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The Phytochrome-Deficient *pcd1* Mutant of Pea Is Unable to Convert Heme to Biliverdin IXα

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We isolated a new pea mutant that was selected on the basis of pale color and elongated internodes in a screen under white light. The mutant was designated *pcd1* for phytochrome chromophore deficient. Light-grown *pcd1* plants have yellow-green foliage with a reduced chlorophyll (Chl) content and an abnormally high Chl *a*/Chl *b* ratio. Etiolated *pcd1* seedlings are developmentally insensitive to far-red light, show a reduced response to red light, and have no spectrophotometrically detectable phytochrome. The phytochrome A apoprotein is present at the wild-type level in etiolated *pcd1* seedlings but is not depleted by red light treatment. Crude phytochrome preparations from etiolated *pcd1* tissue also lack spectral activity but can be assembled with phycocyanobilin, an analog of the endogenous phytochrome chromophore phytochromobilin, to yield a difference spectrum characteristic of an apophytochrome-phycocyanobilin adduct. These results indicate that the *pcd1*-conferred phenotype results from a deficiency in phytochrome chromophore synthesis. Furthermore, etioplast preparations from *pcd1* seedlings can metabolize biliverdin (BV) IXα but not heme to phytochromobilin, indicating that *pcd1* plants are severely impaired in their ability to convert heme to BV IXα. This provides clear evidence that the conversion of heme to BV IXα is an enzymatic process in higher plants and that it is required for synthesis of the phytochrome chromophore and hence for normal photomorphogenesis.

INTRODUCTION

The phytochromes are a family of chromoprotein photoreceptors with a well-established and important role in the mediation of plant developmental responses to light (Kendrick and Kronenberg, 1994). The spectral characteristics of phytochrome derive from an interaction between an apoprotein and a covalently bound linear tetrapyrrole chromophore (Lagarias and Rapoport, 1980). Holophytochrome biosynthesis therefore requires the convergence of two separate pathways, one for synthesis of the apoprotein and another for synthesis of the chromophore precursor, phytochromobilin (PΦB). Several distinct forms of the phytochrome apoprotein exist, and these are products of a small gene family designated *PHYA* to *PHYE* in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994). Assembly of apophytochrome with PΦB is an autocatalytic process that is thought to occur in the cytoplasm (Lagarias and Lagarias, 1989; Terry and Lagarias, 1991). There is no evidence for the involvement of any other protein in phytochrome assembly. Because assembly to phycocyanobilin (PCB), a structural analog of PΦB, proceeds at the same rate for both PHYA (Li and Lagarias, 1992) and PHYB (Kunkel et al., 1993) apoproteins, it is likely that at least these phytochromes (and possibly PHYC as well; Quail et al., 1995) use the same chromophore.

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Figure 1 shows the proposed pathway for PΦB synthesis in higher plants, which has been developed on the basis of metabolism studies (Elich and Lagarias, 1987; Elich et al., 1989; Terry et al., 1995) and by analogy with the pathway for the synthesis of related bilins in algae (Beale, 1993; Terry et al., 1993b). Synthesis of PΦB appears to occur entirely in the plastid (Terry and Lagarias, 1991; Terry et al., 1993b), where it is derived from 5-aminolevulinic acid and is synthesized via a pathway that branches from the synthetic pathway for chlorophyll (Chl). The first committed step for PΦB synthesis is thought to be the oxygenation of heme to biliverdin (BV) IXα, which is subsequently reduced to 3(*Z*)-PΦB. 3(*Z*)-PΦB is further isomerized to 3(*E*)-PΦB, which is considered to be the natural chromophore precursor (Cornejo et al., 1992; Terry et al., 1995). Although much progress has been made recently, understanding of this pathway in higher plants is still far from complete. For example, although ferrochelatase has been cloned (Smith et al., 1994) and PΦB synthase activity has been characterized (Terry and Lagarias, 1991; Terry et al., 1995), direct evidence for heme oxygenase and PΦB isomerase activities has yet to be demonstrated (Terry et al., 1993b, 1995).

Since the advent of a mutant-based approach to plant photomorphogenesis, many mutants that alter phytochrome function have been identified and characterized (Koornneef and Kendrick, 1994). These include mutants deficient in specific

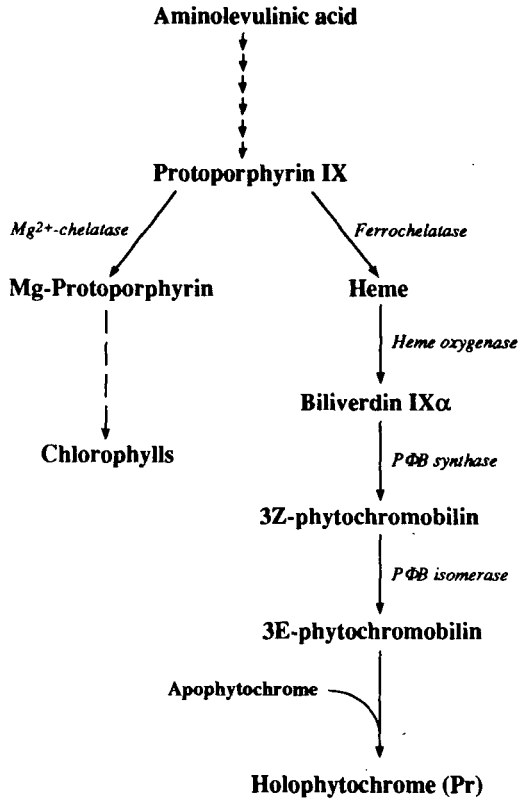

Figure 1. Proposed Pathway for Phytochrome Chromophore Biosynthesis in Higher Plants.

The phytochrome chromophore is synthesized from aminolevulinic acid via a pathway that branches from the pathway for Chl synthesis by the chelation of Fe^{2+} rather than Mg^{2+} to protoporphyrin IX.

phytochromes. Study of these mutants has established that phyA, the light-labile phytochrome abundant in etiolated seedlings, is involved specifically in the mediation of seedling responses to far-red light (FR) (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; van Tuinen et al., 1995a), whereas the light-stable B-type phytochromes appear to control responses to red light (R) (Somers et al., 1991; Devlin et al., 1992; López-Juez et al., 1992; van Tuinen et al., 1995b; Weller et al., 1995).

A third class of mutant, deficient in response to both R and FR, is represented by the long hypocotyl *hy1*, *hy2*, and *hy6* mutants of Arabidopsis (Koornneef et al., 1980; Chory et al., 1989). In view of what is known from phyA- and phyB-specific mutants, the coincidence of R and FR insensitivity in *hy1*, *hy2*, and *hy6* suggests that the lesions in these mutants affect the activity of both phyA and phyB. Etiolated seedlings of *hy1*, *hy2*, and *hy6* have strongly reduced levels of spectrally active phytochrome but wild-type or nearly wild-type levels of PHYA apoprotein (Koornneef et al., 1980; Chory et al., 1989; Parks et al., 1989), suggesting that phytochrome apoprotein is synthesized normally in these mutants but for some reason is not converted into a spectrally active holoprotein. The most plausible explanation, that the mutants are deficient in the phytochrome chromophore, is further supported by the demonstration that exogenously supplied BV can fully or partially restore a wild-type phenotype in the *hy1*, *hy2*, and *hy6* mutants (Parks and Quail, 1991; Nagatani et al., 1993).

