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Far-Red Light Blocks Greening of Arabidopsis Seedlings via a Phytochrome A-Mediated Change in Plastid Development

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We have characterized a far-red-light response that induces a novel pathway for plastid development in Arabidopsis seedlings. This response results in the inability of cotyledons to green upon subsequent white light illumination, and the response is suppressed by exogenous sucrose. Studies with mutants showed that this far-red block of greening is phytochrome A dependent and requires an intact downstream signaling pathway in which FHY1 and FHY3 may be components but in which HY5 is not. This highlights a previously undefined branchpoint in the phytochrome signaling pathway. Ultrastructural analysis showed that the far-red block correlates with both the failure of plastids to accumulate prolamellar bodies and the formation of vesicles in the stroma. We present evidence that the far-red block of greening is the result of severe repression of protochlorophyllide reductase (*POR*) genes by far-red light coupled with irreversible plastid damage. This results in the temporal separation of phytochrome-mediated *POR* repression from light-dependent protochlorophyllide reduction, two processes that normally occur in coordination in white light.

INTRODUCTION

The deetiolation response of dark-grown seedlings is a complex developmental program initiated and maintained by the onset of illumination. This process comprises changes in seedling morphology and physiology mediated by the light-regulated expression of a wide range of genes. In dark-grown seedlings of Arabidopsis, deetiolation is characterized morphologically by a rapid inhibition of hypocotyl elongation, opening and expansion of the cotyledons, and accumulation of the photosynthetic pigment chlorophyll. These immediate responses to illumination are followed by the development of the adult structures of the normal light-grown plant.

The opening and expansion of cotyledons and the inhibition of hypocotyl elongation involve changes in cell shape, size, and organization. Accumulation of chlorophyll, however, signifies a change in the composition and structure of plastids, resulting in the formation of mature chloroplasts. The development of functional chloroplasts is directed largely by the coordinated targeting, import, and assembly of an array of nuclear-encoded components of the developing plastid (reviewed in Soll and Alefsen, 1993). In seedlings germinated and grown in continuous light, mature chloroplasts develop in the cotyledons directly from proplastids, which are in a relatively undifferentiated state (Bowman, 1994).

In seedlings grown in the dark, however, the plastids of the cotyledons develop into non-green etioplasts containing a highly organized paracrystalline membrane system, termed the prolamellar body (PLB; Chory et al., 1989; Ryberg and Sundqvist, 1991). The PLB is composed primarily of lipid and contains ∼25% protein (Lütz and Nordman, 1983), of which the predominant polypeptide is NADPH:protochlorophyllide oxidoreductase (POR; EC 1.6.99.1) (Ikeuchi and Murakami, 1983). This enzyme catalyzes the sole light-dependent step in chlorophyll biosynthesis, which is the photoreduction of protochlorophyllide to chlorophyllide (Griffiths, 1978, 1991). Indeed, the majority of total POR protein in etiolated tissue is localized within the membranes of the PLB and exists as a ternary complex with protochlorophyllide and NADPH (Shaw et al., 1985; Ryberg and Dehesh, 1986; Griffiths, 1991; Ryberg and Sundqvist, 1991). The highly ordered membranes of the PLB therefore provide a pool of protochlorophyllide that is reduced immediately to chlorophyllide when illuminated, allowing the production of chlorophyll.

Although the precise mechanism of this reaction is unclear, it is dependent on the absorption of light by protochlorophyllide itself, which drives the transfer of electrons from NADPH to the substrate; the absorption maximum of the protochlorophyllide–POR–NADPH complex is 638 nm (Griffiths, 1991). After the onset of illumination, which initiates the etioplast-to-chloroplast transition, the activity of POR decreases dramatically via changes in POR protein and *POR* mRNA levels (Mapleston and Griffiths, 1980; Apel, 1981; Santel and Apel, 1981; Batschauer and Apel, 1984; Armstrong et al., 1995). During the development of chloroplasts from seedlings grown in

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continuous light, POR complexes do not accumulate in sufficient amounts to aggregate. Whether seedlings are germinated in the dark or in continuous light, however, the formation of functional chloroplasts during photomorphogenesis is crucially dependent on POR activity because it results in the formation of chlorophyll, which is a prerequisite for the development of thylakoid membranes (Bellemare et al., 1982).

Germination and growth of Arabidopsis seedlings in continuous far-red light (FR) can induce partial photomorphogenic development without the accumulation of chlorophyll. Under these conditions, hypocotyl elongation is inhibited and the cotyledons are open (Whitelam et al., 1993). These responses are characteristic of the classic far-red high-irradiance response (FR-HIR). The extent of such responses is dependent on a number of factors, including wavelength, fluence rate, and duration of illumination by FR (Mancinelli, 1994; Smith, 1995). It is generally accepted that the FR-HIR is mediated by phytochrome (phy) A (Smith, 1995). Recently, Dubell and Mullet (1995a, 1995b) showed that continuous FR irradiation can activate the transcription of chloroplast genes and replication of plastid DNA. Thus, phyA-mediated induction of selected facets of plastid development may occur in the absence of conditions that allow the synthesis of chlorophyll.

It has been shown previously that seedlings of both tomato and Arabidopsis grown under these conditions not only are unable to accumulate chlorophyll in FR but also are unable to green when exposed subsequently to continuous white light (Wc), although the mechanism was not understood (Barnes et al., 1995; Van Tuinen et al., 1995). In this study, we characterize this phenomenon in detail in a range of putative phytochrome signaling mutants, and we show that FR irradiation blocks greening of Arabidopsis via the formation of aberrant plastids in developing cotyledons. This process requires functional phyA and an intact phyA signal transduction pathway, and the process is suppressed by the growth of seedlings in a sucrose-containing medium. We present compelling evidence that FR blocks greening by preventing the accumulation of POR via repression of genes encoding this protein. The irreversible nature of the FR block may be the result of ensuing degradation of plastid components, signified by the formation of vesicles in the stroma.

RESULTS

FR Irradiation of Arabidopsis Seedlings Blocks Greening of Cotyledons via a phyA-Dependent Response

Arabidopsis seedlings were grown for 5 days in the dark or 1 day in darkness followed by 4 days in FR. Consistent with well-established phenotypes in these conditions, seedlings that had been grown for 5 days in the dark were etiolated, possessing elongated hypocotyls and closed cotyledons. Wild-type Landsberg *erecta* (*Ler*), which had been grown for 1 day in darkness followed by 4 days in FR, displayed the expected FR-HIR phenotype—short hypocotyls and open cotyledons. The *phyB* mutant was indistinguishable from the wild type following growth in FR, whereas the FR-insensitive *phyA* mutant was etiolated. Furthermore, the putative phyA signal transduction mutant *fhy1* displayed long hypocotyls but had partially open cotyledons, consistent with previous observations (Whitelam et al., 1993).

Figure 1A shows that following growth of seedlings for an additional 4 days in Wc, all dark-grown seedlings possessed open and expanded green cotyledons. The amounts of chlorophyll accumulated in wild-type, *phyA*, and *fhy1* seedlings were similar, although *phyB* seedlings accumulated less (Figure 1C). By contrast, the open cotyledons of wild-type and *phyB* seedlings grown in FR failed to green after 4 days in Wc (Figure 1B). Chlorophyll accumulation was negligible in the wild type and was undetectable in *phyB* (Figure 1D). Both *phyA* and *fhy1* seedlings grown in FR, however, developed open, expanded cotyledons after 4 days in Wc (Figure 1B), accumulating amounts of chlorophyll that were indistinguishable from those of *phyA* and *fhy1* seedlings grown initially in darkness (Figure 1D). Figure 2 shows that the response of a *phyA–phyB* mutant grown for 1 day in darkness followed by 4 days in FR was similar to that of the *phyA* mutant, with the double mutant retaining the ability to form open cotyledons that accumulated chlorophyll after 4 days in Wc. In the same experiment, we examined the response of the long hypocotyl *hy5* mutant (Koornneef et al., 1980), which is thought to be deficient in a light-signaling component (Chory, 1992). Although *hy5* displayed an elongated hypocotyl after 1 day in darkness followed by 4 days in FR, the cotyledons did not green after an additional 4 days in Wc (Figure 2A) and chlorophyll accumulation was negligible (Figure 2B).

FR Block of Greening Is Dependent on the Duration of FR Irradiation

To investigate the influence of a range of FR exposure times on subsequent greening, *Ler*, *phyA*, *fhy1*, and *phyB* seedlings were grown for 1 day in darkness followed by 1 to 4 days in FR. The ability of seedlings to green was then assayed by quantification of chlorophyll after growth for 4 days in Wc (data not shown). Both wild-type and mutant seedlings grown for 1 day in darkness followed by 1 day in FR greened efficiently during subsequent Wc illumination, but after 1 day in darkness followed by 2 days in FR, wild-type and *phyB* seedlings failed to green. Under these conditions, only *phyA* and *fhy1* seedlings accumulated chlorophyll; these mutants retained the ability to green even after 1 day in darkness followed by 4 days in FR.

