

# Etioplast Differentiation in Arabidopsis: Both PORA and PORB Restore the Prolamellar Body and Photoactive Protochlorophyllide–F655 to the *cop1* Photomorphogenic Mutant

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The etioplast plastid type of dark-grown angiosperms is defined by the accumulation of the chlorophyll (Chl) precursor protochlorophyllide (Pchlde) and the presence of the paracrystalline prolamellar body (PLB) membrane. Both features correlate with the presence of NADPH:Pchlde oxidoreductase (POR), a light-dependent enzyme that reduces photoactive Pchlde–F655 to chlorophyllide and plays a key role in chloroplast differentiation during greening. Two differentially expressed and regulated POR enzymes, PORA and PORB, have recently been discovered in angiosperms. To investigate the hypothesis that etioplast differentiation requires PORA, we have constitutively overexpressed PORA and PORB in the Arabidopsis wild type and in the constitutive photomorphogenic *cop1-18* (previously *det340*) mutant, which is deficient in the PLB and Pchlde–F655. In both genetic backgrounds, POR overexpression increased PLB size, the ratio of Pchlde–F655 to nonphotoactive Pchl[ide]–F632, and the amount of Pchlde–F655. Dramatically, restoration of either PORA or PORB to the *cop1* mutant led to the formation of etioplasts containing an extensive PLB and large amounts of photoactive Pchlde–F655.

## INTRODUCTION

The angiosperm seedling developmental programs determined by the presence or absence of light upon germination are termed photomorphogenesis and skotomorphogenesis, respectively (reviewed in McNellis and Deng, 1995). Photomorphogenesis prepares the seedling for a photoautotrophic lifestyle and encompasses phenomena such as apical hook opening, cotyledon expansion, suppression of hypocotyl elongation, differentiation of proplastids into chloroplasts, and light regulation of gene expression. Skotomorphogenesis, also known as etiolation, entails extensive hypocotyl elongation, an arrest in cotyledon development, and differentiation of proplastids into etioplasts. The achlorophyllous etioplast is defined by the accumulation of the Mg tetrapyrrole protochlorophyllide (Pchlde), a chlorophyll (Chl) precursor, within a paracrystalline lattice of membrane tubules known as the prolamellar body (PLB) (reviewed in Kirk and

Tilney-Bassett, 1978; Henningsen et al., 1993). Here, we investigate the requirements for etioplast differentiation in the dicotyledonous angiosperm Arabidopsis.

The developmental switch from skotomorphogenesis to photomorphogenesis in etiolated angiosperms normally requires light. Photons trigger at least two crucial processes within cells, namely, the strictly light-dependent enzymatic conversion of Pchlde to chlorophyllide (Chlide) in etioplasts and the activation of phytochrome. However, several types of genetic screens have identified recessive Arabidopsis mutants whose constitutive photomorphogenic (*cop*), deetiolated (*det*), and *fusca* (*fus*) phenotypes reflect inappropriate photomorphogenic development in the absence of light (reviewed in von Arnim and Deng, 1996). Mutations in a subset of these *COP/DET/FUS* loci representing 10 independent genes are associated with strongly pleiotropic phenotypes. When such mutant seedlings germinate in darkness, they display light-grown morphologies, partial differentiation of proplastids into lens-shaped pseudochloroplasts that contain rudimentary prothylakoids but lack PLBs, and the light-independent regulation of nuclear genes normally controlled by phytochrome. A similar mutant of pea, designated

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*lip1* for light-independent photomorphogenesis, has also been identified (Frances et al., 1992). Despite their pleiotropic phenotypes, such photomorphogenic mutants retain the normal light requirement for Pchl<sub>ide</sub> reduction and therefore neither synthesize Chl nor develop photosynthetically active chloroplasts in the dark.

The strictly light-dependent reduction of Pchl<sub>ide</sub> to Chl<sub>ide</sub> is catalyzed by NADPH:Pchl<sub>ide</sub> oxidoreductase (POR; EC 1.3.1.33) (Griffiths, 1978; Apel et al., 1980), a nuclear-encoded enzyme that is synthesized as a cytosolic precursor and imported into plastids (Apel, 1981). Pchl<sub>ide</sub> reduction represents the only biochemical step in light-dependent Chl biosynthesis that directly requires photons (reviewed in von Wettstein et al., 1995; Porra, 1997). POR thus plays a central role in the production of Chls that are photochemically indispensable for photosynthesis and structurally required for the stability of most nuclear- and plastid-encoded Chl binding proteins.

However, POR is not only closely associated with chloroplast differentiation but also with Pchl<sub>ide</sub>-protein complex assembly and plastid inner membrane differentiation during etioplast differentiation. Spectroscopic studies of intact etiolated leaves from many plant species have identified three main *in vivo* forms of Pchl<sub>ide</sub> (reviewed in Kirk and Tilney-Bassett, 1978; Sundqvist and Dahlin, 1997). Two pigment forms, designated Pchl<sub>ide</sub>-A638 and Pchl<sub>ide</sub>-A650, reflecting their respective room temperature absorbance maxima, are photoactive (i.e., directly photoreducible by a 1-msec flash). At -196°C, both of these Pchl<sub>ide</sub> forms contribute to one predominant low-temperature (LT) fluorescence emission (F) band, Pchl<sub>ide</sub>-F655, resulting from excitation energy migration from Pchl<sub>ide</sub>-A638 to Pchl<sub>ide</sub>-A650 (Kahn et al., 1970). Pchl<sub>ide</sub>-F655 is normally considered to be the major substrate for light-dependent Chl biosynthesis (Franck and Strzalka, 1992; Sundqvist and Dahlin, 1997). The third *in vivo* Pchl<sub>ide</sub> form is nonphotoactive Pchl<sub>(ide)</sub>-A628/Pchl<sub>(ide)</sub>-F632, which is probably a mixture of several distinct Pchl<sub>ide</sub> and protochlorophyll (Pchl) species.

The presence of photoactive Pchl<sub>ide</sub>-A650/Pchl<sub>ide</sub>-F655 and a structurally intact PLB has long been known to correlate *in vivo* (Butler and Briggs, 1966; Klein and Schiff, 1972). Fractionation of etioplast membranes reveals the PLB to be highly enriched in Pchl<sub>ide</sub>-F655, whereas the prothylakoids that extend outward from the PLB contain a higher ratio of Pchl<sub>(ide)</sub>-F632 to Pchl<sub>ide</sub>-F655 (Ryberg and Sundqvist, 1982). Photoactive Pchl<sub>ide</sub>-F655 and nonphotoactive Pchl<sub>(ide)</sub>-F632 observed in isolated etioplast inner membranes are considered to represent pigment bound in a dark-stable Pchl<sub>ide</sub>:NADPH:POR ternary complex and pigment outside of this complex, respectively (Griffiths, 1978; Oliver and Griffiths, 1982). POR is indeed the most abundant protein of the PLB (Dehesh and Ryberg, 1985; Ryberg and Dehesh, 1986). *In vivo*, Pchl<sub>ide</sub>-F655 is generally believed to arise from aggregation of Pchl<sub>ide</sub>:NADPH:POR ternary complexes within the PLB (Sundqvist and Dahlin, 1997).

Recent experiments have identified two distinct but differentially regulated angiosperm POR enzymes, PORA and PORB, in Arabidopsis (Armstrong et al., 1995) and in barley (Holtorf et al., 1995). *PORA* and *PORB* mRNAs both accumulate in young seedlings, but only *PORB* mRNA is detectable at later stages of plant development. In addition, whereas *PORA* expression is strongly downregulated by phytochrome, *PORB* expression is less light sensitive in Arabidopsis (Armstrong et al., 1995; Frick et al., 1995; Barnes et al., 1996; Runge et al., 1996) or even insensitive in barley (Apel, 1981; Holtorf et al., 1995). Steady state POR levels reflect not only the differential regulation of the *POR* genes but also the light-induced proteolytic instability of PORA and PORB after catalysis (Santel and Apel, 1981; Kay and Griffiths, 1983; Reinbothe et al., 1995) and the unusual Pchl<sub>ide</sub>-dependent uptake of PORA by plastids (Reinbothe et al., 1995). These multiple levels of regulation suggest that PORA and PORB pursue their functions simultaneously during the early stages of greening and chloroplast differentiation, whereas PORB alone maintains Chl biosynthesis in older seedlings and adult plants (Armstrong et al., 1995).