Similar mutants are also known in tomato. Like *hy1*, the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants are insensitive to both R and FR and have strongly reduced levels of spectrally active phytochrome (Koornneef et al., 1985; Parks et al., 1987; Kendrick et al., 1994). Attempts to rescue the *au* mutant by feeding chromophore precursors or structural analogs have proven unsuccessful so far (Kendrick et al., 1994). However, the phenotypic similarity of *au* to the chromophore-deficient Arabidopsis mutants and epistasis of *au* and *yg-2* over a constitutively expressed *PHYA* transgene are consistent with the hypothesis that both *au* and *yg-2* are blocked in phytochrome chromophore biosynthesis (Kendrick et al., 1994).

Thus, among the several candidates for mutations that block phytochrome chromophore synthesis, only the *hy1*, *hy2*, and *hy6* mutants clearly have been shown to be chromophore deficient (Parks and Quail, 1991), and their sites of action remain to be determined. Identification of the precise steps blocked by these mutants will contribute greatly to further elucidation of the details of the pathway for PΦB synthesis and may provide confirmation of the enzymatic nature of certain poorly characterized steps. In addition, *hy1*, *hy2*, *hy6*, and *au* are all widely used as phytochrome-deficient controls in genetic, physiological, and biochemical studies (e.g., Neuhaus et al., 1993; Millar et al., 1995), and further knowledge about the sites of action and potential secondary effects of such mutations therefore will assist in interpretation of these data.

We are using pea as an additional model species for the investigation of the genetic basis of phytochrome action, including phytochrome chromophore biosynthesis. This species has a number of advantages over Arabidopsis and tomato for biochemical studies, including the ease of obtaining large amounts of dark-grown tissue. We have recently isolated a number of pea mutations that appear to affect the pathway for chromophore biosynthesis, and in this study we characterize one such mutant, *pcd1* (for *p*hytochrome *c*hromophore *d*eficient). This mutant is very similar in phenotype to the *au* mutant of tomato and the *hy1* mutant of Arabidopsis. Here, we report that the *pcd1* mutant is deficient in phytochrome chromophore synthesis and that this deficiency results from an inability of mutant plants to convert heme to BV IXα.

RESULTS

Isolation of the *pcd1* Mutant

Pea mutant line S2-14 was selected for its yellow-green foliage and elongated internodes relative to parental cultivar Solara plants in an M_2 generation grown in the glasshouse under a

natural photoperiod. In addition to pale foliage, mutants have slightly elongated internodes, show reduced branching, and flower slightly later than wild-type plants grown under the same conditions. The phenotypes of wild-type and S2-14 plants grown in continuous white light (W) are shown in Figure 2. A cross of S2-14 to cultivar Solara yielded an entirely wild-type F_1 and an F_2 segregation of 49 wild-type plants to 15 pale mutants ($X^2_{(3:1)} = 0.083$, $P > 0.7$). A cross of S2-14 to a standard wild-type line, cultivar Torsdag, returned an F_2 segregation of 82 wild-type plants to 28 pale mutants ($X^2_{(3:1)} = 0.012$, $P > 0.9$). Perfect cosegregation of all characteristic aspects of the S2-14 mutant phenotype in close accord with a 3:1 ratio indicated that this pleiotropic phenotype most probably results from a recessive mutation at a single locus, which we have designated *pcd1*.

pcd1 Mutant Shows Altered Photomorphogenesis

Figure 3 shows the responses of *pcd1* to broad-band monochromatic light. The *pcd1* mutant was dramatically elongated under FR, appearing completely insensitive to FR in terms of both elongation and rate of development (Figures 3A and 3B). Because phyA is the predominant phytochrome mediating responses to continuous FR (Reed et al., 1994; van Tuinen et al., 1995a), this result indicates that *pcd1* is strongly deficient in phyA activity. Dark-grown *pcd1* and wild-type plants were the same height (Figure 3B) and showed no other visible difference, indicating that expression of the *pcd1*-conferred phenotype is dependent on light and therefore that the mutation is truly photomorphogenic in nature. A marked reduction in the response of *pcd1* to R was also apparent, although the mutant appeared to retain some sensitivity to R (Figure 3B). This reduction in sensitivity to R is more severe in *pcd1* than in phyB-deficient *lv* mutants (Weller et al., 1995), suggesting that *pcd1* shows strongly reduced activity of phyB and at least one other R-sensing phytochrome. In contrast, the sensitivity of *pcd1* to W and to blue light was only slightly reduced compared with the wild type (Figures 2 and 3).

We also tested the response of *pcd1* to end-of-day (EOD)–FR treatment. Figure 4 shows that wild-type plants responded strongly to EOD-FR treatment, which leads to increased internode elongation throughout the growth of the plant. In contrast, over the very early internodes, *pcd1* seedlings showed a greatly reduced reponse to EOD-FR. However, with increasing age,

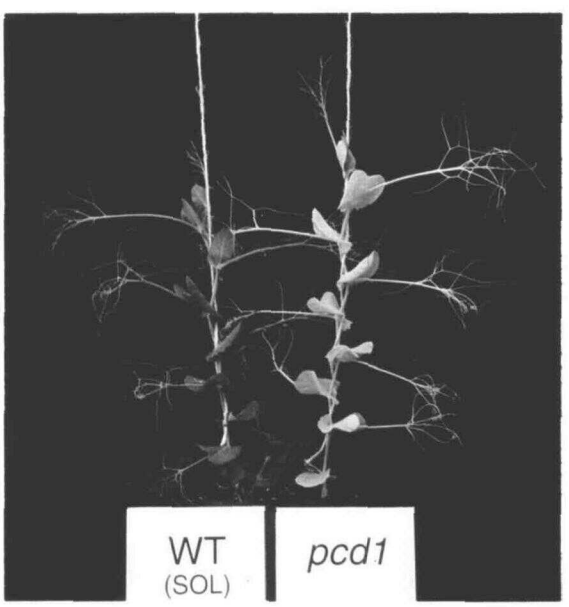

Figure 2. Phenotype of the *pcd1* Mutant.

Plants were grown at 20°C in continuous fluorescent W (150 μmol m^{-2} sec^{-1}) for 30 days. The cultivar Solara (WT [SOL]) background carries Mendel's dwarfing *le* mutation and the *afila* mutation (homeotic conversion of leaflets to tendrils).

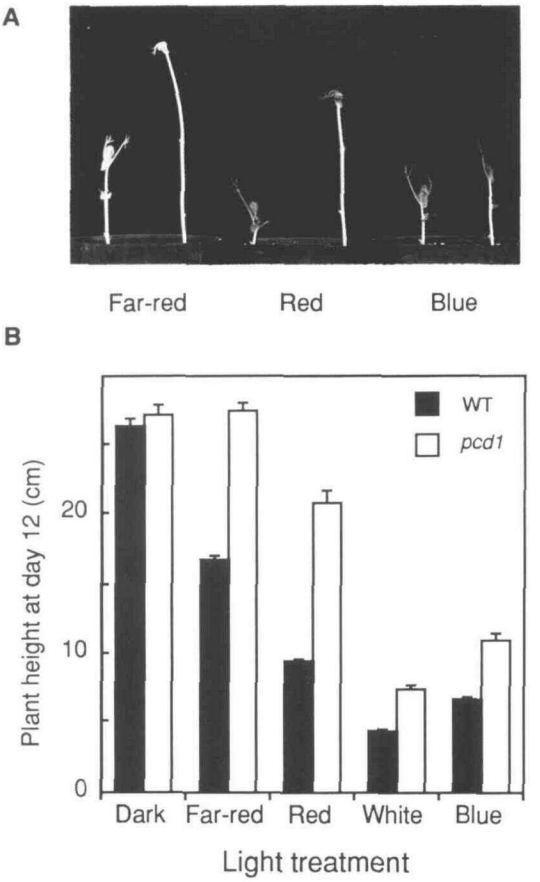

Figure 3. Phenotype of the *pcd1* Mutant in Monochromatic Light.