FR Block Occurs during the FR Irradiation Period

It is possible that the loss of ability to green following treatment with FR was the result of an FR-induced increase in

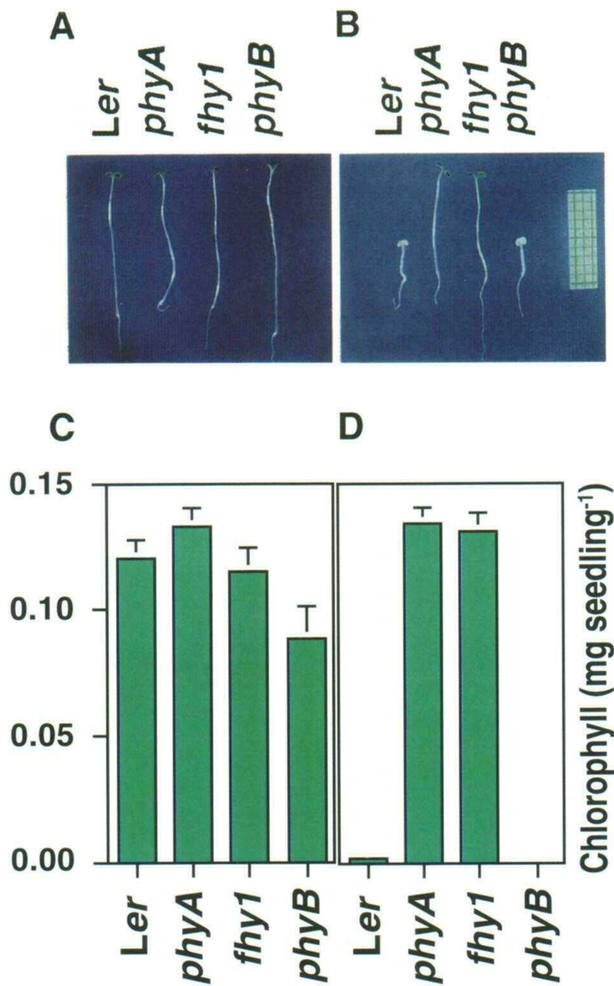

Figure 1. Greening of *Ler*, *phyA*, *fhy1*, and *phyB* Seedlings.

Seedlings were grown on Murashige and Skoog medium for 5 days in darkness or 1 day in darkness followed by 4 days in continuous FR. Seedlings were then transferred to Wc for an additional 4 days before analysis of greening.

(A) Morphology of seedlings grown for 5 days in darkness followed by 4 days in Wc.

(B) Morphology of seedlings grown for 1 day in darkness followed by 4 days in FR and an additional 4 days in Wc.

(C) Chlorophyll content of seedlings treated as given in **(A)**.

(D) Chlorophyll content of seedlings treated as given in **(B)**.

In **(C)** and **(D)**, three replicate samples of 20 seedlings were harvested for chlorophyll measurements. Error bars indicate standard errors. Scale in **(B)** = 10 mm for **(A)** and **(B)**.

sensitivity to Wc, leading to photobleaching. To address this possibility, we investigated the greening response of *Ler*, *phyA*, and *fhy1* seedlings grown for 1 day in darkness and 2 days in FR followed by a range of Wc fluence rates (0.9 to 4.9 Wm^{-2}). Visual inspection of seedlings showed that only *phyA* and *fhy1* mutants retained the ability to green after 1 day in darkness and 2 days in FR, regardless of subsequent Wc fluence rates (data not shown). To investigate further whether conditions subsequent to FR treatment could influence the ability to green, seedlings that had been grown for 1 day in

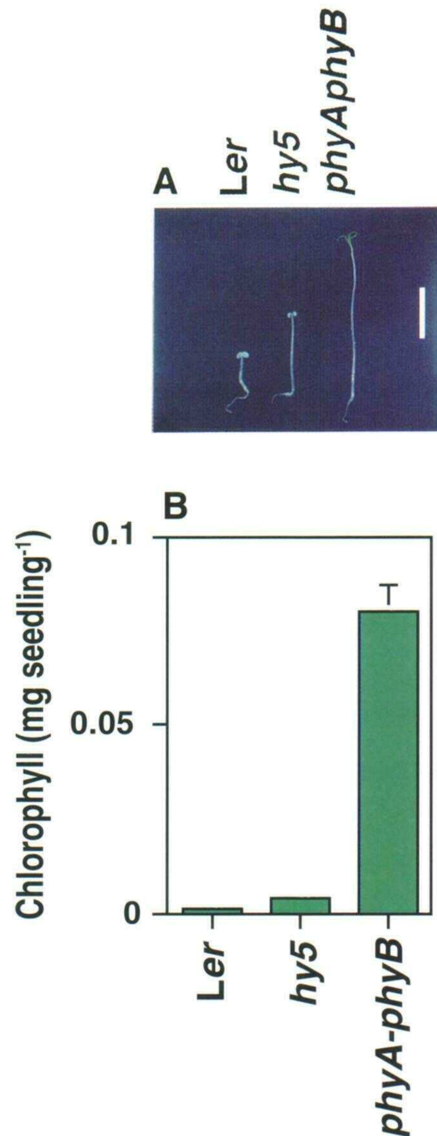

Figure 2. Greening of *Ler*, *hy5*, and *phyA–phyB* Seedlings.

Seedlings were grown on Murashige and Skoog medium for 1 day in darkness followed by 4 days in continuous FR and an additional 4 days in Wc before analysis of greening.

(A) Morphology of seedlings. Bar = 5 mm.

(B) Chlorophyll content of seedlings. Three replicate samples of 20 seedlings were harvested for chlorophyll measurements. Error bars indicate standard errors.

darkness followed by 2 days in FR were transferred back into darkness for 2 days before being grown in Wc. The greening response of seedlings was not affected by this treatment; only *phyA* and *fhy1* seedlings retained the ability to accumulate chlorophyll after 1 day in darkness and 2 days in FR (data not shown). Thus, the FR block of greening is activated during the FR treatment, cannot be reversed by subsequent dark treatment, and is insensitive to Wc fluence rate.

Sucrose Suppresses the FR Block of Greening

Growth of Arabidopsis seedlings on media containing sucrose suppresses the effect of FR irradiation on hypocotyl elongation such that wild-type plants possess longer hypocotyls, resembling those of *fhy* mutants (Whitelam et al., 1993). Therefore, we examined the effect of sucrose on the FR block of greening. Figure 3A shows that L*er*, *phyA*, *fhy1*, and *phyB* seedlings grown on medium containing 90 mM (3% [w/v]) sucrose for 5 days in darkness, followed by 2 days in Wc, developed open cotyledons that accumulated chlorophyll (Figure 3C). The amounts of chlorophyll accumulated in both the wild type and mutants were similar and were approximately fivefold greater than the amounts accumulated in the absence of sucrose (compare Figures 1 and 3, and note the difference in scale). In contrast to seedlings grown in FR in the absence of sucrose (Figure 1B), cotyledons of wild-type and *phyB* seedlings grown for 1 day in darkness followed by 4 days in FR became fully green after 2 days in Wc (Figure 3B). As expected, *phyA* and *fhy1* seedlings also retained the ability to green after similar irradiation with FR. Measurements of chlorophyll levels (Figure 3D) showed that the chlorophyll content of wild-type seedlings grown in FR was almost threefold greater than that of wild-type seedlings that had been grown initially in darkness. Increased chlorophyll content may have been the result of increased cotyledon expansion. To rule out the possibility that sucrose simply promoted the greening response during subsequent growth in Wc, seedlings grown for 1 day in darkness followed by 4 days in FR in the absence of sucrose were transferred to medium containing 90 mM sucrose at the onset of Wc illumination. This treatment did not stimulate greening (data not shown), indicating that the suppressive effect of sucrose was exerted during the period of FR irradiation.