Based primarily on the abundance of PORA in barley etioplasts and the Pchl<sub>ide</sub>-dependent nature of its plastid import, several recent reviews on the topic of Chl biosynthesis have hypothesized specific functions for PORA (von Wettstein et al., 1995; Fujita, 1996; Reinbothe et al., 1996; Porra, 1997), including protection against photooxidative damage by the binding of free Pchl<sub>ide</sub>, structural organization of the PLB, and assembly of photoactive Pchl<sub>ide</sub>-F655.

Experiments performed with nearly achlorophyllous wild-type Arabidopsis seedlings grown in phytochrome A-activating continuous far-red light (FRC) (Frick et al., 1995; Barnes et al., 1996; Runge et al., 1996; Sperling et al., 1997) and with the dark-grown *det340* pleiotropic photomorphogenic mutant (Lebedev et al., 1995) have also been taken as evidence for PORA-specific functions. Both types of seedlings lack PORA and *PORA* mRNA, contain reduced levels of PORB and *PORB* mRNA, are completely or almost completely deficient in photoactive Pchl<sub>ide</sub>-F655 and PLBs, and fail to green normally during subsequent exposure to white (W) light. The FRC-induced block in greening was proposed to result primarily from the absence of PORA and Pchl<sub>ide</sub>-F655 and the overaccumulation of Pchl<sub>(ide)</sub>-F632, a potent sensitizer for photooxidative damage (Frick et al., 1995; Runge et al., 1996). However, more recent experiments have demonstrated that either Arabidopsis PORA or PORB overexpression overcomes the FRC-induced block in greening caused by photooxidative damage to plastids in W light (Sperling et al., 1997). Therefore, protection against Pchl<sub>(ide)</sub>-F632-induced photooxidative damage is a general property of POR rather than a specific function of PORA.

Here, we have applied a transgenic approach to investigate the hypothetical roles of PORA and PORB in the formation of the paracrystalline PLB and the assembly of the photoactive Pchl<sub>ide</sub>-F655 pigment-protein complex during

skotomorphogenesis. PORA and PORB have been constitutively overexpressed in etiolated seedlings of wild-type *Arabidopsis* and in dark-grown seedlings of the PLB-deficient and Pchl<sub>ide</sub>-F655—deficient *det340* mutant. The data obtained mandate a significant revision of recent hypotheses regarding PORA-specific functions in angiosperms.

## RESULTS

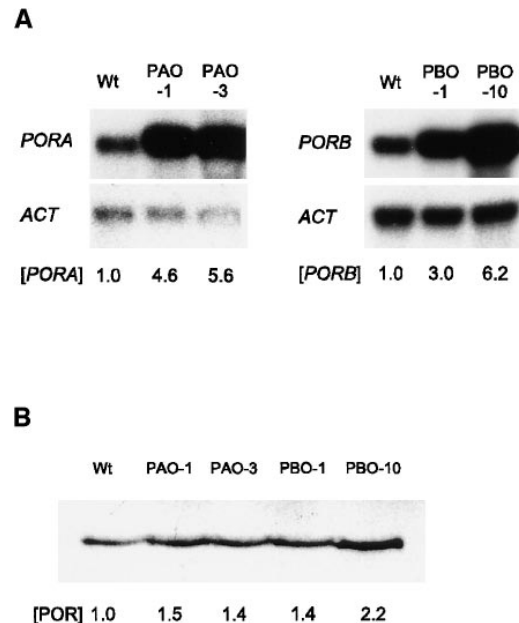
### The *det340* and *cop1* Photomorphogenic Mutations Are Allelic

Genetic complementation tests were performed to examine possible allelism between the recessive *det340* *Arabidopsis* mutant used in this study (Lebedev et al., 1995) and other PLB-deficient pleiotropic photomorphogenic *cop/det/fus* mutants, such as *cop1* (allelic with *fus1*), *det1* (allelic with *fus2*), *cop10* (allelic with *fus9*), and *cop11* (allelic with *fus6*) (Chory et al., 1989; Deng et al., 1991; Castle and Meinke, 1994; McNellis et al., 1994; Miséra et al., 1994; Wei et al., 1994). In crosses using heterozygous *det340* plants as the pollen acceptors and homozygous *det1*, heterozygous *cop10*, or heterozygous *cop11* plants as the pollen donors, all of the resulting seeds were normally pigmented. In addition, the F<sub>1</sub> hybrid seedlings displayed a wild-type morphology when grown either in the dark or in W light, indicating that the mutations are not allelic.

The most informative cross was performed using heterozygous *det340* plants as the pollen acceptors and heterozygous *cop1* plants of a seedling-lethal allele, *cop1-5* (*fus1-1/emb168*) (Deng et al., 1992; Castle and Meinke, 1994; Miséra et al., 1994), as the pollen donors. The resulting population of 180 seeds segregated ~1:3 (28%) for reddish pigmentation characteristic of anthocyanin overaccumulation. Homozygous *cop1-5* seeds and seedlings are purple due to a more severe version of this phenotype (McNellis et al., 1994), whereas heterozygous *cop1-5*, homozygous *det340*, and heterozygous *det340* seeds are normally pigmented. In addition, the F<sub>1</sub> seedlings arising from the reddish seeds displayed a pleiotropic photomorphogenic phenotype when grown in the dark and developed as dwarf plants in the light. In contrast to *cop1-5* homozygotes, these F<sub>1</sub> seedlings were viable beyond the cotyledon stage, although they did not flower or set seed, as was occasionally observed for light-grown *det340* homozygotes. Therefore, the *det340* and *cop1-5* mutations are allelic. We accordingly propose the redesignation of *det340* as *cop1-18*. Comparison of the *cop1-18* phenotype with those of other *cop1* mutants carrying alleles of various strengths indicates that *cop1-18* is a strong mutant allele (McNellis et al., 1994). The *COP1* gene has been isolated, and the COP1 protein has been demonstrated to function as a repressor of photomorphogenesis in etiolated seedlings (Deng et al., 1992).

### Generation of PORA- and PORB-Overexpressing *cop1* Seedlings

Transgenic *Arabidopsis* seedlings that constitutively overexpress either *Arabidopsis* PORA or PORB in a wild-type genetic background have recently been described with respect to their phenotypes in FRC and upon subsequent W light treatment (Sperling et al., 1997). Several of these independent and homozygous PORA-overexpressing (PAO) and PORB-overexpressing (PBO) lines were selected for further phenotypic analysis. As a first step, their *POR* mRNA and POR protein levels were measured in etiolated seedlings, as illustrated in Figure 1. Relative to untransformed etiolated wild-type seedlings, the amounts of *PORA* mRNA in lines PAO-1 and PAO-3 and *PORB* mRNA in lines PBO-1 and PBO-10 were three- to sixfold higher, as demonstrated by

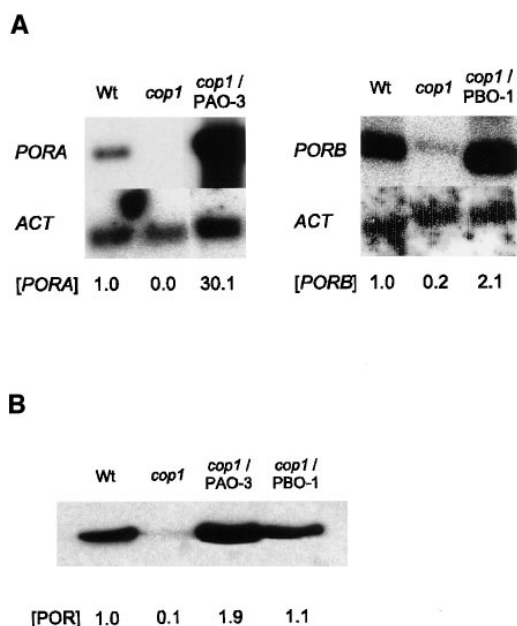


**Figure 1.** *POR* mRNA and POR Amounts in Wild-Type *Arabidopsis* Seedlings That Overexpress PORA or PORB.

**(A)** *PORA* mRNA levels in PAO lines and *PORB* mRNA levels in PBO lines.

**(B)** Total POR protein levels in PAO and PBO lines.

Gel blots were prepared using 5  $\mu$ g of total RNA from etiolated seedlings and probed for the mRNAs indicated at the left. Actin (*ACT*) mRNA provides a control for the sample loading. Numbers at the bottom represent the *ACT*-corrected amounts of the relevant mRNAs from transgenic seedlings, relative to the etiolated wild type (Wt). A gel blot was prepared using 5  $\mu$ g of total protein from extracts of etiolated seedlings, which, independent of genotype, contained similar amounts of total protein on a per seedling basis. Numbers at the bottom represent the amounts of immunoreactive total POR protein detected relative to the etiolated wild type.