Seedlings were grown in the dark or under continuous FR (8 μmol m^{-2} sec^{-1}), R (20 μmol m^{-2} sec^{-1}), blue light (10 μmol m^{-2} sec^{-1}), or fluorescent W (150 μmol m^{-2} sec^{-1}) for 12 days after sowing.
(A) Representative wild-type (left of each pair) and *pcd1* seedlings.
(B) Total plant height ($n \approx 12$ to 15). Error bars represent SE. WT, wild type.

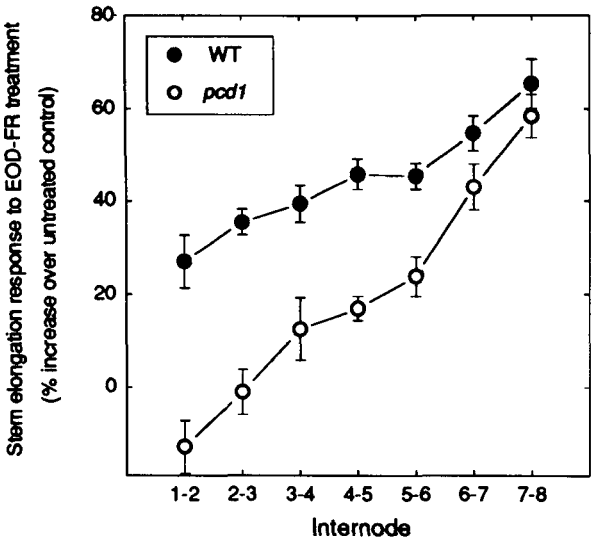

Figure 4. Elongation Response of *pcd1* to EOD-FR Treatment.

Plants were grown for 3 weeks at 20°C in a 12-hr light/dark cycle with or without 20 min of EOD-FR treatment (8 μmol m^{-2} sec^{-1}). The absolute increase in length in response to EOD-FR was 70 ± 4 cm for the wild type (WT) and 48 ± 5 cm for *pcd1* over internodes 1 to 8 (n = 12 to 15). Error bars represent SE.

mutant seedlings gradually recovered the ability to respond, to the extent that newly produced internodes in 3-week-old *pcd1* plants showed the same relative response as those of wild-type plants. It has been shown that phyB is the principal phytochrome controlling the elongation response to EOD-FR, because phyB-deficient mutants of Arabidopsis (Nagatani et al., 1991), Brassica (Devlin et al., 1992), cucumber (López-Juez et al., 1992), and pea (Nagatani et al., 1990) all lack this response. The response of the *pcd1* mutant is different from that of phyB-deficient *lv* mutants, which show no such recovery of EOD-FR response with age (Nagatani et al., 1990). This result shows that *pcd1* is strongly deficient in phyB activity at the seedling stage but recovers phyB activity as the plant matures.

Mature W-grown *pcd1* plants have a yellow-green phenotype (Figure 2), and we noticed that this varied considerably, depending on the conditions under which the plants were grown. We grew *pcd1* plants under a number of different W regimens and found that under all conditions, *pcd1* caused a reduction in Chl content and a substantial increase in the Chl *a*/Chl *b* ratio. This was most pronounced in short photoperiods and least pronounced in plants grown in continuous light. Representative effects of the *pcd1* mutation on foliar Chl levels are shown in Table 1.

These aspects of the *pcd1*-conferred phenotype are also common to photomorphogenic mutants of Arabidopsis (*hy1*, *hy2*, and *hy6*) and tomato (*au* and *yg-2*). All of these mutants are severely deficient in spectrophotometrically detectable phytochrome (Koornneef et al., 1980, 1985; Chory et al., 1989). We therefore examined the phytochrome content of *pcd1* in more detail.

Spectrophotometric and Immunochemical Analysis of Phytochrome in the *pcd1* Mutant

Figure 5A shows representative difference spectra for in vivo phytochrome phototransformation in standard samples of etiolated wild-type and *pcd1* tissue. Although samples from wild-type plants contained phytochrome routinely giving a signal of 40 to 50 units (one unit is 10^{-3} $\Delta\Delta A_{660-730\,nm}$), the signal in *pcd1* samples was below the detection limit of the spectrophotometer, which was ~0.3 units. Etiolated *pcd1* plants therefore contain <1% of the spectrophotometrically detectable phytochrome present in the wild type.

We also examined the phytochrome apoprotein content of the *pcd1* mutant. Immunoblot analysis of crude protein extracts showed that etiolated *pcd1* plants have normal levels of the PHYA apoprotein (Figure 5B). In addition, the depletion of PHYA apoprotein seen in response to irradiation of wild-type seedlings with 4 hr of R was not observed for the *pcd1* mutant (Figure 5B). As the depletion of PHYA after R is dependent on conversion to Pfr, this suggests that PHYA in the *pcd1* mutant does not undergo photoconversion. Both of these results are consistent with the suggestion that phytochrome in *pcd1* plants lacks a chromophore.

Table 1. Chl Content and Chl *a*/Chl *b* Ratio of the *pcd1* Mutant

Photoperiod (hr)	Genotype	Chl Content (mg g fresh weight^{-1})[a]			Chl *a*/*b*
		Chl *a*	Chl *b*	Total Chl	
24	WT[b]	1.67 ± 0.12	0.52 ± 0.04	2.19 ± 0.16	3.2
	pcd1	1.41 ± 0.16	0.17 ± 0.25	1.58 ± 0.18	8.4
8	WT	1.50 ± 0.12	0.38 ± 0.03	1.88 ± 0.14	3.9
	pcd1	0.25 ± 0.01	0.03 ± 0.01	0.28 ± 0.01	12.0

[a] Values are expressed as the mean ± SE of six samples. Tissue samples were taken from stipules because the wild type has no true leaflets.
[b] Wild type.

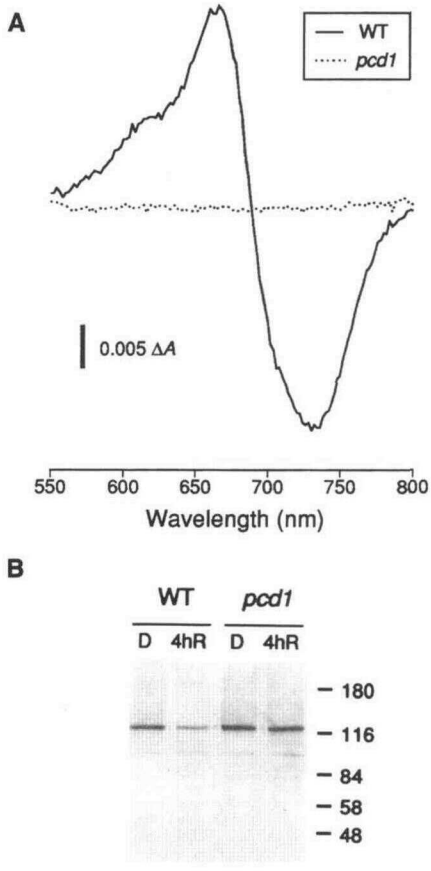

Figure 5. Phytochrome Content of *pcd1* Seedlings.