It is possible that the basis for the mechanism of the FR block was the starvation of seedlings during accelerated development in FR. If so, sucrose would suppress this effect by providing a nutrient source. To test this hypothesis, we repeated the experiment described in Figure 3 but exposed seedlings to longer periods of FR. Figure 4 shows that all seedlings grown for 10 days in darkness in the presence of sucrose were able to green upon subsequent exposure to Wc (Figures 4A and 4C). When seedlings were grown for 1 day in darkness followed by 9 days in FR, only the cotyledons of *phyA* and *fhy1* seedlings were able to green (Figure 4B), although *fhy1* seedlings accumulated less chlorophyll than did seedlings grown

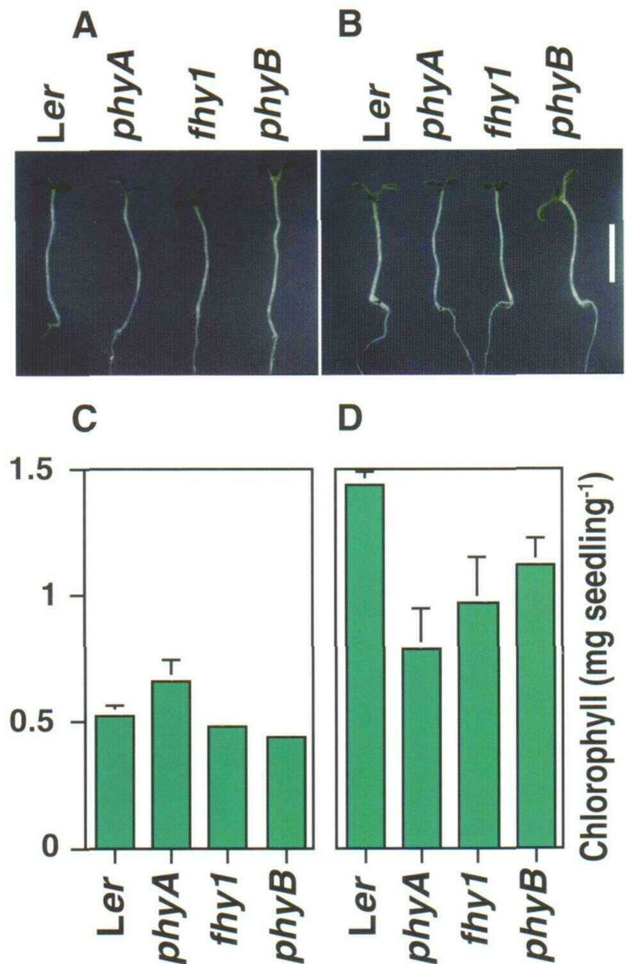

Figure 3. Greening of L*er*, *phyA*, *fhy1*, and *phyB* Seedlings in the Presence of Sucrose.

Seedlings were treated as described in Figure 1, except that seedlings were grown on Murashige and Skoog medium containing 90 mM sucrose.

(A) Morphology of seedlings grown for 5 days in darkness followed by 2 days in Wc.

(B) Morphology of seedlings grown for 1 day in darkness followed by 4 days in FR and an additional 2 days in Wc.

(C) Chlorophyll content of seedlings treated as given in **(A)**.

(D) Chlorophyll content of seedlings treated as given in **(B)**.

In **(C)** and **(D)**, three replicate samples of 20 seedlings were harvested for chlorophyll measurements. Error bars indicate standard errors. Bar in **(B)** = 5 mm for **(A)** and **(B)**.

in darkness (Figure 4D). In these conditions, although the cotyledons of wild-type and *phyB* seedlings were unable to green, these seedlings were able to develop green true leaves upon illumination with Wc (Figure 4B). Clearly, FR irradiation may block subsequent greening of cotyledons even in the pres-

ence of sucrose, suggesting that the FR block of greening is unlikely to result from starvation of seedlings.

FR Irradiation Results in Aberrant Plastid Development

In an effort to understand the effect of FR on developing seedlings, we analyzed the ultrastructure of cotyledon tissue in *Ler*, *phyA*, *fhy1*, and *phyB* seedlings by electron microscopy. In the first experiment, seedlings were grown for 5 days in darkness or 1 day in darkness followed by 4 days in FR, as described for Figure 1. Figures 5A, 5C, 5E, and 5G show that following growth for 5 days in darkness, plastids in the mesophyll cells of cotyledon tissue from all seedlings contained large, highly developed PLBs typical of etioplasts. Clusters of phytoferritin also were visible. Plastids in the cotyledons of wild-type seedlings grown for 1 day in darkness followed by 4 days in FR, however, showed aberrant development (Figure 5B). Plastids did not contain large, regular PLBs, and only a small number of prothylakoid membranes were visible. In addition, unusual vesicles developed in the stroma. These vesicles (100 to 150 nm in diameter) were bound by a single membrane and contained electron-dense material. The absence of a PLB and the formation of vesicles also were observed consistently in plastids from *phyB* seedlings (Figure 5H). By contrast, plastids in the cotyledons of *phyA* and *fhy1* seedlings grown for 1 day in darkness followed by 4 days in FR contained large PLBs similar to those of plants grown continuously in the dark (Figures 5D and 5F, respectively). Furthermore, these plastids did not accumulate aberrant vesicles.

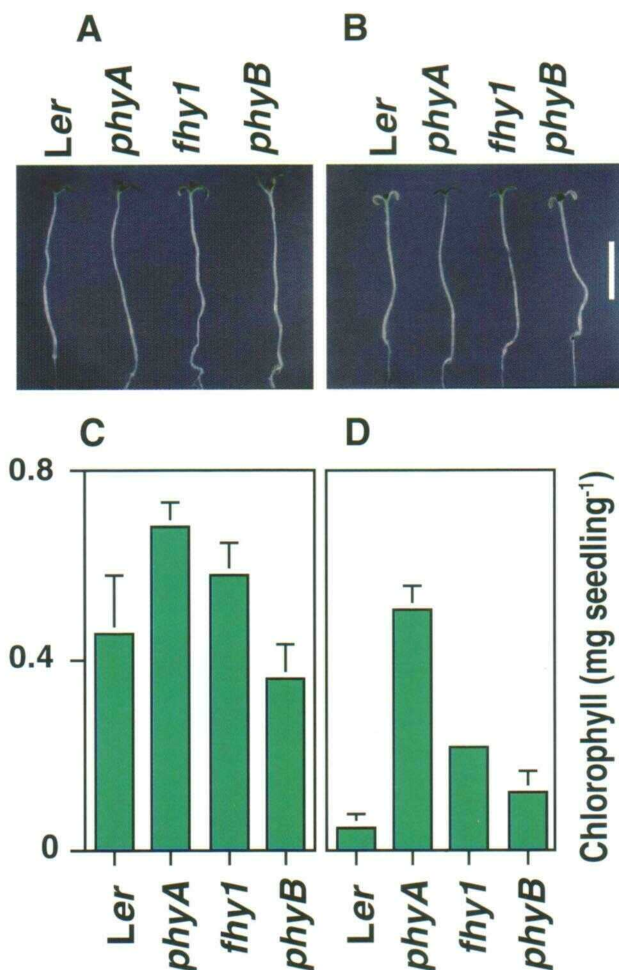

Figure 4. Greening of *Ler*, *phyA*, *fhy1*, and *phyB* Seedlings in the Presence of Sucrose after Prolonged FR Irradiation.

Seedlings were grown on Murashige and Skoog medium containing 90 mM sucrose for 10 days in darkness or 1 day in darkness followed by 9 days in continuous FR, followed by an additional 4 days in Wc.
(A) Morphology of seedlings grown for 10 days in darkness followed by 4 days in Wc.
(B) Morphology of seedlings grown for 1 day in darkness followed by 9 days in continuous FR and an additional 4 days in Wc.
(C) Chlorophyll content of seedlings treated as given in **(A)**.
(D) Chlorophyll content of seedlings treated as given in **(B)**.
In **(C)** and **(D)**, three replicate samples of 20 seedlings were harvested for chlorophyll measurements. Bar in **(B)** = 5 mm for **(A)** and **(B)**.

Figure 6 shows that after growth for 1 day in darkness followed by 4 days in FR, cells possessed large central vacuoles and retained lipid storage bodies, indicating that some endogenous nutrient reserves were available, supporting our previous conclusion that the FR block of greening is not the result of starvation. Plastid number and size were not markedly different in cells from the wild type or mutants (data not shown). Some plastids in wild-type seedlings retained small PLBs, but the structure was irregular, and aberrant vesicle formation was observed in almost all plastids (Figure 6A). A large number of such vesicles were present in plastids from *phyB* seedlings (Figure 6D). In contrast, almost all plastids from *phyA* and *fhy1* seedlings contained PLBs (Figures 6B and 6C).

To investigate the role of sucrose, we performed a similar ultrastructural analysis with seedlings grown on sucrose medium, as described for Figure 3. Figure 7 shows that after growth of seedlings for 5 days in the presence of sucrose, plastids from all seedlings contained starch granules, storage proteins remained within the vacuole, and numerous lipid bodies were present (data not shown). This is in contrast to seedlings grown in the absence of sucrose. Similar to the results obtained with seedlings grown in the absence of sucrose, plastids in the cotyledons of all seedlings grown in the dark contained well-defined PLBs (Figures 7A, 7C, 7E, and 7G). As expected, plastids from *phyA* and *fhy1* seedlings grown in FR closely resembled those of seedlings grown in the dark (Figures 7D and 7F), and plastids in wild-type and *phyB* seedlings grown in FR did not contain large PLBs (Figures 7B and 7H). The aberrant vesicles that accumulated in the plastids of wild-type and *phyB* seedlings grown in the absence of sucrose (Figures 5B, 5H, 6A, and 6C), however, were not present under these conditions.

