1* and *rpt2*, has shown that the response pathways for hypocotyl and root phototropism have some genetically separable components in Arabidopsis (Okada and Shimura, 1992). Nevertheless, because hypocotyl and root tropisms are dependent upon the development of differential growth patterns for phenotypic expression (Firn and Digby, 1980; Iino, 1990), molecules directly involved in basic growth processes might be expected to be common components of the signal–response pathways of different organs. Furthermore, these molecules may be shared with respect to various tropic responses, including phototropism and gravitropism, as demonstrated by analyses of mutants (Khurana and Poff, 1989; Khurana et al., 1989). However, because *nph1* mutants are gravitropically normal, it is unlikely that the pleiotropic nature of the *nph1* mutations with respect to phototropism in different organs under different developmental conditions results from an alteration in a common downstream signaling molecule.

Detailed analysis of the wavelength dependencies for first positive phototropism in etiolated wild-type Arabidopsis seedlings has shown that both blue and green light can induce this response (Steinitz et al., 1985). Furthermore, the blue light–mediated response was shown to exhibit complex fluence rate dependencies as a result of two additive, overlapping photosensor functions: one (PI) absorbing most effectively in the blue region of the electromagnetic spectrum, and the other (PII) absorbing green light most effectively (Konjevic et al., 1989). As mentioned previously, the study of the phototropic response of hypocotyls of etiolated *nph1-2* (previously strain JK224) seedlings showed that, although this mutant is altered with respect to its fluence threshold for blue light–induced first positive phototropism (Khurana and Poff, 1989), it responds, as does the wild type, to first positive fluences of green light (Konjevic et al., 1992). These photophysiological properties led to the proposal that *nph1-2* represents a PI phototropism photoreceptor mutant (Khurana and Poff, 1989; Konjevic et al., 1992). However, data presented here show that in addition to lacking blue light–induced phototropism, etiolated hypocotyls of severe *nph1* alleles (*nph1-1*, *nph1-3*, and *nph1-4*) lack green and UV-A light–induced second positive phototropism as well, indicating that the *NPH1* locus affords an Arabidopsis seedling the ability to perceive not only blue but also UV-A and green light.

The photophysiological differences between the *nph1-2* allele and the null alleles (*nph1-1*, *nph1-3*, and *nph1-4*) may not be surprising when the methods for generating those allelic mutations are considered. The null alleles were all generated by fast neutron bombardment, which most often results in gross chromosomal changes, such as deletions (Rédei and Koncz, 1992). These types of mutations are usually very deleterious because they often result in the severe dysfunction or lack of the protein product of the mutated gene. In contrast, the "leaky" *nph1-2* allele was generated by EMS mutagenesis, which usually causes point mutations (Rédei and Koncz, 1992) and may result in only subtle changes in physiological function of the target protein. The combined results from studies of the *nph1* mutants suggest that the severe alleles of *nph1* completely ablate the holoprotein photoreceptor function, whereas the lesion in *nph1-2* affects the function of a single chromophore within the photoreceptor holoprotein, resulting in an alteration of sensitivity to blue light alone. Therefore, we hypothesize that the *NPH1* gene encodes the apoprotein for a single holoprotein photoreceptor that can use more than one chromophore and that this dual-chromophoric or multichromophoric nature results in wild-type Arabidopsis seedlings exhibiting the complex wavelength sensitivities that have been observed as PI and PII photosensor functions (Konjevic et al., 1989). Alternatively, *NPH1* could encode a nonphotoreceptor protein that is an essential transduction component through which separate PI and PII photoreceptors could operate independently. For example, NPH1 could have individual protein–protein interaction domains that are specific for PI and PII or activities that are separately specific to PI and PII. Both of these possibilities are consistent with the observed differences between leaky and null alleles of *nph1*. However, because multiple alleles have been isolated at each mutant *NPH* locus, except for the *NPH2* locus, and no mutants outside the *nph1* allele series exhibit changes in spectral sensitivity (data not shown) or light-potentiated phosphorylation of the putative NPH1 protein (see following discussion), this alternative hypothesis seems unlikely.