(A) Difference spectrum for in vivo phytochrome phototransformation (Pfr-Pr) in etiolated wild-type (WT) and *pcd1* seedlings.
(B) PHYA apoprotein content in crude protein extracts from wild-type and *pcd1* seedlings grown in complete darkness (D) or given a 4-hr R treatment (4hR; 17 μmol m^{-2} sec^{-1}) before harvest. The positions and molecular masses (kilodaltons) of prestained markers (Sigma) are indicated. Lanes were loaded on an equivalent fresh weight basis. The PHYA apoprotein was detected using an anti–pea phyA monoclonal antibody mAP5 (Nagatani et al., 1984).

pcd1 Mutant Is Deficient in the Phytochrome Chromophore

One possible explanation for the deficiency in phytochrome spectral activity in *pcd1* is that the apophytochrome has undergone a modification that prevents assembly in vivo. To address this, we attempted to assemble apophytochrome extracted from etiolated *pcd1* seedlings. We partially purified the phytochrome apoprotein from *pcd1* plants in the presence of PCB. PCB is a structural analog of the native phytochrome chromophore PΦB and derives from the light-harvesting chromoprotein C-phycocyanin. Previous reports have demonstrated that PCB can substitute for PΦB in the assembly of a spectrally and biologically active phytochrome holoprotein (Elich and Lagarias, 1989; Parks and Quail, 1991) and that this assembly is an autocatalytic process, requiring only apophytochrome and PCB (Lagarias and Lagarias, 1989).

Figure 6 shows that the apophytochrome in *pcd1* extracts can assemble with PCB to give a spectrally active phytochrome, when PCB is included in the extraction buffer. The difference spectrum for this holophytochrome has absorption peaks in the R (652 nm) and FR (716 nm) that are blue shifted by 16 and 14 nm, respectively, relative to the corresponding peaks for phytochrome in wild-type samples. These are very close to the peak values previously reported for a pea apophytochrome A–PCB adduct (Deforce et al., 1993). We typically recovered 65 to 80% of wild-type spectral activity in *pcd1* extracts incubated with PCB, based on equivalent tissue fresh weight. Apophytochrome that was extracted from *pcd1* tissue in the absence of PCB, but subsequently assembled with PCB added to the partially purified extract, resulted in a holoprotein with spectral characteristics identical to those shown in Figure 6 (data not shown). However, yields were considerably lower (~25%), indicating that the apoprotein was not stable during the extraction procedure. These results clearly show that the loss of spectral activity of the phytochrome in *pcd1* is due to a deficiency of the endogenous chromophore rather than incompetence of the apoprotein to bind chromophore.

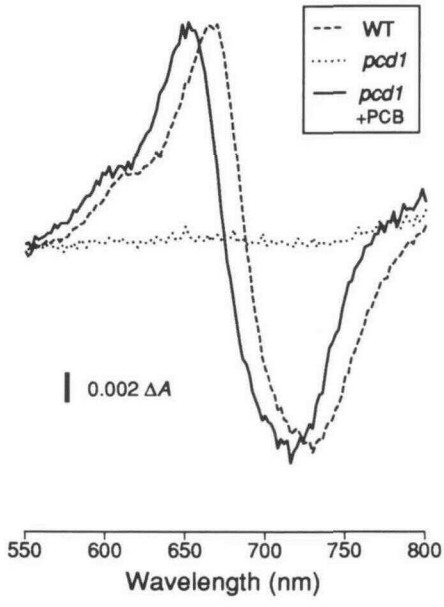

Figure 6. In Vitro Assembly of the *pcd1* Phytochrome Apoprotein with PCB.

Difference spectra for phytochrome (Pfr-Pr) in extracts from wild-type (WT), *pcd1*, and *pcd1* tissue extracted in the presence of 3 μM PCB (*pcd1*+PCB) are shown. The wild-type spectrum has been scaled down for ease of comparison, and the scale bar applies to *pcd1* and *pcd1*+PCB spectra only.

BV Restores Phytochrome Spectral Activity to *pcd1* in Vivo

We then examined the possibility that the *pcd1* mutation might lead in some other way to the failure or prevention of assembly in vivo. To test this hypothesis, we attempted to recover holophytochrome in vivo by feeding chromophore precursors to intact seedlings. It has been shown previously that both exogenously supplied PCB and BV can restore a wild-type level of spectrally active phytochrome and a wild-type light-grown phenotype in the chromophore-deficient mutant *hy1* of Arabidopsis (Parks and Quail, 1991). We tested whether the *pcd1* mutant might be rescued in a similar manner by supplying these chromophore precursors to germinating *pcd1* seed. Although we tried several methods of supplying PCB and BV to germinating seedlings, no restoration of light-induced inhibition of stem elongation or phytochrome spectral activity was seen (data not shown). However, because the ineffectiveness of these compounds could have resulted from problems with uptake from the growing medium, we attempted an alternative method of floating excised tissue segments in a solution of BV (Elich and Lagarias, 1987; Elich et al., 1989). Because pea shoot tissue treated in this way retains substantial amounts of BV that cannot be washed out, it was impossible to obtain valid in vivo measurements of phytochrome. We therefore partially purified phytochrome from BV-treated explants and measured spectral activity in vitro. PCB was not used because the possibility of assembly during the extraction procedure could not be excluded.

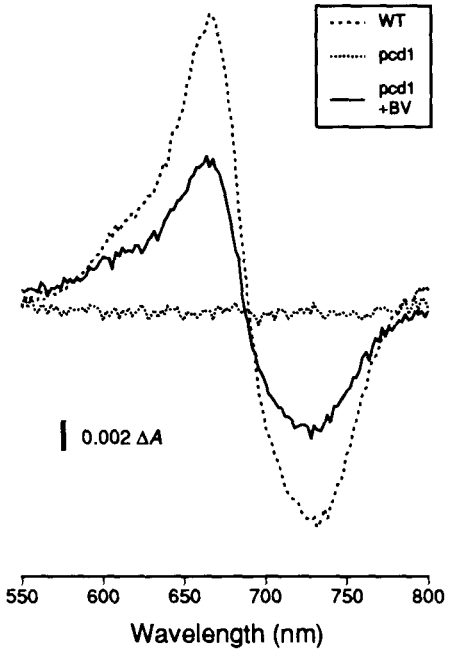

Figure 7. Recovery of Phytochrome Spectral Activity in *pcd1* Extracts Incubated with BV.

Difference spectra (Pfr-Pr) for phytochrome in extracts from wild-type (WT), *pcd1*, and BV-incubated *pcd1* tissue (*pcd1*+BV) are shown. Apical explant sections were floated in 300 μM BV or buffer alone for 6 hr at room temperature. The wild-type spectrum has been scaled down for ease of comparison. The scale bar applies to *pcd1* and *pcd1*+BV spectra only.

The difference spectrum of the newly synthesized holophytochrome is shown in Figure 7. This spectrum shows no substantial deviation from the spectrum of phytochrome extracted from wild-type tissue either in shape or in wavelength of the ΔA peaks (666 and 732 nm for both samples). Feeding of the natural BV isomer, BV IXα, gave an identical result (data not shown). The amount of holoprotein in *pcd1* after BV incubation was ~27% of that found in wild-type explants (based on equivalent tissue fresh weight); this recovery compares favorably with the value reported for gabaculine-treated oat coleoptiles (34%), using a higher concentration of BV (500 μM; Elich and Lagarias, 1987).