To examine further whether the absence of a PLB and formation of aberrant vesicles correlated with the loss of ability

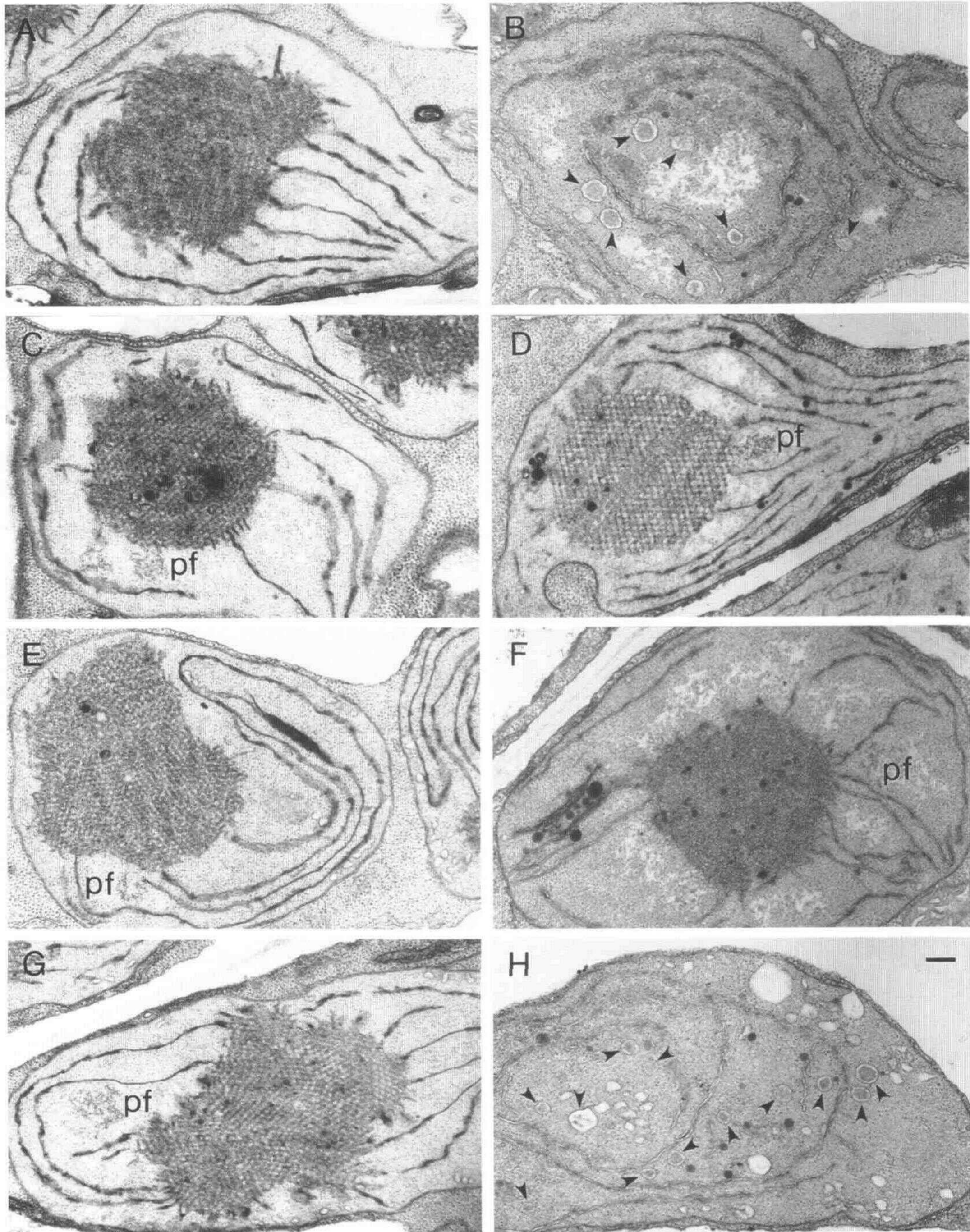

Figure 5. Plastids in Mesophyll Cells of Cotyledons.

Ler, *phyA*, *fhy1*, and *phyB* seedlings were grown for 5 days in darkness or for 1 day in darkness followed by 4 days in FR on Murashige and Skoog medium in the absence of sucrose.

(A) and **(B)** L*er* in dark and FR.

(C) and **(D)** *phyA* in dark and FR.

(E) and **(F)** *fhy1* in dark and FR.

(G) and **(H)** *phyB* in dark and FR.

Magnification for **(A)** to **(H)** is ×21,250; bar in **(H)** = 200 nm. pf indicates phytoferritin; arrowheads indicate aberrant vesicles.

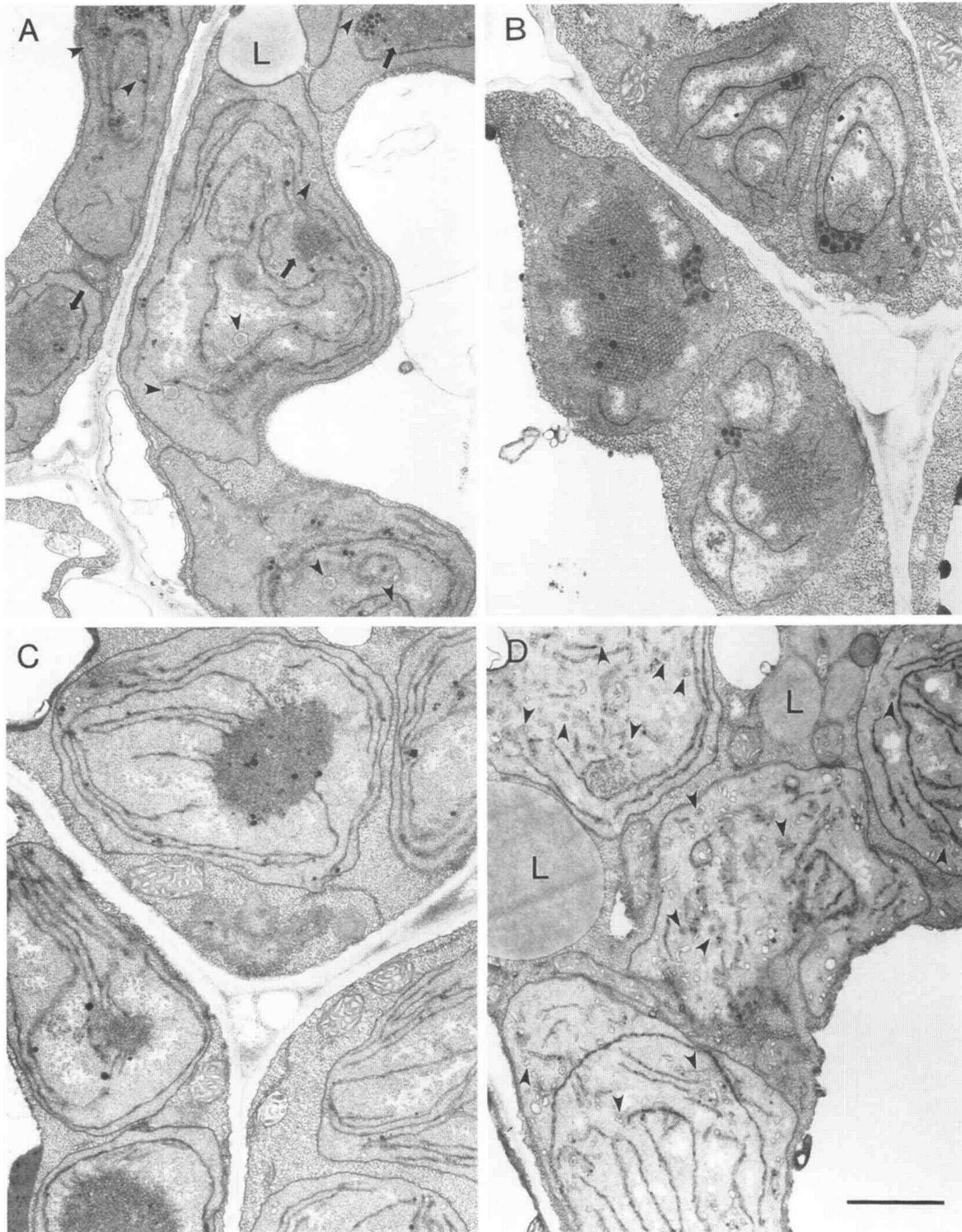

Figure 6. Lower Magnification of Mesophyll Cells in Cotyledons.

Ler, *phyA*, *fhy1*, and *phyB* seedlings were grown for 1 day in darkness followed by continuous FR for 4 days on Murashige and Skoog medium in the absence of sucrose.