To our knowledge, the existence of green light–sensitive systems for phototropism has only been observed in two higher plants other than Arabidopsis: lettuce (Steinitz et al., 1985) and *Celosia cristata* (Atkins, 1936). However, very similar blue and UV-A light dependencies for phototropism have been observed for all higher plants examined (for review, see Briggs and Iino, 1983; Iino, 1990). Because blue/UV-A light–sensitive phototropism appears to have been evolutionarily conserved in higher plants, whereas green light–induced phototropism has not been, and because there is no obvious adaptive advantage for these three plants to use green light as a directional light cue, it is reasonable to hypothesize that the green light sensitivity has arisen by a spurious modification of the existing blue/UV-A light–sensitive system. Such a modification could

be as simple as the ability of the photoreceptor apoprotein to utilize two chemical forms of the same chromophore molecule. For example, if the phototropism photoreceptor is a flavoprotein, as suggested previously (see Briggs and Iino, 1983; Iino, 1990), the apoprotein could bind a particular flavin moiety in both an oxidized and a semireduced form, thus giving the holoprotein absorptive properties in the UV-A/blue regions of the spectrum in the oxidized form and in the blue/green regions in the reduced form (Schmidt, 1987; Song, 1987).

Photoreceptors that use more than one chromophore for blue light responses are not unknown. For example, the DNA photolyases catalyze the light-dependent repair of UV light–dimerized DNA through the concerted action of two chromophores (for review, see Sancar, 1994). One of the chromophores is an invariant flavin adenine dinucleotide (FAD) molecule (Iwatsuki et al., 1980); the second can be either a deazaflavin (Eker et al., 1981) or pterin derivative (Johnson et al., 1988). It has been shown in vitro under saturating light conditions that light absorption by either the FAD or the deazaflavin/pterin molecule can result in the excision of pyrimidine dimers (Jorns et al., 1990). However, in the natural environment, both probably act together, with the photoreduced FAD required for catalysis (Kim et al., 1993) and the deazaflavin/pterin chromophore acting as a light-harvesting antenna pigment (Eker et al., 1988; Jorns et al., 1990), thus effectively broadening the action spectrum for the response over what is attributable to each chromophore alone. Although it appears that the PI and PII chromophores for the phototropic response of Arabidopsis act in an independent and additive manner, with neither functioning as an antenna pigment (Konjevic et al., 1989), the physiological consequence of having two chromophores within a single holoprotein is similar to that for the two-chromophore photolyase enzyme: the broadening of the spectral sensitivity for the response.

Previous studies have suggested that the photoreceptor apoprotein for the phototropic response of higher plants is plasma membrane associated (see Vierstra and Poff, 1984; Short and Briggs, 1994). A potential candidate for this plasmalemma-associated photoreceptor molecule is a protein that becomes rapidly phosphorylated in response to blue light irradiation (for review, see Short and Briggs, 1994). The phosphorylation response has been observed in all monocotyledonous and dicotyledonous species examined, and the molecular mass of the phosphoprotein ranges from 100 to 130 kD, depending upon the species examined (Reymond et al., 1992a; Hager and Brich, 1993). In Arabidopsis, this protein has an apparent molecular mass of ~120 kD, as observed in crude microsomal membranes isolated from etiolated wild-type seedlings (Reymond et al., 1992a, 1992b). Reymond et al. (1992b) have shown that membranes from the *nph1-2* mutant exhibit severely reduced blue light–dependent in vitro phosphorylation of the 120-kD target protein compared to wild-type membranes. In our study, we observed that microsomal membranes from the *nph1-2* mutant exhibited no reproducible phosphorylation of the 120-kD protein. Moreover, we determined that this lack of phosphorylation, and therefore the severely reduced levels of phosphorylation observed by Reymond et al. (1992b), resulted from *nph1-2* membranes having no more than 10% of the 120-kD protein observed in wild-type membranes.

Despite the fact that the *nph1-1*, *nph1-3*, and *nph1-4* mutants are clearly distinct from *nph1-2*, based on photophysiological analyses (as discussed previously), all of the *nph1* mutants are indistinguishable from each other biochemically: specifically, no *nph1* allele has more than 10% of the wild-type levels of the 120-kD protein. However, the severe reduction or absence of the 120-kD protein in the *nph1* mutants, along with the photophysiological evidence that the mutation in *nph1-2* affects a blue light photoreceptor and the correlative evidence that the 120-kD phosphoprotein may be an autophosphorylating photoreceptor (for review, see Short and Briggs, 1994), make it probable that the NPH1 protein, or putative phototropism photoreceptor apoprotein (see previous discussion), and the 120-kD protein are one and the same. Additional support for this hypothesis is provided by the observation that the abundance of the 120-kD protein is not altered in any other mutant *nph* loci currently identified. However, direct testing of this hypothesis awaits the cloning of the *NPH1* gene.