Because BV itself does not assemble in vitro with apophytochrome to give a spectrally active holoprotein (Li and Lagarias, 1992), the recovery of spectral activity in *pcd1* indicates that BV has been converted to PΦB and that assembly of holophytochrome has occurred in vivo. This result therefore shows that apophytochrome synthesis and assembly are normal in *pcd1* and that the mutant is deficient in phytochrome chromophore synthesis. In addition, the recovery of signal obtained with BV suggests that the block in chromophore synthesis imposed by *pcd1* occurs most probably before BV.

pcd1 Mutant Is Not Deficient in Heme

As shown in Figure 1, the immediate precursor of BV IXα in PΦB synthesis is thought to be heme. Because the *pcd1* mutation is sufficiently severe to prevent detectable PΦB synthesis, a block in the pathway before heme also would be expected to result in reduced heme levels. Previous attempts to recover phytochrome spectral activity from chromophore-depleted oat explants incubated with heme were reported to be unsuccessful (Elich et al., 1989); therefore, we did not attempt a similar experiment. Instead, to address whether the *pcd1* mutation might have its effect before heme formation, we quantitated total noncovalently bound heme (as an estimate of total cellular heme) in wild-type and *pcd1* seedlings. As shown in Table 2, we found no significant difference in the level of noncovalently bound heme in wild-type and *pcd1* seedlings. Hemes are also a component of all cytochromes and therefore are essential to the basic cellular functions of photosynthesis and respiration. Heme deficiency therefore might be expected to seriously compromise the general vigor of the plant. However, consistent with the heme quantitation data, *pcd1* plants are generally healthy plants and are not noticeably less vigorous than wild-type plants (see also Figure 2). These results, when taken together with the fact that *pcd1* plants are able to utilize BV (Figure 7), provide indirect evidence that the *pcd1* mutation may prevent the conversion of heme to BV IXα.

Table 2. Quantitation of Noncovalently Bound Heme in *pcd1* Seedlings

Genotype	Heme[a] nmol (g fresh weight)$^{-1}$	pmol (seedling)$^{-1}$
WT[b]	0.56 ± 0.15	67.2 ± 16.0
pcd1	0.55 ± 0.12	70.8 ± 14.3

[a] Values are expressed as mean ± SE of four replicate measurements.
[b] Wild type.

pcd1 Mutant Is Unable to Convert Heme to BV IXα

To address specifically the question of whether the *pcd1* mutant is unable to convert heme to BV IXα and PΦB, we used a recently developed assay system for the detection of PΦB (Terry et al., 1995). Isolated etioplasts were incubated with heme in the presence of an NADPH regenerating system, and the products were analyzed by reverse phase HPLC. Figure 8 shows that wild-type plastids converted heme to a major product with a retention time of ~11 min (trace C). The retention time of this peak is consistent with its identification as either BV IXα or 3(*Z*)-PΦB, which coelute in this solvent system (Terry et al., 1995).

To identify this peak unequivocally, we reexamined the products using a modified HPLC solvent system that can resolve BV IXα from 3(*Z*)-PΦB (Terry et al., 1995). This HPLC trace is shown in Figure 9A. Under these conditions, the original single peak resolved into two (Figure 9A, labeled *a* and *b*). Coinjection studies with authentic BV IXα and 3(*Z*)-PΦB samples provisonally identified peaks *a* and *b* (Figure 9A) as 3(*Z*)-PΦB and BV IXα, respectively (data not shown). The low yield of peak *b* precluded more rigorous identification, but peak *a* was purified and analyzed further.

Figure 9B shows an absorption spectrum of peak *a*, with BV IXα also shown for comparison. The spectrum of peak *a* is red shifted in comparison with BV IXα, consistent with its identification as 3(*Z*)-PΦB (Terry et al., 1995). For final verification of the identity of peak *a*, we attempted an assembly reaction with partially purified apophytochrome derived from the *pcd1* mutant. It has been demonstrated previously that 3(*Z*)-PΦB will assemble with apophytochrome, although it is not clear whether isomerization to the 3(*E*)-isomer is required for the assembly to proceed (Terry et al., 1995). As shown in Figure 9C, assembly of peak *a* with pea apophytochrome resulted in a holophytochrome with a difference spectrum that is almost identical to the wild-type pea phytochrome under these conditions (cf. Figures 6 and 7). This result confirms that the major product from the incubation of wild-type plastids with heme is 3(*Z*)-PΦB.

Incubation of wild-type plastids with heme also resulted in the synthesis of a product with a retention time of ~19 min (Figure 8, trace C). This was identified as 3(*E*)-PΦB by coinjection with the authentic standard (data not shown). Because the major product was 3(*Z*)-PΦB, the additional synthesis of 3(*E*)-PΦB would be expected (Terry et al., 1995). Incubation of wild-type plastids in the absence of heme also resulted in the synthesis of 3(*Z*)-PΦB, though the yield was greatly reduced (Figure 8, trace A). This may reflect synthesis from endogenous chromophore precursors. Incubations of heme without plastids resulted in some coupled oxidation of the heme, leading to the appearance of a number of small peaks, one of which was identified as BV IXα (data not shown). When plastids isolated from *pcd1* seedlings were incubated with heme, the 3(*Z*)-PΦB peak was almost completely absent, and no 3(*E*)-PΦB was detected (Figure 8, trace D). No peaks were discernible from incubations of *pcd1* plastids in the absence of heme (trace B). These results clearly demonstrate that isolated *pcd1* plastids are unable to convert heme to 3(*Z*)-PΦB.

To confirm that *pcd1* can synthesize PΦB from BV IXα and that isolated plastids from *pcd1* are functionally active, we also examined these steps in the same plastid preparations. The metabolism of BV IXα to PΦB has previously been well characterized using oat etioplasts (Terry et al., 1995). As shown in

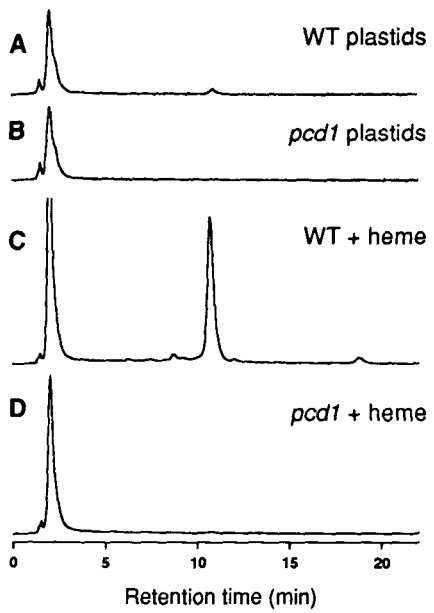

Figure 8. HPLC Analysis of Heme Metabolism in Wild-Type and *pcd1* Plastids.

(A) to **(D)** Plastids from etiolated seedlings of wild type (WT; traces **[A]** and **[C]**) and *pcd1* (**[B]** and **[D]**) were incubated in the presence (**[C]** and **[D]**) or absence (**[A]** and **[B]**) of 10 μM heme. Incubations were for 3 hr at 28°C and included an NADPH regenerating system. The final protein concentrations were 1.17 mg/mL for the wild type and 1.16 mg/mL for *pcd1*. The products were analyzed by reverse phase HPLC using a solvent system of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]), and absorbance was monitored at 380 nm. The absorbance scale for the samples containing plastids only (**[A]** and **[B]**) is half that of the samples from incubations with heme (**[C]** and **[D]**).