**Figure 2.** *POR* mRNA and POR Amounts in *cop1* Seedlings That Overexpress PORA or PORB.

(A) *PORA* and *PORB* mRNA levels in the *cop1*/PAO-3 and *cop1*/PBO-1 lines, respectively.

(B) Total POR protein levels in the *cop1*/PAO-3 and *cop1*/PBO-1 lines.

RNA and protein gel blots of dark-grown seedlings displaying the *cop1* mutant morphology were prepared and quantitated as described in Figure 1. Wt, wild type.

gel blot analysis (Figure 1A). Using protein gel blot analysis, we detected PORA and PORB in the wild type as a single immunoreactive signal with an apparent molecular mass of ~36 kD (Figure 1B), which is consistent with the processing of the cytosolic POR precursors during plastid import. The amounts of total POR in PAO and PBO seedlings were up to twofold higher on a total protein basis than in the corresponding wild-type control.

To extend our study of the effects of PORA or PORB overexpression to a severely POR-deficient background, the *POR* transgenes were introduced into the *cop1-18* mutant. For this purpose, the transgenic lines PAO-3 and PBO-1, whose etiolated seedlings accumulate approximately equal amounts of total POR protein (Figure 1B), were crossed with plants heterozygous for the *cop1* mutation. This strategy ultimately resulted in the generation of new transgenic lines designated *cop1*/PAO-3 and *cop1*/PBO-1, which were propagated as *cop1* heterozygotes. The *POR* mRNA and POR protein amounts in homozygous mutants isolated from segregating populations of dark-grown *cop1*, *cop1*/PAO-3, and *cop1*/PBO-1 seedlings were determined, as shown in Figure 2. The *cop1* mutant did not detectably accumulate *PORA*

mRNA and contained at least fivefold less *PORB* mRNA than was observed in the etiolated wild-type control (Figure 2A). The residual amount of PORB present in dark-grown *cop1* seedlings was also ~10-fold lower on a total protein basis than the total POR content of the etiolated control (Figure 2B). In contrast, the *cop1*/PAO-3 and *cop1*/PBO-1 seedlings accumulated much higher levels of the *PORA* and *PORB* mRNAs, respectively, than were found in the *cop1* mutant and at least twofold more of the respective *POR* mRNAs than were present in etiolated wild-type seedlings. Although line *cop1*/PAO-3 contained a surprisingly large amount of *PORA* mRNA, the amount of POR protein accumulated was only twofold that of the etiolated control. Importantly for the phenotypic analysis, the *cop1*/PAO-3 and *cop1*/PBO-1 seedlings contained ~19- and 11-fold more total POR, respectively, than did the *cop1* mutant.

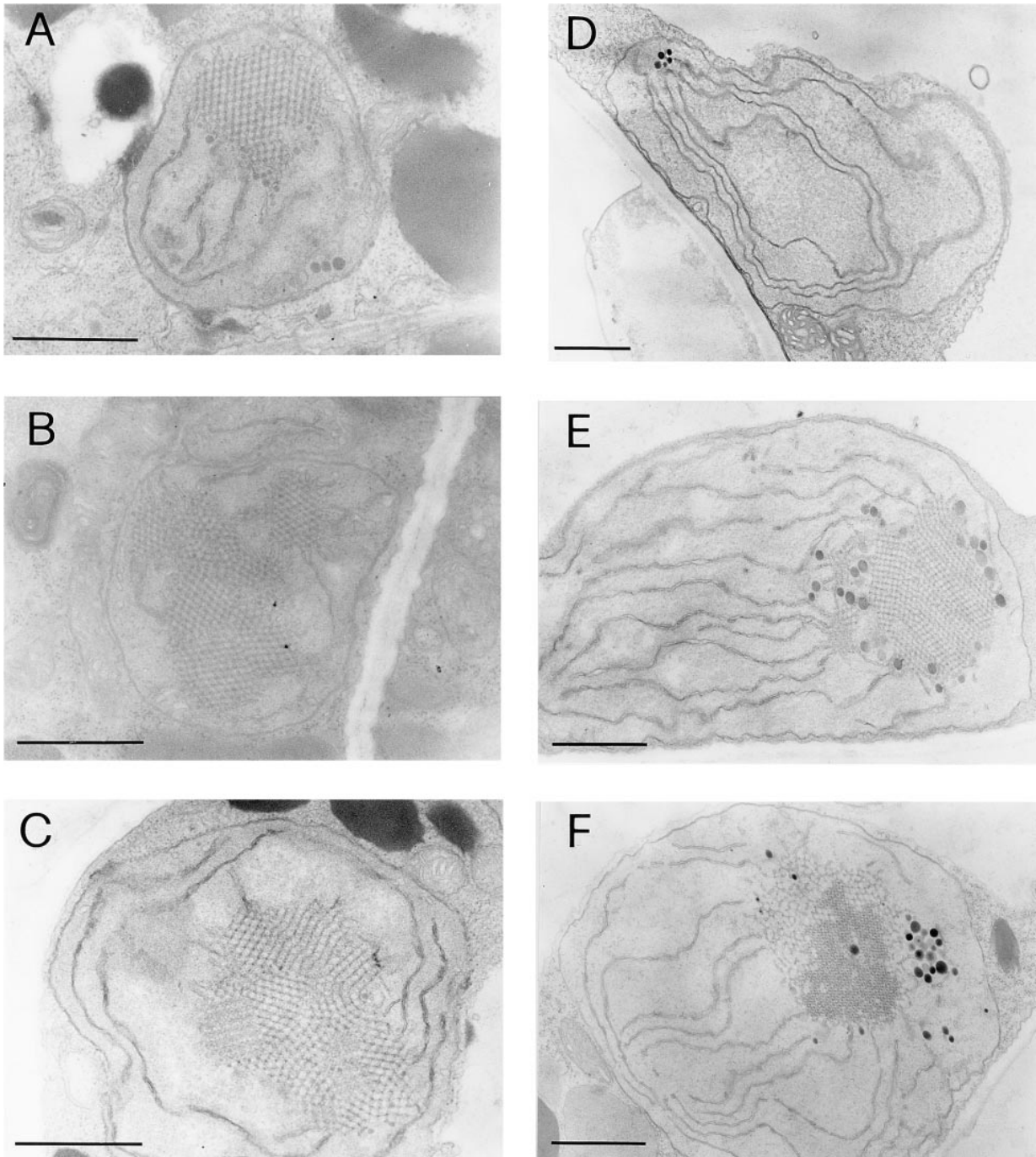
### Both PORA and PORB Restore the Prolamellar Body to *cop1* Plastids

To investigate the involvement of PORA and PORB in PLB formation, ultrathin sections of cotyledon cells were examined by transmission electron microscopy. Figure 3 presents this analysis of plastid ultrastructure for the untransformed etiolated wild-type and transgenic lines PAO-3 and PBO-1 as well as for the dark-grown *cop1* mutant and the transgenic lines *cop1*/PAO-3 and *cop1*/PBO-1. Typical etioplasts from the wild-type, PAO-3, and PBO-1 seedlings were irregularly spherical or ellipsoidal. Thus, POR overexpression had no apparent effect on plastid shape. On the other hand, the POR-overexpressing transgenic lines did contain PLBs that were consistently larger than those found in the wild type (Figures 3A to 3C).