Although currently we cannot determine how much, if any, of the NPH1 protein is present in any of the *nph1* mutants, we interpreted the biochemical and genetic data as indicating that very little of NPH1 present in etiolated wild-type Arabidopsis seedlings is required for normal phototropic responsiveness because at least 90% of that protein can be eliminated with only minor physiological consequence, as was seen with *nph1-2*. However, because we have isolated three *nph1* alleles that completely lack phototropic responses, it is possible that some critical threshold level of NPH1 is needed for phototropic sensitivity and that, although we cannot distinguish the null alleles from the weak *nph1-2* allele by SDS-PAGE analysis, the null mutants probably contain NPH1 in amounts below this threshold level, if they contain any NPH1 at all. We are currently generating antibodies against NPH1 purified from microsomal membranes of etiolated wild-type Arabidopsis seedlings and will be able to determine more precisely how much protein is present in each of the *nph1* alleles.

Mutations that result in the lack of a phototropic photoreceptor could result in a null phototropism phenotype under all light conditions and would be independent of the developmental state, as was observed with the *nph1-1*, *nph1-3*, and *nph1-4* alleles. However, it is not immediately obvious how a lesion in the *NPH1* locus that results in a change in the sensitivity to particular wavelengths of light, such as that observed for the *nph1-2* mutant, could result in such a dramatic reduction in the level of NPH1. One possibility consistent with the proposed effect of the lesion in *nph1-2* is that a mutation that eliminates or severely reduces the ability of the NPH1 apoprotein to bind a particular chromophore could in turn result in the degradation of the apoprotein if chromophore binding is necessary for apoprotein stability. Mutants of maize (Greene et al., 1988) and rice (Terao and Katoh, 1989) with chlorophyll *b* deficiencies represent systems in which chromophore deficiency apparently results in apoprotein instability. These mutants have increased

turnover rates for the apoproteins of the light-harvesting chlorophyll *a/b* proteins I and II that parallel the extent of chlorophyll *b* deficiency despite normal levels of translation for the apoproteins and proper integration into the thylakoid membranes. There is also evidence that this type of instability can occur with flavoproteins. For example, an FAD binding acyl coenzyme A dehydrogenase from rat liver mitochondria has been shown to exhibit more rapid degradation in riboflavin-deficient mitochondria than in control and riboflavin-repleted mitochondria, indicating that the apoenzyme requires the flavin cofactor for stability (Nagao and Tanaka, 1992).

The studies of the *nph1* mutant allele series, presented here and elsewhere (Khurana and Poff, 1989; Konjevic et al., 1992; Reymond et al., 1992b), support the hypothesis that the *NPH1* locus encodes the apoprotein for a dual-chromophoric or multichromophoric holoprotein photoreceptor that regulates all of the phototropic responses of Arabidopsis. It also appears that there are no functionally redundant photosensory systems operating during the primary phototropic response, because mutations in the *NPH1* gene can result in phototropic nulls. Furthermore, the phototropic photoperception system is distinct from the photosystem mediating blue light–dependent hypocotyl growth inhibition, as demonstrated by biochemical and genetic analysis of *nph1* and *hy4* mutants. Finally, it is likely that the apoprotein for the putative NPH1 photoreceptor is a 120-kD plasma membrane–associated phosphoprotein. We are currently using both molecular mapping and immunological approaches to clone the *NPH1* locus. We will then be able to determine unequivocally if *NPH1* indeed encodes a functional photoreceptor for phototropism. Furthermore, molecular identification of the *NPH1* gene may provide insights into other blue light–regulated processes and aid in the unraveling of the complex network of signaling pathways involved in photomorphogenesis.

METHODS

Plant Materials, Growth Conditions, and Light Sources

Arabidopsis thaliana Columbia ecotype carrying the homozygous recessive *glabrous1* (*gl1*) mutation (Koornneef et al., 1982) was the parental strain of the fast neutron–mutagenized seed lots, and the Wassilewskija ecotype was the parental strain of the T-DNA insertion lots (Feldmann, 1992). Mutant strains JK218 and JK224 were isolated from ethyl methanesulfonate (EMS)–mutagenized seed of the Estland ecotype (Khurana and Poff, 1989). The *hy4-2.23N* allele was isolated from fast neutron–mutagenized seed of the Landsberg *erecta* ecotype (Koornneef et al., 1980), whereas the *hy4-105* allele was isolated from EMS-mutagenized seed of the Columbia ecotype (J.C. Young and R.P. Hangarter, unpublished results).