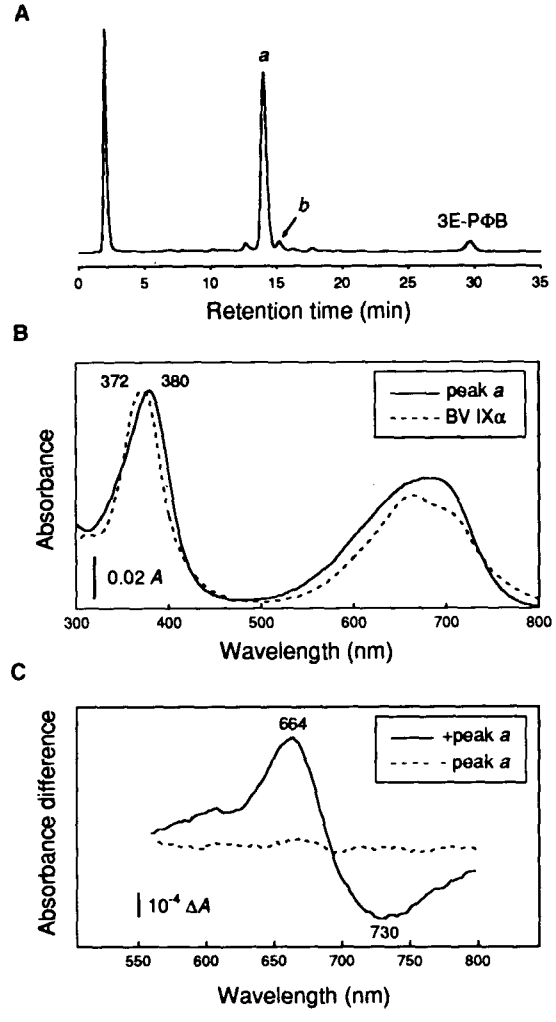

Figure 9. Analysis of the Major Product from the Incubation of Heme with Wild-Type Plastids.

(A) Reverse phase HPLC analysis of the products of incubating 10 μM heme with wild-type etioplasts. Incubations were for 3 hr at 28°C and also included an NADPH regenerating system. The final protein concentration was 0.84 mg/mL. The solvent system used was ethanol–acetone–100 mM formic acid (25:65:10 [v/v]), and absorbance was monitored at 380 nm. The identity of 3(*E*)-PΦB was confirmed by coinjection studies. Peak *a* was collected from two identical injections, one of which is shown.

(B) Absorbance spectra of peak *a* and BV IXα in acetonitrile–0.1% TFA (60:40 [v/v]).

(C) Phytochrome difference spectrum (Pfr-Pr) for apophytochrome assembled with peak *a* (+peak *a*). The phytochrome apoprotein was purified partially from *pcd1* tissue (10 g fresh weight), and the contents of peak *a* were added to the clarified extract to give a final bilin concentration of 0.4 μM (calculated using the absorption coefficient of 3(*Z*)-PΦB at 382 nm). Spectra were recorded after incubation of extracts on ice for 30 min and have been smoothed mathematically. The control extract (−peak *a*) was obtained from 2.5 g fresh weight.

Figure 10, both wild-type and *pcd1* plastids converted BV IXα to 3(*Z*)- and 3(*E*)-PΦB, the products confirmed by coinjection studies and absorption spectroscopy (data not shown). This result thus confirms that the conversion of BV IXα to 3(*Z*)- and 3(*E*)-PΦB is normal in *pcd1* and that this mutant is deficient specifically in the ability to synthesize BV IXα from heme.

DISCUSSION

pcd1 Is Unable to Convert Heme to BV IXα

Heme has been proposed to be the precursor of BV IXα in the biosynthesis of the phytochrome chromophore. The proposal is based on analogy with the pathway for phycobilin biosynthesis in the red alga *Cyanidium caldarium* (Beale, 1993; Terry et al., 1993b) and is supported by two pieces of experimental evidence. Isolated cucumber etioplasts can use heme, but not Mg-protoporphyrin, to synthesize PΦB detected by assembly to apophytochrome (Terry et al., 1993b). Second, application of an inhibitor of ferrochelatase, which is required for heme synthesis (see Figure 1), results in a reduction in spectrophotometrically detectable phytochrome in embryonic axes of pea (Konomi et al., 1993). In our assays, the major product after the incubation of heme with wild-type plastids was 3(*Z*)-PΦB, whereas BV IXα was present only in trace amounts (Figures 8 and 9). Plastids from *pcd1* seedlings were unable to synthesize 3(*Z*)-PΦB from heme (Figure 8), although the same plastid preparation could convert BV IXα to 3(*Z*)-PΦB

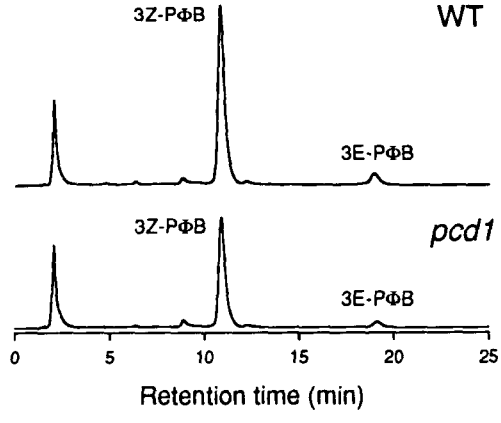

Figure 10. HPLC Analysis of BV IXα Metabolism by Wild-Type and *pcd1* Plastids.

Plastids from etiolated seedlings of the wild type (WT) and *pcd1* were incubated with 8 μM BV IXα. Incubations were for 3 hr at 28°C and included an NADPH regenerating system. The final protein concentrations were 1.17 mg/mL for the wild type and 1.16 mg/mL for *pcd1*. The products were analyzed by reverse phase HPLC, using a solvent system of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]), and absorbance was monitored at 380 nm. The identities of 3(*Z*)- and 3(*E*)-PΦB were confirmed by coinjection studies.

(Figure 10). Together with the demonstrations that *pcd1* seedlings are not heme deficient (Table 2) and that BV can restore holophytochrome to *pcd1* in vivo (Figure 7), these results clearly demonstrate that *pcd1* is unable to convert heme to BV IXα. These experiments therefore confirm the proposal that heme is an intermediate in the synthesis of the phytochrome chromophore.

We consider it probable that the *pcd1* mutation lies in the catalytic subunit of the enzyme accomplishing the conversion of heme to BV IXα. Unfortunately, there are no molecular probes available for this enzyme, and it was not possible to test this hypothesis directly. The enzymatic conversion of heme to BV IXα in higher plants is thought to be accomplished by the enzyme heme oxygenase, as it is in mammals and red algae (Cornejo and Beale, 1988; Maines, 1988; Beale, 1993). This activity has not been measured previously in higher plants, in part due to the problem of distinguishing facile, nonenzymatic-coupled oxidation of heme from the enzyme-catalyzed reaction (Terry et al., 1993b). The absence of this reaction in *pcd1* provides direct evidence for this step being enzyme catalyzed in higher plants. However, because the reaction proceeds to 3(*Z*)-PΦB, no further characterization has been performed, and the nature of the reaction from heme to BV IXα remains unknown. The reason that 3(*Z*)-PΦB, and not BV IXα, is the major product after the incubation of heme with isolated plastids is also unknown. However, the formation of BV IXα does not require PΦB synthase activity because, in mutants unable to synthesize 3(*Z*)-PΦB from BV IXα, incubation of heme results in BV IXα accumulation (M.J. Terry, J.L. Weller, J.B. Reid, and R.E. Kendrick, unpublished data).