Dark-grown *cop1*, *cop1*/PAO-3, and *cop1*/PBO-1 seedlings displayed no obvious differences in their overall or cotyledon morphologies due to POR overexpression. With respect to plastid ultrastructure, the lens-shaped plastids of the *cop1* mutant contained unstacked prothylakoids rather than PLBs (Figure 3D; Deng et al., 1991; Lebedev et al., 1995). The transgenic *cop1*/PAO-3 and *cop1*/PBO-1 seedlings, however, displayed dramatically different plastid inner membrane architectures from that of the untransformed mutant (Figures 3E and 3F). Either PORA or PORB overexpression led to the formation of paracrystalline PLBs similar to those observed in the etiolated *Arabidopsis* wild type (Figure 3A and data not shown).

### PORA or PORB Overexpression Increases the Ratio of Photoactive Pchl<sub>a</sub>-F655 to Nonphotoactive Pchl<sub>a</sub>(ide)-F632 in Wild-Type Seedlings

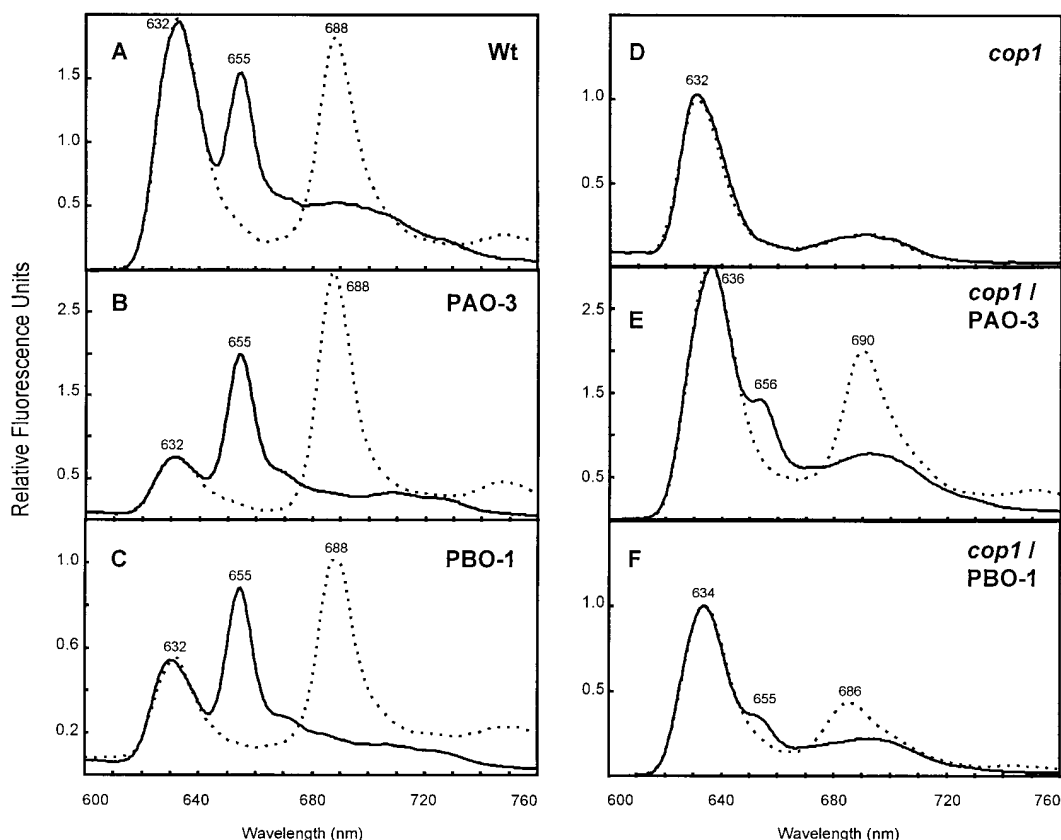
LT fluorescence spectroscopy at -196°C was performed to determine the effect of PORA or PORB overexpression on the relative in situ levels of photoactive Pchl<sub>a</sub>-F655 and



**Figure 3.** Plastid Ultrastructure in Cotyledons of POR-Overexpressing Seedlings.

- (A) Untransformed wild type.
- (B) PAO-3 line.
- (C) PBO-1 line.
- (D) *cop1* mutant.
- (E) *cop1*/PAO-3 line.
- (F) *cop1*/PBO-1 line.

The ultrastructure of cotyledon plastids from etiolated wild-type and dark-grown *cop1* seedlings of various POR genotypes was examined using electron microscopy. Note the appearance of paracrystalline PLB membranes upon overexpression of PORA (E) or PORB (F) in the *cop1* genetic background. Magnifications were  $\times 20,000$  for (A) to (C),  $\times 12,000$  for (D), and  $\times 15,000$  for (E) and (F). Bars in (A) to (F) = 1  $\mu\text{m}$ .



**Figure 4.** Photoactive and Nonphotoactive Pchl(ide) Pigment Forms in Cotyledons of POR-Overexpressing Seedlings.

- (A) Untransformed wild type (Wt).  
 (B) PAO-3 line.  
 (C) PBO-1 line.  
 (D) *cop1* mutant.  
 (E) *cop1*/PAO-3 line.  
 (F) *cop1*/PBO-1 line.

In situ LT fluorescence spectra of unilluminated (solid curves) and flash-illuminated (dotted curves) cotyledons of etiolated wild-type and dark-grown *cop1* seedlings were recorded. Fluorescence emission bands observed with an excitation wavelength of 440 nm include nonphotoactive Pchl(ide)-F632, photoactive Pchl(ide)-F655 representing the Pchl(ide):NADPH:POR ternary complex, and, after flash treatment, Chlide-F690. The broad preflash fluorescence emission centered at 693 nm, which is most evident in the *cop1* genetic background ([D] to [F]), represents a vibrational sublevel of Pchl(ide)-F632 (Böddi et al., 1992). Note the presence of a preflash shoulder at 655 to 656 nm and a postflash Chlide peak at 686 to 690 nm upon the overexpression of either PORA (E) or PORB (F) in the *cop1* mutant.

nonphotoactive Pchl(ide)-F632 in plastids of intact etiolated *Arabidopsis* cotyledons. This method avoids the POR-mediated photoenzymatic reduction of Pchl(ide), which occurs at a high yield at temperatures as low as  $-77^{\circ}\text{C}$  (Smith and Benitez, 1954). A single saturating 1-msec light flash applied to excised cotyledons briefly warmed from  $-196^{\circ}\text{C}$  to, for example,  $-20^{\circ}\text{C}$  reduces all of the photoactive Pchl(ide) to Chlide. The first long-lived Chlide product has an LT fluorescence emission band at  $\sim 686$  to 690 nm (Chlide-F690) and

appears a few microseconds after the flash (Sironval and Brouers, 1970; Franck and Mathis, 1980).

Spectra of cotyledons from the etiolated wild type and from transgenic lines PAO-3 and PBO-1 were recorded both before and after flash illumination, as shown in Figure 4. Overexpression of either PORA or PORB in these seedlings significantly increased the preflash ratio of Pchl(ide)-F655 to Pchl(ide)-F632 (Figures 4A to 4C). In addition, the accumulated Pchl(ide)-F655 was quantitatively converted to Chlide-F690

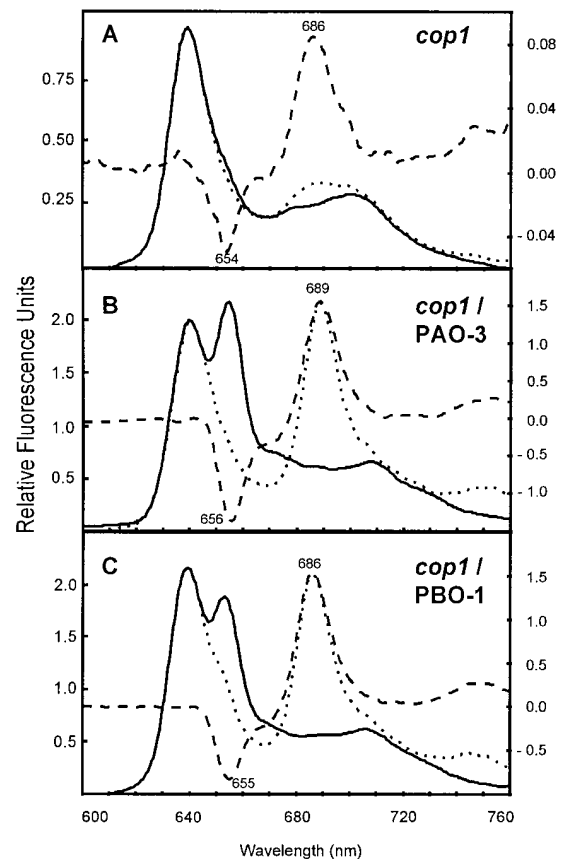
by a 1-msec flash, indicating that it was indeed enzymatically active.