(A) *Ler.*

(B) *phyA.*

(C) *fhy1.*

(D) *phyB.*

Magnification for **(A)** to **(D)** is ×14,025; bar in **(D)** = 1 μm. L indicates lipid bodies; arrowheads indicate aberrant vesicles; arrows indicate PLBs.

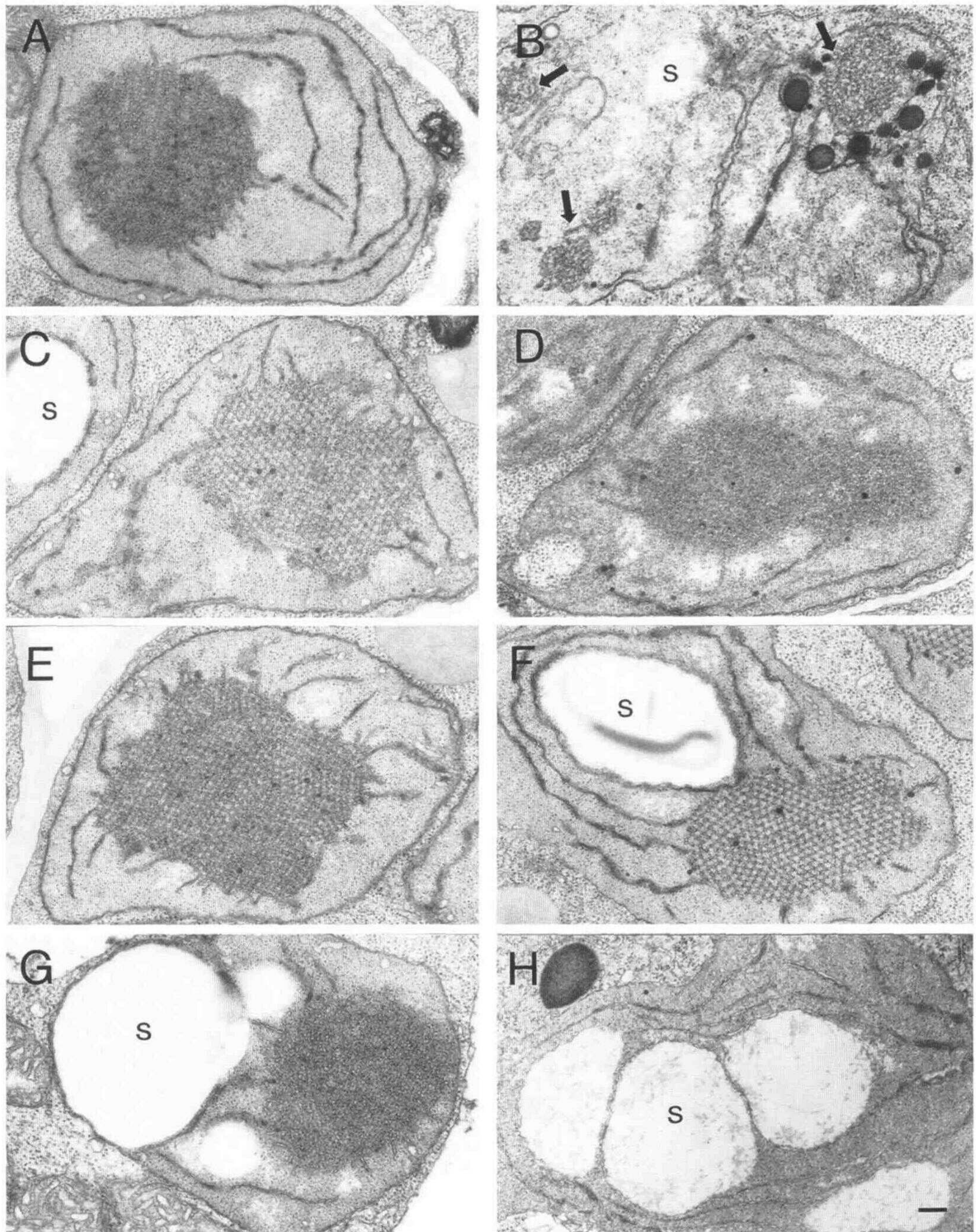

Figure 7. Plastids in Mesophyll Cells of Cotyledons from Seedlings Grown in the Presence of Sucrose.

Ler, *phyA*, *fhy1*, and *phyB* seedlings were grown for 5 days in darkness or for 1 day in darkness followed by continuous FR for 4 days on Murashige and Skoog medium containing 90 mM sucrose.

(A) and **(B)** Ler in dark and FR.

(C) and **(D)** *phyA* in dark and FR.

(E) and **(F)** *fhy1* in dark and FR.

(G) and **(H)** *phyB* in dark and FR.

Magnification for **(A)** to **(H)** is ×21,250; bar in **(H)** = 200 nm. s indicates starch granules; arrows indicate small PLBs.

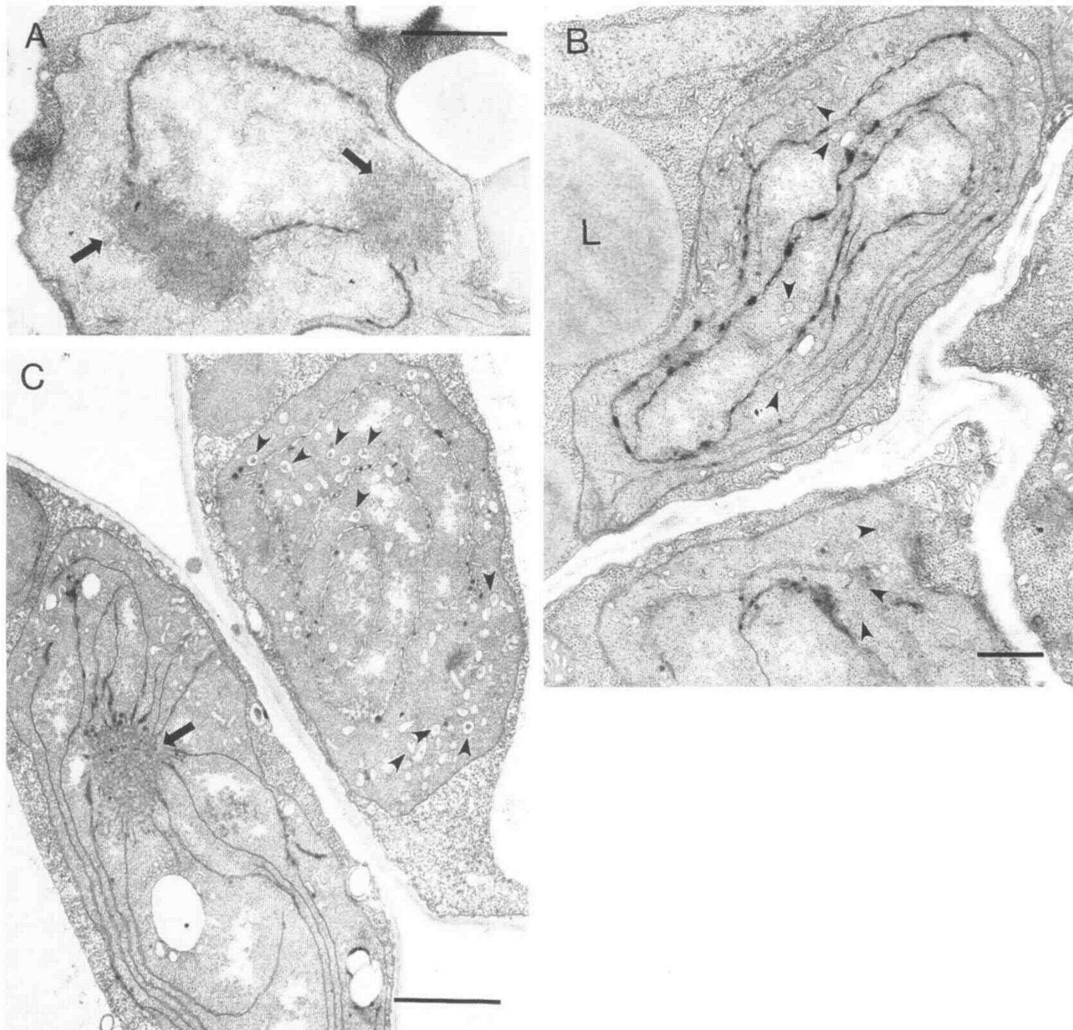

Figure 8. Time Course Experiment for Plastid Development in Wild-Type Seedlings.