Phenotype of the *pcd1* Mutant

Most of the observed aspects of the *pcd1*-conferred phenotype may be explained readily in terms of the deficiency in active phytochrome that results from the demonstrated block in PΦB synthesis. The mutant is clearly lacking in both phyA and phyB responses at the seedling stage and is at least partially deficient in a response to R, which is retained in phyB-deficient *lv* mutants (Weller et al., 1995). This implies that the PΦB deficiency results in a reduction in the level of activity of at least two and possibly all phytochromes in pea. One notable aspect of the *pcd1*-conferred phenotype is the gradual recovery of the phyB-mediated EOD-FR response. This suggests that more active phyB is present in the mature tissues of *pcd1* plants and thus implies that more PΦB has been synthesized. This might result from slight leakiness of *pcd1* or might reflect the presence of a *PCD1* homolog, which is expressed at higher levels in mature tissue. The location of the *pcd1* lesion after the branch point for Chl and PΦB synthesis shows that the pale phenotype of the mutant does not result from a direct impairment in Chl synthesis. However, phytochrome has well-documented effects on plastid development and on the synthesis of Chl (Kasemir, 1983) and Chl *a*/*b* binding proteins (Batschauer et al., 1994), and it is possible that one or more of these effects account for the pale phenotype. In addition, negative feedback effects of free heme on aminolevulinic acid synthesis have been proposed (Chereskin and Castelfranco, 1982). Although there was no difference in noncovalently bound heme (Table 2), it is possible that an accumulation of free heme in the *pcd1* mutant might occur, and this could also contribute to the chlorotic phenotype.

Relationship of *pcd1* to Other Mutants

Mutants similar in phenotype to *pcd1* have been identified in several other species. Mutants *au* and *yg-2* of tomato, *hy1*, *hy2*, and *hy6* of Arabidopsis, and *pew1* and *pew2* of *Nicotiana plumbaginifolia* are all pale, are elongated in FR and R, and have reduced levels of spectrally active phytochrome (Koornneef et al., 1980, 1985; Chory et al., 1989; Kraepiel et al., 1994). These mutants are all considered to be chromophore-deficient mutants, although this has been conclusively proven only for the *hy* mutants (Parks and Quail, 1991; Nagatani et al., 1993).

Although pale, the *au* and *yg-2* mutants are relatively healthy and vigorous, suggesting that these plants, like *pcd1*, are not deficient in heme (Kendrick et al., 1994). The lesions in *au* and *yg-2* are therefore also likely to affect steps after the formation of heme. Again, although the *hy1*, *hy2*, and *hy6* mutants have reduced Chl levels (Chory et al., 1989) and would be expected to have impaired photosynthetic ability, all three mutants are generally healthy. These mutants are therefore also unlikely to be deficient in heme, suggesting that they may be blocked after heme formation as well. Recent preliminary evidence from heme and BV IXα metabolism studies in *au* and *yg-2* has confirmed that both mutants are in fact blocked in PΦB synthesis after this point (M.J. Terry and R.E. Kendrick, unpublished data). The *hy1* mutant is clearly rescued by BV (Parks and Quail, 1991); therefore, it seems probable that this mutation may affect the same step as that altered by *pcd1*—preventing the conversion of heme to BV IXα. By the same reasoning, *hy2* has also been suggested to lie before BV (Parks and Quail, 1991). However, the incomplete rescue of *hy2* on BV (Parks and Quail, 1991) may indicate that the mutation is in fact after BV IXα. Such a mutation, if leaky, might still allow synthesis of significant amounts of PΦB in the presence of BV at a sufficiently high concentration. However, further investigation of the pathway for PΦB synthesis in *hy1* and *hy2* clearly is necessary to address these questions.

The results presented in this study identify the biochemical basis of the *pcd1*-conferred phenotype. They demonstrate conclusively that the phenotype results from impairment of a specific, committed step in phytochrome chromophore biosynthesis. Furthermore, they establish that heme is an intermediate in the synthesis of the phytochrome chromophore. The feasibility of addressing this kind of biochemical problem in pea highlights an advantage of this species for a mutant-based approach to understanding the function of phytochrome in higher plants. The present study should assist greatly in the investigation of similar mutants in other species less amenable to

biochemical investigation and should lead ultimately to a better understanding of phytochrome chromophore synthesis and its role in plant photomorphogenesis.

METHODS

Plant Material and Growth Conditions

The original *pcd1* (for *p*hytochrome *c*hromophore *d*eficient) mutant line S2-14 was derived from the pea (*Pisum sativum*) cultivar Solara at INRA Station de Genetique et d'Amelioration des Plantes (Versailles, France). Imbibed seeds of cultivar Solara were mutagenized by soaking in 4 mM ethyl methanesulfonate (EMS) for 4 hr at room temperature. The S2-14 mutant was identified in an M_2 population grown in a glasshouse under natural daylength conditions. The mutant line was bred by single plant selection for three generations before backcrossing to cultivar Solara. All *pcd1* material in this study derived from bulked *pcd1* segregates in the F_4 and F_5 generations of this cross.

For subsequent experiments, all seeds were sown either in a 1:1 mix of dolerite chips and vermiculite topped with potting soil (genetics and physiological experiments) or in vermiculite alone (biochemical experiments). Plants for all genetic experiments were grown in a heated glasshouse under natural daylight extended to 18 hr with light from a mixed fluorescent/incandescent source. The initial screen under monochromatic light and plants for chlorophyll (Chl) extractions were grown in growth cabinets at 20°C, using light sources as described by Weller et al. (1995). Light sources for the end-of-day far-red light (EOD-FR) experiment were identical to those described by Weller and Reid (1993). All other plants were grown in growth cabinets at 25°C. The red light (R) and FR sources used in other experiments and the green safety light used for manipulation of etiolated plants and during all biochemical experiments have been described by Nagatani et al. (1993). The fluence rates and spectra for all light sources were measured using a LiCor spectroradiometer (LiCor Corp., Lincoln, NE).

Protein Extraction and Immunoblotting

Crude protein extracts were prepared from dark-grown or R-treated seedlings and subjected to PAGE as described previously by Weller et al. (1995). The PHYA apoprotein was detected immunochemically (Weller et al., 1995), using the anti-pea phyA monoclonal antibody mAP5 (Nagatani et al., 1984). Protein was quantitated by the method of Bradford (1976), using Bio-Rad protein assay reagent and BSA as a standard.

Reagent Preparation

Biliverdin (BV) IXα was obtained from Porphyrin Products, Inc. (Logan, UT) and further purified by C18 reverse phase HPLC using a Shimadzu (Tokyo, Japan) LC10AS liquid chromatograph system and a Supelcosil LC-18 ODS column (4.6 × 25 cm; 5-mm particle diameter; Supelco Inc., Bellefonte, PA). The mobile phase consisted of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]; mobile phase A) with a flow rate of 1.5 mL/min, and the column eluate was monitored at 380 nm. HPLC-purified BV IXα was concentrated by diluting four times with 0.1% (v/v) trifluoro-acetic acid (TFA) and applying to a C18 Bond Elut column (1 mL; Varian, Harbor City, CA). After washing with 0.1% TFA, BV IXα was eluted with acetonitrile/0.1% TFA (3:2 [v/v]) and dried in vacuo. For BV feeding experiments to apical segments, BV (a mixture of isomers) was obtained from Sigma.