### Both PORA and PORB Reconstitute Photoactive Pchl<sub>ide</sub>-F655 in *cop1* Seedlings

Analogous LT fluorescence emission measurements were performed with dark-grown *cop1*, *cop1/PAO-3*, and *cop1/PBO-1* seedlings. Nonphotoactive Pchl<sub>ide</sub>-F632 was the only obvious pigment form either preflash or postflash in the *cop1* mutant (Figure 4D; Lebedev et al., 1995). In contrast, a noticeable preflash Pchl<sub>ide</sub> fluorescence emission shoulder at ~655 nm was evident in both *cop1/PAO-3* and *cop1/PBO-1* cotyledons (Figures 4E and 4F). The appearance of this shoulder also coincided with a slight redshift in the Pchl<sub>ide</sub>-F632 emission band that probably results from its spectral overlap with the 655-nm component. After flash illumination of *cop1/PAO-3* and *cop1/PBO-1* cotyledons, the 655-nm emission shoulder disappeared and was replaced by a Chlide emission band at 686 to 690 nm.

To further investigate the nature of the reconstituted photoactive Pchl<sub>ide</sub> in the *cop1* mutant, additional LT fluorescence emission spectra were collected using an excitation wavelength of 465 nm rather than 440 nm. This approach exploits differences in excitation spectra of different pigment forms to enhance the relative fluorescence due to photoactive Pchl<sub>ide</sub>-F655 while minimizing the background fluorescence emission due to the large amounts of nonphotoactive Pchl<sub>ide</sub>-F632 present in the *cop1* mutant (see below). As demonstrated in Figure 5 and in contrast to the spectrum presented in Figure 4D, excitation of *cop1* cotyledons at 465 nm yielded slightly different preflash and postflash emission spectra in the region of 655 nm. In addition, small amounts of Chlide-F690 were formed by flash illumination. "Postflash minus preflash" difference spectra were therefore calculated to highlight more precisely the fluorescence emission maxima of the photoactive Pchl<sub>ide</sub> substrate and the Chlide product of the photoenzymatic reduction. The difference spectra made even more clear that *cop1* seedlings contain a small but clearly detectable amount of Pchl<sub>ide</sub>-F655 that can be reduced to Chlide-F690 (Figure 5A). Furthermore, the putative Pchl<sub>ide</sub>-F655 pigment forms observed as shoulders in the emission spectra of *cop1/PAO-3* and *cop1/PBO-1* cotyledons excited at 440 nm (Figures 4E and 4F) became much more prominent upon excitation at 465 nm (Figures 5B and 5C). As in the *cop1* mutant, these PORA- or PORB-dependent Pchl<sub>ide</sub>-F655 species could be converted by flash treatment to Chlide-F690. Importantly, none of the in situ LT fluorescence data that we have collected (Figures 4 and 5; U. Sperling and F. Franck, unpublished data) provide evidence for the preferential involvement of either PORA or PORB in the assembly of a novel photoactive Pchl<sub>ide</sub> species other than Pchl<sub>ide</sub>-F655 or for a novel photoenzymatic reduction product other than Chlide-F690 (see Discussion).

Quantitative comparisons between the amounts of specific Pchl<sub>ide</sub>(ide) species detected by in situ LT fluorescence emission measurements are complicated by excitation energy migration between different pigment forms (Kahn et al., 1970) and by the large difference in cotyledon size between etiolated wild-type and dark-grown *cop1* seedlings. Therefore, to determine whether overexpression of PORA or PORB in the *cop1* genetic background reconstituted Pchl<sub>ide</sub>-F655 to wild-type levels, it was necessary to quantitate the preflash



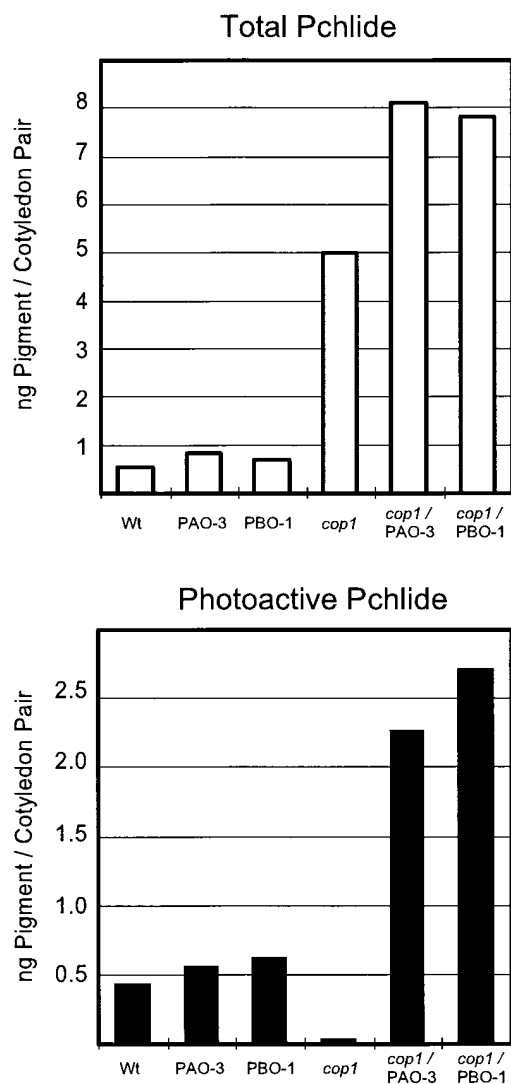
**Figure 5.** Direct Detection of Photoactive Pchl<sub>ide</sub>-F655 in the *cop1* Mutant and in POR-Overexpressing *cop1* Seedlings.

(A) *cop1* mutant.

(B) *cop1/PAO-3* line.

(C) *cop1/PBO-1* line.

In situ LT fluorescence spectra of unilluminated (solid curves) and flash-illuminated (dotted curves) cotyledons of dark-grown seedlings were collected using an excitation wavelength of 465 nm. The fluorescence emission bands are as described in Figure 4. Relative fluorescence intensities are given by the lefthand y-axis. In the difference spectra (dashed curves, righthand y-axis), note the presence of a negative peak at 654 to 656 nm and a positive peak at ~686 to 689 nm upon the overexpression of either PORA (B) or PORB (C), and to a much lesser extent in the *cop1* mutant itself (A).



**Figure 6.** Quantitative Determination of Total Pchl(ide) and Photoactive Pchl(ide) Pigment Forms in POR-Overexpressing Seedlings.

Room temperature fluorescence spectra of the acetone-extracted total pigments from unilluminated and flash-illuminated cotyledons of seedlings were collected. Total Pchl(ide) was calculated from the preflash fluorescence emission band at 634 nm. Photoactive Pchl(ide)-F655 was determined based on the postflash emission at 672 nm that results from its quantitative photoenzymatic reduction to Chlide. Pigment amounts are given on a per-cotyledon-pair basis. Wt, wild type.

and postflash amounts of Pchl(ide) and Chlide by fluorometric measurements of pigment concentrations after acetone extraction of cotyledons. The Chlide concentration after one flash was taken as a quantitative measure of Pchl(ide)-F655 before the flash, because this Pchl(ide) form was the only

photoactive pigment species detected in the LT emission spectra (Figures 4 and 5).

As seen in Figure 6, overexpression of either PORA in line PAO-3 or PORB in line PBO-1 led to modest but reproducible increases (up to 1.5-fold on a per-cotyledon-pair basis) in total extractable Pchl(ide), relative to the etiolated wild-type control. Similar increases in the amounts of photoactive Pchl(ide)-F655 in PAO-3 and PBO-1 seedlings were also observed. The dark-grown *cop1* mutant contained approximately ninefold more total Pchl(ide) but ~10-fold less photoactive Pchl(ide)-F655 than did the etiolated wild type (Figure 6; Lebedev et al., 1995). POR overexpression in both the *cop1*/PAO-3 and *cop1*/PBO-1 transgenic lines increased total Pchl(ide) accumulation ~1.5-fold with respect to the *cop1* mutant. Significantly, virtually all of this increase was due to photoactive Pchl(ide)-F655. The transgenic lines *cop1*/PAO-3 and *cop1*/PBO-1 accumulated approximately five- to sixfold more of this pigment form on a per-cotyledon-pair basis than did the etiolated wild type. Thus, overexpression of either PORA or PORB fully reconstituted photoactive Pchl(ide)-F655 in the *cop1* genetic background.