Wild-type (*Ler*) seedlings were grown on Murashige and Skoog medium in the absence of sucrose for 1 day in the dark followed by 1 to 3 days of FR.
(A) One day of darkness followed by 1 day of FR.
(B) One day of darkness followed by 2 days of FR.
(C) One day of darkness followed by 3 days of FR.
Magnification for **(A)** and **(C)** is ×14,025; bars = 1 μm. Magnification for **(B)** is ×17,000; bar = 500 nm. L indicates lipid bodies; arrowheads indicate aberrant vesicles; arrows indicate PLBs.

to green, we performed an ultrastructural analysis of plastids from wild-type seedlings grown for 1 day in darkness followed by 1 to 3 days in FR in the absence of sucrose. Figure 8A shows that after 1 day in darkness followed by 1 day in FR, plastids contained small but regular PLBs, which presumably accumulated during the first day of growth in the dark. Furthermore, plastid size and number were low, and cells of wild-type seedlings were occupied by partially digested protein bodies and numerous lipid bodies (data not shown). After 1 day in darkness followed by 2 days in FR, the formation of a small number of aberrant vesicles was visible (Figure 8B). Analysis of tissue after 1 day in darkness followed by 3 days in FR showed that plastids did retain small, irregular PLBs, although aberrant vesicles were abundant at this stage. Results, from experiments in which sucrose was included in the medium (Figure 7), suggest that the absence of an extensive PLB is necessary but not sufficient for the FR block of greening to occur. Results from Figures 7 and 8 demonstrate the correlation between the loss of ability to green and the appearance of aberrant vesicles in the stroma.

FR Irradiation Blocks the Accumulation of POR Protein

Our ultrastructural analysis suggested that the absence of large PLBs is necessary for the FR block to occur. Furthermore, the time course experiment (Figure 8) indicated that the absence of PLBs in seedlings grown in FR is mostly the result of a failure to accumulate these membrane systems rather than a degradation of extensive, preexisting PLBs. Therefore, we analyzed the accumulation of POR, the major protein component of the PLB, in *Ler*, *phyA*, *fhy1*, and *phyB* seedlings grown in darkness or in FR by protein gel blotting. Figure 9 shows that anti-POR antibodies cross-reacted with a well-defined band migrating at 36 kD (molecular mass markers not shown). This band presumably contains the two closely migrating POR proteins reported by Armstrong et al. (1995). Figure 9 shows that POR protein levels were low in all seedlings grown for 2 days in darkness but accumulated after continued growth for 3 or 5 days in darkness. Similarly, seedlings grown for 1 day in darkness followed by 1 day in FR (equivalent in age to seedlings grown for 2 days in darkness) contained low amounts of POR protein. In contrast to wild-type seedlings grown in darkness, the accumulation of POR protein in FR was lower, and this pattern was more marked in *phyB* seedlings.

The accumulation of POR protein in *phyA* and *fhy1* seedlings grown in FR, however, was similar to that observed when seedlings were grown in the dark. The failure of wild-type and *phyB* seedlings to accumulate large amounts of POR protein correlated with the absence of large PLBs in FR-grown seedlings. The residual amounts of POR were perhaps indicative of the small, irregular PLBs observed in some wild-type plastids (Figure 8C). Analysis of POR protein accumulation in seedlings grown in medium containing 90 mM sucrose showed that sucrose did not markedly affect the levels of POR protein accumulated in the wild type or mutants grown in darkness or continuous FR (data not shown). This was consistent with the observation that growth of seedlings in the presence of sucrose did not affect the pattern of PLB formation in wild-type and mutant seedlings in the dark or in FR (Figure 7).

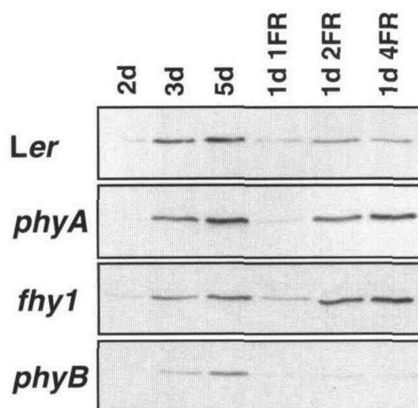

Figure 9. Analysis of POR Protein.

Ler, *phyA*, *fhy1*, and *phyB* seedlings were grown on Murashige and Skoog medium for 2, 3, or 5 days in darkness (2d, 3d, and 5d) or for 1 day in the dark followed by 1, 2, or 4 days in continuous FR (1d 1FR, 1d 2FR, and 1d 4FR). Seedlings were harvested, and POR protein levels were assayed immunologically using protein gel blot analysis. Equal loading of proteins in each lane was verified by staining of duplicate gels.

FR Irradiation Represses the Expression of *POR* Genes

It is possible that the block in POR protein accumulation and PLB formation in FR was the result of a decrease in POR synthesis or an increase in protein turnover. Therefore, we used RNA gel blot analysis to examine the expression of the genes encoding POR (*PORA* and *PORB*) in seedlings grown in the same conditions as those for the protein gel blot analysis. To monitor the time dependence of the FR block of greening, a sample of seedlings grown in each condition was transferred to Wc for 4 days and greening was assessed. As expected, greening of wild-type and *phyB* seedlings was blocked after 1 day in darkness followed by 2 days in FR but not after 1 day in darkness followed by 1 day in FR (data not shown). Figure 10 shows that *PORA* and *PORB* mRNA was abundant in *Ler*, *phyA*, *fhy1*, and *phyB* seedlings grown for 2 days in darkness, after which time both transcripts declined to low levels. There was no appreciable difference between the dark response of *PORA* and *PORB* transcripts, and quantification by PhosphorImager analysis (normalized to 18S rRNA hybridizations) showed that after growth for 5 days in darkness, *PORA* and *PORB* mRNA had declined to 10 to 20% of the levels present after 2 days in darkness.

Some RNA degradation was observed in the sample from *fhy1* seedlings grown for 5 days in darkness, and the small upper band visible in all *PORA* gel blots was the result of nonspecific cross-reaction with 18S rRNA. The high rate of POR protein accumulation between 2 and 3 days in darkness (Figure 9) correlated well with high transcript levels present during this time (Figure 10). When wild-type seedlings were grown for 1 day in darkness followed by FR, *POR* gene expression was repressed dramatically. *PORA* expression was repressed strongly after 1 day in darkness followed by 1 day in FR (Figure 10), with mRNA levels declining to 5% of those present after 2 days in darkness. At the same time, *PORB* mRNA remained detectable, with mRNA levels being ~25% of those after 2 days in darkness. After 1 day in darkness followed by 2 days in FR, *PORB* mRNA declined to 5 to 10% and was mostly undetectable in RNA gel blots (Figure 10).

This pattern of response was similar in *phyB* seedlings, with *PORA* being repressed strongly after 1 day in darkness followed by 1 day in FR and *PORB* mRNA declining to 10% after 1 day in darkness followed by 2 days in FR. Because the FR block of greening was effective in wild-type and *phyB* seed-

lings only after 1 day in darkness followed by 2 days in FR, we concluded that there is a correlation between the presence of *PORB* mRNA and the capacity to develop chloroplasts. In *phyA* and *fhy1* seedlings, the pattern of *PORA* and *PORB* gene expression in FR resembled that observed in darkness, with transcript levels from both genes declining to low levels after growth for 1 day in darkness followed by 4 days in FR.

Sucrose Promotes *POR* Gene Expression in the Dark

Because growth of seedlings in medium containing 90 mM sucrose suppressed the FR block of greening (Figure 3) but did not elicit any marked effect on PLB formation (Figure 7) or POR protein levels, we investigated the effect of sucrose on *POR* gene expression. Comparison of *POR* mRNA levels in seedlings grown in darkness showed that seedlings grown on media containing sucrose possessed higher levels of *PORA* and *PORB* mRNA than did seedlings grown in the absence of sucrose (Figure 10). Quantification of mRNA levels by PhosphorImager analysis showed that this increase was approximately threefold and was similar in all seedlings. Sucrose, however, did not block the repression of *POR* gene expression by FR. In the wild type, after 1 day in darkness followed by 4 days in FR in the presence of sucrose, *PORA* mRNA declined to 5% of levels after 2 days in darkness in the absence of sucrose, but *PORB* mRNA was clearly detectable, with mRNA levels declining to ~20%. This response was very similar in the *phyB* mutant. Thus, sucrose promotes *POR* gene expression in darkness, ensuring that *PORB* transcript is present throughout the whole FR irradiation period, after which plants are exposed to Wc and are able to develop chloroplasts.