Phycocyanobilin (PCB) was purified from *Spirulina platensis* as described previously by Terry et al. (1993a). The sample contained >90% 3(*E*)-PCB when analyzed by HPLC. 3(*E*)-phytochromobilin (3[*E*]-PΦB) was a gift from J.C. Lagarias (University of California, Davis, CA). 3(*Z*)-PΦB was purified by HPLC after conversion of BV IXα to PΦB by isolated pea etioplasts (see below; Terry et al., 1995). HPLC conditions were identical to those for BV IXα purification, except that the mobile phase was changed to ethanol–acetone–100 mM formic acid (25:65:10 [v/v]; mobile phase B) to increase the resolution between BV IXα and 3(*Z*)-PΦB (Terry et al., 1995). All bilins were prepared as 1 mM stock solutions in DMSO, using the following molar absorption coefficients: 66,200 M cm^{-1} at 377 nm for BV IXα (McDonagh and Palma, 1980), 47,900 M cm^{-1} at 374 nm for 3(*E*)-PCB (Cole et al., 1967), and 64,570 M cm^{-1} at 386 nm and 38,020 M cm^{-1} at 382 nm for 3(*E*)-PΦB and 3(*Z*)-PΦB, respectively (Weller and Gossauer, 1980). Absorption spectrophotometry of bilin and heme samples was performed using a spectrophotometer (model U-3410; Hitachi, Tokyo, Japan).

A stock solution of heme was prepared by dissolving hemin chloride (Sigma) in 0.1 M NaOH and adjusting to pH 7.7 with 1 N HCl. The final heme concentration was 1 mM.

Unless otherwise stated, all chemicals and reagents were purchased from Sigma, Kanto Chemical Co. (Tokyo, Japan), and Nakalai Tesque (Kyoto, Japan).

Phytochrome Assembly in Vivo

For the experiments involving feeding of BV to floating shoot tips, 40 to 50 apical segments (2 cm long) from 5-day-old seedlings (~3 g fresh weight) were harvested in green safe-light under buffer (15 mM Hepes, NaOH, pH 7.4) and transferred to Petri dishes containing 300 μM of BV IXα in the same buffer. The apical segments were then cut into smaller segments, 3 to 4 mm in length, and floated with gentle shaking for 6 hr in darkness. After floating, the tissue was rinsed several times, blotted dry, weighed, frozen in liquid nitrogen, and stored at −80°C until phytochrome extraction.

Phytochrome Extraction and Assembly in Vitro

For each extraction, 40 to 50 apical segments (2 cm long) from 5-day-old etiolated seedlings (~3 g fresh weight) were harvested and homogenized in liquid nitrogen. The homogenate was suspended in a 3:1 (v/w) volume of extraction buffer (50 mM Tris, 100 mM ammonium sulfate, 25% [v/v] ethylene glycol, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM diethyldithiocarbamate, 142 mM β-mercaptoethanol, 4 mM cysteine, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, adjusted with HCl to pH 8.3 at 5°C) and extracted with gentle stirring for 30 min at 4°C in the presence or absence of 3 μM PCB. After centrifugation for 30 min at 200,000*g*, saturated ammonium sulfate (0.725:1 [v/v]) was added to the supernatant, and the extract was gently stirred for an additional 30 min. The ammonium sulfate pellet was collected by centrifugation (30 min at 30,000*g*) and resuspended in 1 mL of TEGE buffer (25 mM Tris, 2 mM EDTA, 25% ethylene glycol [v/v], 2 mM PMSF, 1 mM DTT, 2 μg/mL leupeptin, adjusted with HCl to pH 7.8 at 5°C). The extract was then clarified by centrifugation (15 min at 200,000*g*) before spectrophotometric assay. Extracts were maintained at or below 4°C throughout the extraction. For assembly experiments with extracted apophyto-

chrome samples, the bilins were added to the clarified extract and incubated on ice for 30 min.

Spectrophotometric Assay for Phytochrome

All spectrophotometric determinations were made using a recording difference spectrophotometer (model 3410; Hitachi). Standard samples for in vivo spectrophotometric phytochrome determinations were prepared as described previously by Weller et al. (1995). All samples and extracts were kept at 4°C during measurements. Actinic beams for phytochrome photoconversion were obtained by filtering light from a xenon lamp (150 W; Ushio Inc., Tokyo, Japan) through a 658- or a 748-nm interference filter (Vacuum Optics Corp., Tokyo, Japan). Phytochrome was photoconverted by exposure to 60 sec of R and 90 sec of FR (in vivo determinations) or 180 sec of R and 300 sec of FR (in vitro), irradiations sufficient to induce essentially full photoconversion, as determined by kinetic measurements.

Assays for PΦB Synthesis

PΦB synthesis from heme and BV IXα was assayed in isolated pea etioplasts essentially as described previously for PΦB synthase assays in oat etioplasts (Terry et al., 1995). Etioplasts were isolated by differential centrifugation from 8-day-old dark-grown seedlings as described by Terry and Lagarias (1991) with the following modifications. PVP (0.5% [w/v]) was added to the homogenization medium, and the final crude plastid pellet was washed once with assay buffer stock (20 mM Tes, 10 mM Hepes, NaOH, pH 7.7, containing 500 mM sorbitol) before use.

PΦB synthesis assays were performed in 20 mM Tes, 10 mM Hepes, NaOH buffer, pH 7.7, containing 500 mM sorbitol, 1 mM PMSF, 0.5 mM DTT, 2 μM leupeptin, 3000 units/mL catalase, and an NADPH regenerating system (1.2 mM $NADP^+$, 10 mM glucose 6-phosphate, 1.5 units/mL glucose 6-phosphate dehydrogenase). The reaction was initiated by the addition of either heme (10 μM final concentration) or BV IXα (8 μM). For the assay with BV IXα, an argon treatment was used to deplete oxygen. The reaction volume was 0.5 mL, and reaction mixtures were incubated in the dark at 28°C with shaking. After a 3-hr incubation, samples were purified partially using a Bond Elut column (see above) as previously described by Terry et al. (1995). The elution volume was 1 mL. HPLC analysis of bilins was performed as described above using mobile phase A (Figures 8 and 10) or B (Figure 9).

Tetrapyrrole Quantitation

Heme Quantitation

Total noncovalently bound heme was extracted from the top 2 to 3 cm of 8-day-old dark-grown seedlings, essentially as described by Thomas and Weinstein (1990). Harvested tissue (5 g fresh weight) was homogenized in 20 mL of cold 90% (v/v) acetone containing 10 mM NH_4OH. After centrifugation at 4000*g* for 2 min, the pellet was washed twice more in the same volume. Noncovalently bound hemes were extracted twice with 10 mL of cold 80% (v/v) acetone containing 5% (v/v) HCl. The heme extracts were pooled, transferred to chloroform–butanol (2:1 [v/v]), and washed twice with water. The heme was then concentrated by application to a DEAE–Sepharose column (Bond Elut DEA, 3 mL; Varian) with an equal volume of 95% (v/v) ethanol. After washes with chloroform–butanol (2:1) and 95% ethanol, the heme was eluted in 2 mL of ethanol–acetic acid–water (70:17:13 [v/v]). The concentration was determined by absorption spectroscopy (see above), using the molar absorption coefficient of 144,000 M cm^{-1} at 398 nm calculated for air-oxidized protoheme in this solvent (Weinstein and Beale, 1983).

Chl Quantitation

Two 6-mm discs were punched from the sixth pair of stipules of 4-week-old plants and extracted in 2 mL of dimethylformamide for 24 hr at 4°C. Chl levels in the extracts were determined spectrophotometrically according to Innskeep and Bloom (1985).

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