## DISCUSSION

### PORA and PORB Are the Central Determinants of Etioplast Differentiation

The exact roles of PORA and PORB in etioplast differentiation during skotomorphogenesis have hitherto been the subject of speculation. Before the discovery of multiple POR enzymes, bulk POR had been identified in the plastids of etiolated monocotyledonous angiosperm seedlings in conjunction with the paracrystalline PLB and photoactive Pchl(ide)-F655 (Ryberg and Sundqvist, 1982; Dehesh and Ryberg, 1985; Ryberg and Dehesh, 1986). PORA and PORB were later found to occur in roughly equal amounts in etiolated Arabidopsis seedlings (Armstrong et al., 1995), although PORA was much more abundant than PORB in etiolated barley (Holtorf et al., 1995). Recent reviews have interpreted the latter observation as evidence of an essential role for PORA in the formation of the PLB and the assembly of photoactive Pchl(ide)-F655 (von Wettstein et al., 1995; Fujita, 1996; Reinbothe et al., 1996; Porra, 1997). In addition, data obtained with FRC-grown Arabidopsis wild-type (Runge et al., 1996) and dark-grown *cop1* seedlings (Lebedev et al., 1995) established a correlation between the loss of detectable PORA, Pchl(ide)-F655, and, in the case of the *cop1* mutant, the PLB. As discussed below, however, both PORA and PORB can in vivo reconstitute the PLB and photoactive Pchl(ide)-F655, which are the two distinguishing features of the etioplast plastid type (Kirk and Tilney-Bassett, 1978; Henningsen et al., 1993).



### Prolamellar Body Formation in Plastids Depends on Total POR Content

In contrast to the hypothesis that PLB formation is an exclusive property of PORA, some recent evidence has hinted at the involvement of PORB in this process. For example, plastids of FRC-grown wild-type *Arabidopsis* seedlings that completely lack PORA still retain some PORB and possess small PLBs (Frick et al., 1995; Barnes et al., 1996; Runge et al., 1996). Overexpression of either PORB or PORA in such seedlings seems to enhance PLB formation (Sperling et al., 1997). Here, we also observed that PLB formation is enhanced in etioplasts of wild-type seedlings that overexpress either PORA or PORB (Figures 3A to 3C). The presence of preexisting PLBs in both etiolated and FRC-grown untransformed wild-type seedlings complicates the interpretation of these results, however.

PLB-deficient pleiotropic photomorphogenic *cop/det/fus* mutants of *Arabidopsis* (von Arnim and Deng, 1996) therefore offer an attractive experimental system in which to study the roles of PORA and PORB in etioplast inner membrane formation. In the homozygous *cop1-18* mutant, PORA was undetectable, and the amount of PORB was substantially lower than in the etiolated wild type because of down-regulation of the respective *POR* mRNAs (Figure 2; Lebedev et al., 1995). Like other pleiotropic photomorphogenic mutants that carry strong *cop/det/fus* alleles (Wei et al., 1994), dark-grown *cop1* seedlings lack PLBs (Figure 3D; Deng et al., 1991; Lebedev et al., 1995). Constitutive overexpression of either PORA or PORB restored the paracrystalline PLB to *cop1* plastids (Figures 3E and 3F). POR is therefore not only the most abundant protein of the PLB (Dehesh and Ryberg, 1985; Ryberg and Dehesh, 1986) but also an active participant in membrane assembly. Both POR enzymes can apparently mobilize lipids, Pchl<sub>ide</sub>, and NADPH to create the paracrystalline PLB. The structural requirements for Pchl<sub>ide</sub> and NADPH are underscored by the observations that plastids of dark-grown angiosperm mutants that lack Pchl<sub>ide</sub> contain no PLBs (Henningsson et al., 1993; Runge et al., 1995) and that NADPH is required to maintain the structural integrity of isolated PLBs (Ryberg and Sundqvist, 1988).

By extension of the results obtained with the *cop1-18* mutant (this work; Lebedev et al., 1995), POR depletion seems likely to be the cause of PLB deficiency in other pleiotropic photomorphogenic mutants of *Arabidopsis* (von Arnim and Deng, 1996). Indeed, dark-grown seedlings of the PLB-deficient *det1* mutant (Chory et al., 1989) display drastically reduced amounts of the POR polypeptides and *POR* mRNAs (G. Frick, B. van Cleve, and G.A. Armstrong, unpublished data). The light-independent activation of certain phytochrome signaling pathways in such pleiotropic photomorphogenic mutants apparently results in POR levels too low to allow detectable PLB formation. Similarly, the fact that cytokinin treatment of dark-grown wild-type seedlings leads to the differentiation of plastids that lack PLBs (Chory et al.,

1994) may also reflect a severe POR deficiency. On the other hand, plastids of FRC-grown *Arabidopsis* seedlings, which are not as severely POR deficient as the *cop1-18* mutant, still possess small PLBs (Barnes et al., 1996; Runge et al., 1996; Sperling et al., 1997). Certain dark-grown pleiotropic photomorphogenic mutants that carry weak alleles of *cop1*, *cop9*, and *cop11* also retain remnants of the PLB (Wei and Deng, 1992; Castle and Meinke, 1994; McNellis et al., 1994). Finally, although the PLB is most closely associated with etioplast development during skotomorphogenesis, this membrane system sometimes coexists with Chl-containing thylakoids in chloroplasts from redarkened and low light-adapted angiosperms (Kirk and Tilney-Bassett, 1978; Henningsson et al., 1993; Sundqvist and Dahlin, 1997). Intriguingly, the PLB-deficient *lip1* photomorphogenic mutant of pea (Frances et al., 1992) has been reported to contain substantial amounts of POR, although this phenotype remains to be characterized in detail (Sundqvist et al., 1995). In general, however, the presence of an extensive PLB in different angiosperm plastid types probably reflects a high steady state POR content.

### Both PORA and PORB Allow Assembly of Pigment-Protein Complexes Containing Photoactive Pchl<sub>ide</sub>-F655

The presence of photoactive Pchl<sub>ide</sub>-F655 in conjunction with the PLB distinguishes the etioplast from other plastid types (Kirk and Tilney-Bassett, 1978; Henningsson et al., 1993). The previously reported absence of photoactive Pchl<sub>ide</sub>-F655 and PORA in the *Arabidopsis cop1* mutant led to the notion of novel, PORB-specific pigment forms, designated Pchl<sub>ide</sub>-F635 and Pchl<sub>ide</sub>-F642, that are phototransformable (i.e., photoreducible during continuous illumination) (Lebedev et al., 1995). These putative PORB-dependent pigment forms were postulated to sustain Chl biosynthesis even in the absence of Pchl<sub>ide</sub>-F655. Indeed, a typically minor photoactive Pchl<sub>ide</sub>-F642 pigment species is well known in various etiolated angiosperms (Kahn et al., 1970; Böddi et al., 1992). This photoactive Pchl<sub>ide</sub> species is generally believed to arise from a fraction of Pchl<sub>ide</sub>-A638 that fluoresces directly rather than contributing to the Pchl<sub>ide</sub>-F655 emission band (Kahn et al., 1970) and may represent a not highly aggregated pigment form located in prothylakoids or at the periphery of the PLB (Klein and Schiff, 1972; Sundqvist and Dahlin, 1997). In addition to photoactive Pchl<sub>ide</sub>-F642, one example of a novel phototransformable Pchl<sub>ide</sub> species is provided by the *lip1* mutant of pea (Frances et al., 1992). This mutant accumulates phototransformable Pchl<sub>ide</sub>-F633 and synthesizes Chl, despite its apparent lack of Pchl<sub>ide</sub>-F655 (Sundqvist et al., 1995). One cannot exclude, however, that Chl synthesis observed during continuous illumination of the *lip1* and *cop1* mutants in these studies (Lebedev et al., 1995; Sundqvist et al., 1995) actually proceeds through

undetected photoactive Pchl<sub>ide</sub>-F655 that is continuously regenerated from the phototransformable Pchl<sub>ide</sub> pool.