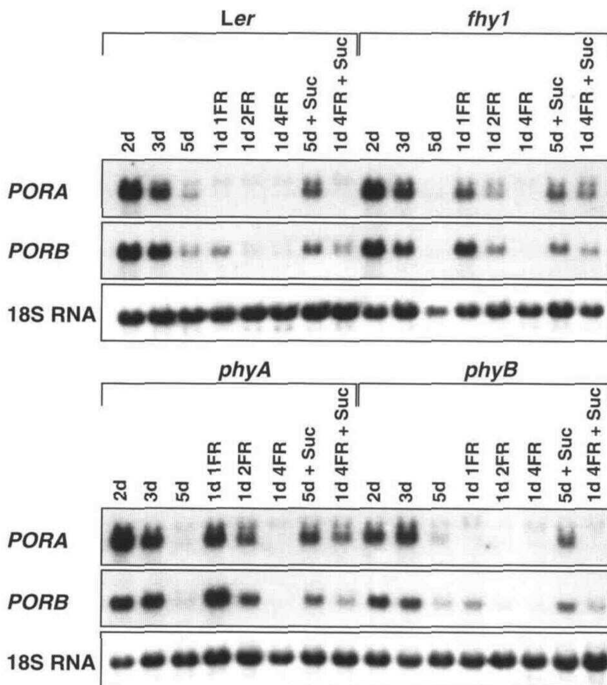

Figure 10. RNA Gel Blot Analysis of *PORA* and *PORB* Expression.

Ler, *phyA*, *fhy1*, and *phyB* seedlings were grown on Murashige and Skoog medium for 2, 3, or 5 days in darkness (2d, 3d, and 5d) or for 1 day in the dark followed by 1, 2, or 4 days in continuous FR (1d 1FR, 1d 2FR, and 1d 4FR). Additional seedlings were germinated and grown on Murashige and Skoog medium containing 90 mM sucrose for 5 days in darkness (5d + Suc) or 1 day in the dark followed by 4 days in continuous FR (1d 4FR + Suc). RNA gel blot analysis was carried out, and membranes were probed with gene-specific probes for *PORA* and *PORB*. As a loading control for total RNA, membranes were reprobed for 18S RNA.

DISCUSSION

FR Block of Greening Defines a Branchpoint in phyA Phototransduction Pathways

We have characterized an FR-induced response in Arabidopsis seedlings that results in the arrest of subsequent plastid development in the cotyledons. Analysis of this FR block of greening showed that *phyA* and *phyA–phyB* mutants were resistant to the damaging effects of FR irradiation, unlike *phyB* mutants, which were sensitive. From these results, we can conclude that the mechanism underpinning the FR block of greening is mediated by phyA and not by phyB. These results are consistent with a wealth of evidence showing that responses in FR are mediated solely by phyA (Smith, 1995). Our results with Arabidopsis are similar to those of Van Tuinen et al. (1995), who showed that wild-type tomato seedlings exposed to FR also lose the ability to accumulate chlorophyll upon transfer to white light. The phyA-deficient *fri* mutants of tomato (Kendrick et al., 1994) are insensitive to FR treatment and retain the ability to accumulate chlorophyll when transferred to Wc, indicating that the phyA-dependent mechanisms that regulate this process may be conserved between species.

The putative phyA signal transduction mutant *fhy1* (Whitelam et al., 1993) was also resistant to the FR block of greening (Figure 1), and additional experiments showed that *fhy3* displayed a similar response (S.A. Barnes, unpublished data). Therefore, this phenomenon is dependent on an intact downstream phyA signal transduction pathway in which FHY1 and FHY3 may be components. Moreover, we have shown that although *hy5*, a putative downstream signal transduction mutant (Koornneef et al., 1980; Chory, 1992), possesses elongated hypocotyls in FR, similar to the *fhy* mutants, it is sensitive to the FR block of greening (Figure 2). Thus, the signal that initiates the FR block of greening is transduced by a pathway in which FHY1 and FHY3 but not HY5 are components. Therefore, the FR block of greening defines a branchpoint in the

phyA signal transduction pathway that was undetectable using hypocotyl elongation assays.

Mechanism of the FR Block

Analysis of the ultrastructure of cotyledons from Arabidopsis seedlings correlated a number of FR-induced changes in plastid morphology with the onset of the FR block of greening (Figures 5 to 8). In all cases, the FR block of greening was accompanied by the absence of a large, distinct PLB and the appearance of aberrant, single membrane–bound vesicles in the stroma. The major protein constituent of the PLB is protochlorophyllide reductase (POR), which catalyzes the sole light-dependent step in chlorophyll biosynthesis. Therefore, it is likely that depletion of POR activity would result in an inability to green. We showed that FR irradiation prevented the accumulation of POR protein to the high levels present in dark-grown seedlings (Figure 9) and that the expression of *PORA* and *PORB* genes was repressed dramatically by FR (Figure 10). This repression was absent in *phyA* and *fhy1* mutants, demonstrating that the regulation of *POR* genes in FR is mediated by phyA and the FHY1 signaling component. In the natural light environment, repression of *POR* genes occurs in coordination with the direct, POR-mediated photoreduction of protochlorophyllide. By irradiating seedlings with FR, we have imposed a temporal separation on these processes, resulting in a depletion of POR activity before subsequent exposure to favorable light conditions. A number of observations, however, suggest that although a depletion of organized PLBs is necessary for the FR block of greening to occur, it is not sufficient to explain *all* of the characteristics of the FR block.

Greening of Cotyledons in Arabidopsis

Dark-grown seedlings accumulate large PLBs, providing a readily available pool of POR complexed with the enzymatic substrates that allow immediate chlorophyll biosynthesis when illuminated. Seedlings germinated and grown in Wc never accumulate large quantities of POR, although they must necessarily contain sufficient POR activity to allow greening. The paradox arising from the repression of *POR* gene expression by Wc when chlorophyll biosynthesis occurs was addressed by Armstrong et al. (1995). They suggested that the lower sensitivity of *PORB* expression to repression by light is sufficient to account for continued chlorophyll biosynthesis under these conditions.

In our experiments, seedlings grown for 5 days in darkness greened efficiently in subsequent Wc. Our analysis with gene-specific probes showed that both *PORA* and *PORB* were expressed abundantly in the wild type and mutants grown in the dark, with mRNA levels for both genes declining similarly with seedling age to very low levels by 5 days in darkness (Figure 10). Although maximal *POR* expression was observed after growth for 2 days in the dark, followed by a steady decline, POR protein levels were high after growth for 3 and 5 days in darkness (Figure 9). This suggests that *POR* gene expression early in development is necessary for accumulation of the PLB; the accumulated PLB then persists after mRNA levels decline and is able to mediate subsequent greening of dark-grown seedlings in the light, a classic etioplast-to-chloroplast transition. Similarly, in FR, the expression of *PORA* and *PORB* in *phyA* and *fhy1* mutants closely resembled the expression pattern in the dark (Figure 10), and seedlings accumulated POR protein (Figure 9) and large PLBs (Figure 5). Therefore, these mutants mimic dark-grown seedlings and are able to green after FR irradiation.

After growth for 1 day in darkness followed by 1 day in FR, wild-type seedlings greened efficiently in Wc, but after 1 day in darkness followed by 2 days in FR there was a dramatic decline in the capacity to green, coinciding with the repression of *PORB* expression to low levels. This suggests that the ability to green is dependent on the expression of *POR* genes at the onset of Wc illumination. Because *PORB* is less sensitive than *PORA* to repression, the loss of greening capacity correlates with the repression of this transcript in particular. A simple lack of *POR* transcript and POR protein cannot, however, explain all characteristics of the FR block of greening. Protein gel blot analysis showed that the POR protein was still detectable in wild-type seedlings after 1 day in darkness followed by 2 days in FR (Figure 9), and ultrastructural analysis showed that small, irregular PLBs were still present after 1 day in darkness followed by 3 days in FR. This POR protein could presumably mediate chlorophyll accumulation.

The striking difference between seedlings grown for 1 day in darkness followed by 1 or 2 days in FR, however, is the onset of vesicle formation in the stroma by 1 day in darkness followed by 2 days in FR (Figure 8). These vesicles may be a morphological manifestation of the onset of degradative processes within the plastid. Therefore, despite the presence of small amounts of POR protein and PLBs, the plastids may be damaged irreversibly, in contrast to seedlings grown for 2 days in darkness containing similar amounts of POR protein but no vesicles. Similarly, after growth for 1 day in darkness followed by 4 days in FR in the presence of sucrose, seedlings also retained the ability to green (Figure 3) as opposed to seedlings grown in the absence of sucrose. Although sucrose did not increase the accumulation of POR or the formation of PLBs (Figure 7), it promoted *POR* gene expression by threefold in the dark and in FR. In particular, *PORB* mRNA was present in seedlings exposed to 4 days of FR, in contrast to seedlings grown similarly in the absence of sucrose. Similar to seedlings grown for 1 day in darkness followed by 1 day in FR in the absence of sucrose, this *PORB* expression presumably allowed sufficient POR synthesis for chlorophyll biosynthesis. Moreover, sucrose suppressed the formation of vesicles in plastids (Figure 7). Thus, the onset of the FR block of greening may be a combination of *PORB* gene expression declining to levels insufficient to allow fresh POR synthesis

to occur at the onset of Wc illumination and an induction of vesicle formation within the plastid.