For all experiments, seeds were surface sterilized and planted in Petri dishes in a random fashion (for mutant screening and etiolated seedling phototropism experiments) or in rows (for gravitropism and de-etiolation experiments) on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) with 1.0% agar (w/v) as described by Liscum and Hangarter (1993a, 1993b). Cold treatment and red light exposure to induce uniform germination were as described by Liscum et al. (1992). After induction of germination, Petri dishes were handled in several different ways, depending upon the experiment. For screening and characterization of the phototropic response in the etiolated seedlings, plates were incubated in darkness for 71.5 hr at 24 ± 2°C and then transferred to unilateral light conditions of the quality and for the duration indicated. Seedlings for membrane preparations were harvested immediately after the 71.5-hr dark growth period. For gravitropism experiments, plates were oriented in a vertical position and placed in darkness for 71.5 hr to allow seedlings to grow along the surface of the agar. Following the 3-day growth period, plates were rotated 90°, on edge, under a dim green safelight (Short et al., 1992; Liscum and Hangarter, 1993b) and returned to darkness where the seedlings were allowed to develop gravitropic curvature for the indicated times. For de-etiolated seedling experiments, plates were oriented in a vertical position and placed in darkness at 24 ± 2°C for 23.5 hr, transferred to white light given from above for 48 hr to induce de-etiolation, and then transferred to unilateral blue light for 48 hr. When appropriate, seedlings (7 to 10 days old) were transferred to Supersoil (Rod McLellan Co., South San Francisco, CA) saturated with nutrient solution (Estelle and Somerville, 1987) and grown to maturity under constant illumination at 23 ± 2°C. Potted plants were watered three times per week, once with nutrient solution (Estelle and Somerville, 1987).

Light for potted plants (150 ± 5 μmol m^{-2} sec^{-1}) was provided by Octron 4100K fluorescent bulbs (032/741; Sylvania, Danvers, MA). White light for de-etiolation was provided by four cool-white fluorescent bulbs (F36T12/CWHO; Sylvania). Red light for induction of germination (10 μmol m^{-2} sec^{-1}) and cold-room manipulations (1.0 ± 0.5 μmol m^{-2} sec^{-1}) was obtained as described by Short and Briggs (1990). Blue light for mutant screening and physiological analyses was obtained by filtering light from one fluorescent black light bulb (F15T8-BL; General Electric [GE], Cleveland, OH) through one layer of Rohm and Haas blue plexiglass (No. 2424; AIN Plastics, San Jose, CA). This light source resulted in a spectrum with a wavelength maximum (λ_{max}) at 436 nm and a 10-nm half bandwidth. Blue light for the induction of hypocotyl growth inhibition was obtained by filtering light from cool-white fluorescent bulbs (F36T12/CWHO; Sylvania) through one layer of Rohm and Haas blue plexiglass (No. 2424) and four layers of cheesecloth as a neutral density filter. Blue light for genetic analysis of F_1 progeny of crosses between strain JK224 and nonphototropic hypocotyl (*nph*) mutants was obtained by filtering light from one 300-W ELH bulb (Sylvania) through a 448-nm interference filter with a 10-nm half bandwidth. Blue light for in vitro phosphorylations was as described by Short and Briggs (1990). (The fluence rate of these last two light sources was adjusted by use of neutral density filters.) Green light was obtained by filtering light from two green fluorescent bulbs (F40G; Sylvania) through one layer each of Rohm and Haas blue plexiglass (No. 2424) and green plexiglass (No. 2092; AIN Plastics). This light source resulted in a spectral output with a λ_{max} at 520 nm and a 30-nm half bandwidth. UV-A light was obtained by filtering light from one fluorescent black light bulb (GE-F15T8-BLB) through two layers of yellow Roscolux No. 10 (Holz-Mueller, San Francisco, CA). The resulting spectral output had a λ_{max} at 360 nm and a 30-nm half bandwidth. Spectral qualities were measured with a Li1800 portable spectroradiometer (LiCor, Inc., Lincoln, NE). Unless otherwise noted, fluence rates were adjusted by changing the distance between plant material and the light source. Fluence rates of the blue, green, and red light sources were measured with a Li185A quantum photometer (LiCor, Inc.); the fluence rate of the UV-A source was measured with a bismuth–silver thermopile (Eppley Laboratory Inc., Newport, RI).