By improving the sensitivity of the method for collecting LT fluorescence spectra, we have been able to directly detect traces of photoactive Pchl<sub>ide</sub>-F655 in the dark-grown *cop1* mutant (Figures 5A and 6). This result is in fact consistent with a previous quantitative pigment determination (see Table 1 in Lebedev et al., 1995), although not with the interpretation thereof. More importantly, we have demonstrated restoration of Pchl<sub>ide</sub>-F655 to *cop1* seedlings upon overexpression of either PORA or PORB (Figures 4E, 4F, 5B, and 5C).

On the other hand, we find no evidence for the appearance of other photoactive Pchl<sub>ide</sub> forms in any of the POR-overexpressing transgenic wild-type or *cop1* seedlings studied. A comparison of the emission spectra of *cop1* mutant, *cop1*/PAO-3, and *cop1*/PBO-1 cotyledons generated using different excitation wavelengths indicates that the Pchl<sub>ide</sub>-F632 emission band is heterogeneous, as reflected in the redshifts of the maxima to 640 nm when the excitation wavelength was changed from 440 to 465 nm (Figures 4 and 5). However, the shapes of the emission spectra in this region were unaffected by a 1-msec flash, and the difference spectra did not reveal any photoactive Pchl<sub>ide</sub> form other than Pchl<sub>ide</sub>-F655, even in PORB-overexpressing *cop1*/PBO-1 seedlings (Figure 5C). In addition, flash illumination of Pchl<sub>ide</sub>-F655—containing cotyledons of any genotype always resulted in the formation of Chl<sub>ide</sub>-F690. This pigment form is the first long-lived product of photoenzymatic Pchl<sub>ide</sub> reduction in various angiosperms (Sironval and Brouers, 1970; Franck and Mathis, 1980), including the *Arabidopsis* wild type (Lebedev et al., 1995; Runge et al., 1995, 1996). Therefore, we conclude that PORA and PORB give rise to qualitatively similar *in vivo* aggregates of Pchl<sub>ide</sub>-protein complexes, reflected in the presence of Pchl<sub>ide</sub>-F655. Whether these complexes are spectroscopically and functionally equivalent in all respects is under investigation.

In retrospect, previous data obtained with dark-grown *cop1* mutant and FRC-grown wild-type seedlings that had suggested a specific role for PORA in the assembly of Pchl<sub>ide</sub>-F655 actually reflect not only the loss of detectable PORA but also a substantial decrease in the amount of PORB (Lebedev et al., 1995; Runge et al., 1996). The residual amounts of Pchl<sub>ide</sub>-F655 in FRC-grown seedlings (Runge et al., 1996) and its almost complete absence in the dark-grown *cop1* mutant (Figure 5A) mirror the phenotypes seen at the level of the PLB. This point is reinforced by the strict correlation between the POR-dependent formation of the PLB and the presence of photoactive Pchl<sub>ide</sub>-F655 in POR-overexpressing transgenic *Arabidopsis* seedlings (Figures 3 to 5; Sperling et al., 1997; U. Sperling, unpublished data).

Despite the dramatic effect of POR overexpression on the assembly of photoactive Pchl<sub>ide</sub>-F655, introduction of neither PORA nor PORB into the *cop1* genetic background significantly influences the amount of nonphotoactive Pchl<sub>ide</sub>-F632 (Figures 5 and 6). The extreme abundance of this

Pchl<sub>ide</sub> species in dark-grown *cop1* mutant seedlings probably results from the constitutive upregulation of the capacity for the synthesis of 5-aminolevulinate. The availability of this rate-limiting C<sub>5</sub> biosynthetic precursor of Pchl<sub>ide</sub> and Chl is normally regulated by an unknown mechanism in etiolated angiosperms and increases upon illumination in a phytochrome-dependent manner (reviewed in Beale and Weinstein, 1991).

Nonphotoactive Pchl<sub>ide</sub>-F632 represents a poorly defined mixture of Pchl<sub>ide</sub> and Pchl pigment species that probably occur both in protein-bound and unbound forms. Although the plastidic localization of Pchl<sub>ide</sub>-F632 in the *cop1* mutant is not known, at least some fraction of this pigment form likely resides in the envelope membranes. Such a localization would be consistent with PLB formation in PORA-overexpressing *cop1*/PAO-3 seedlings (Figure 3E) if, by analogy to barley PORA (Reinbothe et al., 1995), the plastid uptake of *Arabidopsis* PORA is Pchl<sub>ide</sub> dependent. The prothylakoid membranes of *cop1* plastids (Figure 3C) constitute another probable site for Pchl<sub>ide</sub>-F632 accumulation, based on previous etioplast membrane fractionation studies of wheat seedlings (Ryberg and Sundqvist, 1982).

#### POR and the Appearance of Etioplasts during the Evolution of Photosynthetic Organisms

The POR-mediated photoenzymatic reduction of Pchl<sub>ide</sub> to Chl<sub>ide</sub> during light-dependent Chl biosynthesis occurs not only in angiosperms but in all oxygenic photosynthetic organisms thus far examined, including gymnosperms, algae, and cyanobacteria (Kirk and Tilney-Bassett, 1978; von Wettstein et al., 1995; Fujita, 1996). Unlike angiosperms, most gymnosperms, algae, and cyanobacteria also green in the dark. This process requires a light-independent (i.e., dark-active) Pchl<sub>ide</sub> oxidoreductase, referred to as DPOR, that is absent in angiosperms and whose three plastid-encoded subunits are structurally unrelated to nuclear-encoded POR (Fujita, 1996).

Interestingly, the paracrystalline PLB and photoactive Pchl<sub>ide</sub>-F655 that define the etioplast are not ubiquitous among POR-containing organisms but are restricted to angiosperms and to certain gymnosperms that green poorly in the dark (Kirk and Tilney-Bassett, 1978; Selstam and Widell, 1986; Mariani et al., 1990; von Wettstein et al., 1995). Seedlings of such gymnosperms possess etiochloroplasts in which Pchl<sub>ide</sub>-F655, PLBs, and Chl-containing thylakoids coexist. One recent hypothesis has suggested that the evolution of the etioplast coincided with the appearance of multiple forms of POR in photosynthetic organisms (von Wettstein et al., 1995). Indeed, not only do the angiosperms *Arabidopsis* and barley possess the distinct and differentially regulated PORA and PORB genes (Armstrong et al., 1995; Holtorf et al., 1995), but species of pine, a gymnosperm, also contain at least two POR genes (Spano et al., 1992; Forreiter and Apel, 1993). Consistent with this hypothesis, unicellular

photosynthetic organisms, such as the cyanobacterium *Synechocystis* sp PCC 6803 and the green alga *Chlamydomonas reinhardtii*, lack PLBs and possess only one *POR* gene (Suzuki and Bauer, 1995; Li and Timko, 1996).

Our data demonstrate, however, that the formation of the PLB and the assembly of Pchl<sub>ide</sub>-F655 are properties common to both PORA and PORB. Therefore, a PLB-specific POR enzyme that arose through *POR* gene duplication cannot be invoked to explain the origin of the etioplast during evolution. In addition, only one *POR* gene has thus far been isolated from pea and cucumber, which are both angiosperms (Sundqvist and Dahlin, 1997). Although *POR* gene duplication in some higher plants may indeed have increased total POR content and hence promoted the appearance of PLBs and photoactive Pchl<sub>ide</sub>-F655 in plastids of dark-grown seedlings, multiple *POR* genes are evidently not a prerequisite for etioplast formation. The additional factors that have determined the evolutionary distribution of the etioplast among POR-containing organisms therefore remain to be identified.