The onset of vesiculation may signify irreversible degradation of plastids, which is also indicated by the irreversible nature of the FR block of greening. When seedlings were transferred back into darkness following FR treatment, they presumably could have reaccumulated POR, allowing greening upon subsequent transfer to Wc. Indeed, Armstrong et al. (1995) have shown that seedlings illuminated for 2 days could reaccumulate *POR* mRNA following 2 days of darkness. Our experiments, however, showed that seedlings treated with 2 days of FR followed by darkness were unable to green in Wc, suggesting that the FR block is irreversible and indicating a permanent arrest of plastid development.

There is considerable evidence that the rapid decline in POR activity upon illumination of etioplasts is the result of proteolysis (e.g., Santel and Apel, 1981; Kay and Griffiths, 1983). Furthermore, it has been established that growth of Arabidopsis seedlings in the dark leads to a gradual loss of ability to green upon transfer to Wc (Nagatani et al., 1993). El Amrani et al. (1995) have studied the effects of prolonged growth of pea seedlings in the dark and proposed that the loss of greening capacity is associated with increased aminopeptidase activity, resulting in the degradation of PLBs. Recently, Reinbothe et al. (1995) have shown that the degradation of POR, which occurs upon illumination, is mediated by a light-induced, nuclear-encoded protease that is specific for POR. The proplastids that exist in the embryonic cotyledons, however, do not contain PLBs (Bowman, 1994), and our microscopic analysis of seedlings grown for 1 day in darkness followed by 1 day in FR (Figure 8) showed that these young plastids had accumulated only small PLBs. Therefore, the primary effect of FR in our experiments is likely to be that of blocking the accumulation of PLBs rather than mediating the degradation of preexisting PLBs, and this conclusion was confirmed by our protein gel blot analysis (Figure 9). Furthermore, the protease activity characterized by Reinbothe et al. (1995) is dependent on the presence of chlorophyllide, which is not synthesized in FR-grown plants. Therefore, the vesicle formation we observed is unlikely to signify a POR-specific proteolysis and may be the result of more general degradative processes perhaps related to the synthesis of numerous chloroplast components that are turned over rapidly in the absence of chlorophyll (Bellemare et al., 1982).

Clearly, POR is not the sole determinant of chlorophyll content and plastid stability in higher plants. Indeed, Zhang et al. (1992, 1994) have suggested a role for the ankyrin repeat-containing gene *AKR* in the integrity of chloroplasts in Arabidopsis. Decreases in *AKR* expression in plants transformed with antisense and sense *AKR* constructs resulted in bleaching of white light-grown transformants due to the loss of chlorophyll. Plants exhibited this bleaching phenotype only at later stages in development and were normal at the cotyledon stage, suggesting that this phenomenon is different from the processes involved in the FR block of greening. The role of *AKR* therefore may be in the senescence of Arabidopsis chloroplasts at later stages of development and not in the early events of chloroplast biogenesis.

Conclusions and Future Prospects

We have presented evidence that the FR block of greening in the cotyledons of Arabidopsis seedlings is the result of a temporal separation of the phyA-dependent repression of *POR* genes from the white light-dependent photoreduction of protochlorophyllide. In the natural light environment, these processes normally occur in coordination. The irreversible nature of the FR block of greening suggests that permanent arrest of plastid development may occur because of degradative processes, indicated by vesicle formation in the stroma. Proteolytic degradation is not, however, necessary to explain the depletion of POR protein, and vesiculation may represent a more general breakdown of plastid components. This process may be triggered by a depletion of POR itself or by an independent phyA-mediated induction. Both may be suppressed by exogenous sucrose. Further investigation of vesicle formation will address these possibilities.

The question of a physiological role for the FR block of greening is an intriguing point. Pure FR does not exist in the natural environment, and illumination of seedlings with light containing a low R:FR ratio (representing deep canopy shade) fails to block greening (G.C. Whitelam, unpublished results). Therefore, it may be unlikely that the FR block of greening plays a role in the ecology of seedling growth in the natural environment. Regardless of the physiological role of the FR block of greening, we have characterized a process dependent on both phyA and the phyA signal transduction pathway. We used this phenomenon to define a novel branchpoint in the phytochrome signal transduction pathway and to demonstrate the requirement for coordination of early molecular events in the deetiolation process. Based on our conclusions, we have developed a strategy to screen for other mutants deficient in phyA signaling, and we are characterizing mutants that retain the ability to green after FR irradiation.

METHODS

Growth of Plant Material

Landsberg *erecta* (*Ler*) and *phyA* (*phyA-201*; Nagatani et al., 1993), *phyB* (*phyB-1*; Somers et al., 1991; Reed et al., 1993), *fhy1* and *fhy3* (Whitelam et al., 1993), *hy5* (Koornneef et al., 1980), and *phyA-phyB* (*phyA-201phyB-5*; a gift of J. Chory, Salk Institute, San Diego, CA) seeds were surface sterilized and sown onto sterile Murashige and Skoog medium (containing 0 or 90 mM sucrose). Seeds were incubated at 4°C in darkness for 4 days, followed by irradiation for 30 min with continuous white light (Wc) (~2.5 W m^{-2}) provided by cool-white fluorescent bulbs (Gro-Lux F96T12/GRO; Osram Sylvania, Danvers, MA) to promote germination. Plates were transferred to darkness, and seedlings were allowed

to grow for 1 day at 22°C before transfer to far-red light (FR) or continued growth in darkness. FR was provided by F48T12/232/VHO fluorescent bulbs (Osram Sylvania) and filtered through one layer of plexiglass (model 067894; West Lake Plastics, Lenni, PA), yielding an incident FR fluence rate of ~1.0 W m^{-2} unless stated otherwise in the text. During greening experiments, plants were subsequently transferred to Wc (fluence rate ~2.5 W m^{-2} unless stated otherwise in the text). FR fluence rates were measured using a radiometer (model IL-1400A; International Light Inc., Newburyport, MA). Wc fluence rates were measured using an Li-189 radiometer with an Li-190SA quantum sensor (Li-Corp. Inc., Lincoln, NE).

Chlorophyll Measurements

Seedlings were harvested and homogenized in 80% acetone, followed by centrifugation to remove debris. Chlorophyll was determined spectrophotometrically in three replicate samples of 20 seedlings, using the method described by Chory et al. (1991).

Electron Microscopy

Cotyledons were excised and prefixed in 4% paraformaldehyde, 5% glutaraldehyde, 0.1 M $CaCl_2$, 0.1 M cacodylate buffer, pH 7.2, for 3 hr on ice. After washing with the same buffer for 1.5 hr, postfixation was performed in 2% osmium tetroxide, 0.1 M cacodylate buffer for 1 hr on ice. The samples were dehydrated serially in ethanol and propylene oxide and embedded in Epon-Araldite resin (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were cut, stained by uranium and lead, then observed with an electron microscope (model 100C; Jeol Ltd., Tokyo, Japan). At least three plants were examined for each treatment.

Analysis of POR Protein Levels

Frozen seedlings were homogenized and incubated in sample buffer (10% [w/v] glycerol, 10% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue in 80 mM Tris-HCl, pH 8.8) at 65°C for 20 min before centrifugation to remove insoluble debris. Proteins were separated by SDS-PAGE (*resolving gels contained 15% polyacrylamide*). Equal loading of samples was assessed by staining of duplicate gels with Coomassie Brilliant Blue R 250. Proteins were transferred to nylon membranes, and immunodetection was performed using the ECL Western blotting analysis system as described by the manufacturer (Amersham International). Pea anti-POR antibodies were used at a dilution of 1:1000.

RNA Analysis

RNA extraction was performed essentially as described by Kuhlemeier et al. (1988). For RNA gel blot analysis, 10 µg of total RNA was electrophoresed through a denaturing (formaldehyde) 1.2% agarose gel, and RNA was transferred to a Duralon-UV membrane (Stratagene). Hybridization conditions were essentially as described by Lam et al. (1989). cDNA clones encoding *PORA* and *PORB* were obtained by reverse transcriptase–polymerase chain reaction (PCR) amplification of 1 µg of total RNA isolated from 4-day-old dark-grown seedlings by using primers described by Armstrong et al. (1995). The amplification products were cloned in a pT7 Blue vector and sequenced to confirm the identity of *PORA* and *PORB* cDNA. Subsequently, gene-specific probes for *PORA* and *PORB* were obtained by PCR amplification, using primers described by Armstrong et al. (1995). As a loading control for total RNA, we probed blots with an 18S rDNA fragment cloned from *Arabidopsis thaliana* (Takahashi et al., 1995). DNA probes were labeled with ^{32}P-dATP or ^{32}P-dCTP by using the Megaprime DNA labeling kit (Amersham). Quantification of hybridized probes was carried out using a PhosphorImager (model 400E; Molecular Dynamics, Sunnyvale, CA).

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