Isolation of Mutants and Genetic Analysis

Fast neutron–mutagenized M_2 seeds were obtained from Lehle Seeds (Round Rock, TX). Approximately 140,000 M_2 seeds arising from 13 independent parental groups were screened. T-DNA insertion seed stocks (T_4 generation) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH; ~5500 lines) and E. I. DuPont de Nemours and Co. (Wilmington, DE; ~6000 lines). Seedlings were scored visually for hypocotyl phototropism after 10 to 12 hr of continuous blue light (0.1 μmol m^{-2} sec^{-1}). Seedlings exhibiting an altered phototropic response were returned to blue light for an additional 10 to 12 hr, at which time seedlings still maintaining an altered phototropic response were transferred to soil and allowed to self-fertilize. Using this process, 137 (M_2 or T_4) putative phototropic null mutants were isolated. The resulting M_3, or T_5, seed was collected and retested for the mutant phenotype. Nine heritable, fertile mutants were recovered from these putative mutant populations. (Two mutant lines [P5600.1 and P5600.6] isolated from the same T-DNA insertion parental pool were assumed to be siblings, and thus only one, P5600.1 [*nph3-2*], was used in subsequent studies.) Each of these mutants was crossed to the wild type in a reciprocal manner and pairwise to each other to determine patterns of inheritance and allelism, respectively.

The phototropic response of F_1 seedlings arising from crosses between strain JK224 and the *nph* mutants was assessed at the level of first positive curvature because strain JK224 exhibits an alteration only in its first positive phototropic response (Khurana and Poff, 1989). Specifically, phototropism in these F_1 seedlings was induced by exposure to five pulses of blue light at a fluence of 0.2 μmol m^{-2} sec^{-1}, given at 15-min intervals, which results in large first positive curvatures as described by Steinitz and Poff (1986). Curvature was then measured as described by Khurana and Poff (1989). For all other F_1 seedlings and all F_2 progeny, phototropic curvature was induced by a 10-hr exposure to continuous blue light. In these cases, curvature was assayed by visual inspection because the mutant phenotype could be clearly distinguished from a wild-type response (see Figure 1).

Measurement of Tropic Responses

The phototropic response of etiolated hypocotyls was induced by 10 hr of continuous blue, green, or UV-A light. After light exposure, seedlings were carefully removed from the agar medium and placed onto transparent tape. Curvatures were measured (in degrees) from tracings made of projected images of the tape-immobilized seedlings as described by Steinitz and Poff (1986). For hypocotyl and root phototropism in de-etiolated seedlings and hypocotyl gravitropism in etiolated seedlings, plates with seedlings growing along the surface of the agar medium were placed horizontally on an overhead enlarger and projected after appropriate growth periods. Enlarged images of seedlings were traced, and the curvatures of the hypocotyl and root were determined (in degrees) from those tracings.

Microsomal Membrane Preparation, in Vitro Phosphorylation Assays, and Gel Electrophoresis

Seedlings were harvested from agar medium with forceps. Tissue was ground with a mortar and pestle, and microsomal membranes were prepared as described by Short et al. (1993), except that 5 mM ε-aminocaproic acid and 1 mM benzamidine were included in the homogenization buffer, and *N*-methyl-D-glucosamine and 1,2-*bis*(*o*-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetraacetic acid were left out of both the homogenization and resuspension buffers. In addition, the microsomal membranes were pelleted by centrifugation at 100,000*g* for 1 hr and 15 min rather than for 30 min. All manipulations were performed at 4°C under dim red light. For in vitro phosphorylations, 5 μg of microsomal membranes (on protein basis) were solubilized with Triton X-100 (20:1, detergent/protein) in phosphorylation buffer (37.5 mM Tris–2-[*N*-morpholino]ethanesulfonic acid, pH 7.5, 5.3 mM $MgSO_4$, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 5 mM ε-aminocaproic acid, 1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL antipain) on ice for 1 min prior to irradiation or mock irradiation. Irradiations were done on ice at a total fluence of 3300 μmol m^{-2} (1 min at a fluence rate of 55 μmol m^{-2} sec^{-1}). Dark control samples were mock irradiated on ice for 1 min. Following irradiation or mock irradiation, γ-^{32}P-ATP was added to the buffer to a final concentration of 500 μM (specific activity, 1.3 Ci/mmol). (Total volume of reactions was 10 μL after the addition of ATP.) The reaction was vortexed for ~1 sec and allowed to incubate at 24 ± 2°C for 2 min; an equal volume of 2 × sample buffer (125 mM Tris-HCl, pH 6.8, 8 M urea, 10% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue) was added to stop the reaction. Entire samples were electrophoresed on 7.5% SDS–polyacrylamide gels (Laemmli, 1970) until a 116-kD prestained marker (β-galactosidase) was run 4.5 to 5 cm into the separating gel. Gels were then silver stained (Porro et al., 1982), dried, and autoradiographed.

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