## METHODS

### Generation of Transgenic Plants and Plant Growth Conditions

Detailed descriptions of the plasmid vectors used for the constitutive overexpression of PORA or PORB in *Arabidopsis thaliana* plants and the generation of the homozygous POR-overexpressing transgenic lines studied here have been presented elsewhere (Sperling et al., 1997). PORA-overexpressing (PAO) and PORB-overexpressing (PBO) transformants of the *Arabidopsis* ecotype Columbia derivative C24 wild type were regularly propagated on kanamycin-containing (50 µg/mL) Murashige-Skoog (MS) agar Petri plates to select for the presence of the transgene. Homozygous plants of the PAO-3 and PBO-1 transgenic lines were crossed with the homozygous constitutive photomorphogenic *cop1* mutant using the transgenic plants as pollen donors and the mutant as the pollen acceptor. The F<sub>1</sub> progeny were selected with kanamycin, transferred to soil, and allowed to mature and self-fertilize. The resulting seeds were sown on MS medium, and a number of kanamycin-resistant F<sub>2</sub> plants that were wild type in appearance were propagated to identify *cop1* heterozygous seed families in the following generation. F<sub>3</sub> seedling populations were analyzed with respect to their segregation patterns for kanamycin resistance during growth in the light and for the appearance of *cop1* seedling morphology during growth in the dark. This procedure allowed us to identify F<sub>2</sub> and F<sub>3</sub> seed families that were both heterozygous for the *cop1* mutation and either homozygous for PORA overexpression (*cop1*/PAO-3) or heterozygous for PORB overexpression (*cop1*/PBO-1). These transgenic lines were propagated as *cop1* heterozygotes to maximize their seed set.

To obtain etiolated wild-type, PAO, and PBO seedlings and dark-grown *cop1*, *cop1*/PAO-3, and *cop1*/PBO-1 seedlings, surface-sterilized seeds were sown on MS agar, which was supplemented with 2% (w/v) sucrose in the case of the *cop1* heterozygotes and the accompanying etiolated wild-type controls. The seeds were placed in the dark at 4°C for 24 hr, exposed at 22°C to 1 hr of white (W) light

with a fluence rate of 160 µE m<sup>-2</sup> sec<sup>-1</sup> provided by TLD 36W/84 cool-white fluorescent lamps (Philips Electronics, Paris, France), and thereafter returned to darkness at room temperature for 4 days. Plant material was harvested under a green safety light and either processed immediately for electron microscopy and fluorescence measurements or frozen in liquid nitrogen and stored at -80°C until further analysis of RNA and protein contents.

### mRNA and Protein Analyses

RNA and protein samples were isolated and analyzed as described elsewhere (Armstrong et al., 1995; Sperling et al., 1997). Briefly, 4-day-old dark-grown *Arabidopsis* seedlings were used as a source of material to prepare RNA and protein gel blots, respectively. The signals obtained after hybridization of gel blots containing total *Arabidopsis* RNA with 5' α-<sup>32</sup>P-dATP-labeled gene-specific DNA probes for *PORA*, *PORB*, or with an unspecific probe for the actin (*ACT*) gene family were quantitated using a PhosphorImager (Molecular Dynamics, Krefeld, Germany) and associated software. Total POR protein was detected by exposing gel blots incubated with a 1:500 dilution of a polyclonal anti-*Arabidopsis* POR antiserum and subsequently processed with a BM chemiluminescence western blotting kit (Boehringer Mannheim, Rotkreuz, Switzerland) to BIOMAX MR film (Eastman Kodak, Rochester, NY). Images obtained in this fashion were quantitated by using the PhosphorImager.

### Transmission Electron Microscopy

Cotyledons of 4-day-old dark-grown seedlings were harvested, fixed, and embedded, and their plastid ultrastructure was examined by transmission electron microscopy, as described previously (Lebedev et al., 1995; Sperling et al., 1997). In the case of dark-grown *cop1*/PBO-1 seedlings, which were heterozygous for the *PORB* transgene, one cotyledon of each pair was harvested from several different plants and embedded in Spurr's resin (Fluka Chemie, Buchs, Switzerland). The seedlings from which cotyledons had been removed were thereafter transferred to a kanamycin-containing Petri plate and maintained under 16-hr-day/8-hr-night cycles for 1 week at a W light fluence rate of 80 µE m<sup>-2</sup> sec<sup>-1</sup> to assay for antibiotic resistance. Only the excised cotyledons prepared from seedlings ultimately demonstrated to be kanamycin resistant were analyzed further by electron microscopy.

### In Situ Low-Temperature Fluorescence Spectroscopy

In situ low-temperature (LT) fluorescence emission spectra of 4-day-old dark-grown seedlings were collected before and after a light flash from a photographic flash attachment to convert photoactive Pchl<sub>ide</sub> to Chl<sub>ide</sub>. For each set of preflash and postflash measurements, one (*cop1*, *cop1*/PAO-3, and *cop1*/PBO-1) or three (wild type, PAO-3, and PBO-1) pairs of cotyledons were harvested, immobilized on a copper strip, and immediately frozen in liquid nitrogen. The strip was then placed in a homemade LT fluorescence accessory mounted on an LS 50 luminescence spectrophotometer (Perkin-Elmer Cetus, Bucks, UK) and adjusted such that the cotyledons were positioned ~1 cm above the level of the liquid nitrogen and maintained at a temperature of -190 to -180°C for the duration of the measurement. To allow a direct comparison of fluorometrically detectable pigment forms before and after flash treatment, we removed the immobilized

cotyledons from the liquid nitrogen, allowed them to reach  $-20^{\circ}\text{C}$ , subjected them to a 1-msec flash from a distance of 2 cm, and immediately returned them to liquid nitrogen. Postflash fluorescence emission measurements were then collected.

Emission spectra were recorded using excitation and emission slit widths of 10 and 5 nm, respectively, and an excitation wavelength of 440 nm to detect both Pchl(ide) and Chlide pigment species. When indicated, the excitation wavelength was set at 465 nm to enhance the relative fluorescence intensity of photoactive Pchl(ide)-F655 (Ignatov et al., 1983). The excitation and emission beams were passed through two OG 590 or two BG 14 glass cutoff filters (Spindler and Hoyer, Göttingen, Germany), respectively. The spectra obtained were corrected to account for the baseline and the photomultiplier sensitivity at different wavelengths, smoothed, and normalized with respect to the reflected stray light intensity at 610 nm. Difference spectra were calculated between the preflash and postflash measurements normalized with respect to the nonphotoactive Pchl(ide) fluorescence at 632 nm. LT fluorescence emission measurements were repeated at least three times using independent sets of cotyledons for each of the seedling genotypes examined.

#### Measurements of Total Pchl(ide) and Photoactive Pchl(ide) Concentrations

Quantitative analyses of Pchl(ide) content in 4-day-old dark-grown seedlings and Pchl(ide) and Chlide contents in flash-treated dark-grown seedlings were performed. Total pigments were extracted in the presence of 1 mL of 80% acetone/20% water (v/v) from three pairs of cotyledons of etiolated wild-type, PAO-3, and PBO-1 seedlings or one pair of cotyledons from dark-grown *cop1*, *cop1/PAO-3*, and *cop1/PBO-1* seedlings. Room temperature fluorescence emission spectra of the pigment extracts were recorded with the LS 50 luminescence spectrophotometer set at an excitation wavelength of 433 nm. The excitation and emission slit widths were 10 and 5 nm, respectively. Room temperature fluorescence emission measurements were repeated at least three times using independent sets of cotyledons for each of the seedling genotypes examined.

The total Pchl(ide) content of dark-grown cotyledons was calculated from the fluorescence emission intensity at 634 nm. Calibration was performed using an absorption coefficient of  $30.4 \text{ mM}^{-1} \text{ cm}^{-1}$  (Brouers and Michel-Wolwertz, 1983). The concentration of photoactive Pchl(ide) was obtained from the pigment extracts of flash-treated cotyledons by measuring the Chlide fluorescence intensity at 672 nm, after subtraction of the Pchl(ide) fluorescence emission at that wavelength. The Chlide fluorescence intensity was then converted to the fluorescence intensity of an equal amount of Pchl(ide) using a coefficient that was established by comparing Pchl(ide) and Chlide fluorescence intensities of pigment extracts of barley etioplasts before and after a 1-msec light flash (data not shown).

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**Etioplast Differentiation in Arabidopsis: Both PORA and PORB Restore the Prolamellar Body and Photoactive Protochlorophyllide–F655 to the *cop1* Photomorphogenic Mutant**

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