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ORIGINAL ARTICLE

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Altered etioplast development in phytochrome chromophore-deficient mutants

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Abstract Inhibition of chromophore synthesis in the phytochrome-deficient *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants of tomato (*Solanum lycopersicum* L.) results in a severe reduction of protochlorophyllide (Pchlide) accumulation in dark-grown hypocotyls. Experiments with apophytochrome-deficient mutants indicate that the inhibition of Pchlide accumulation results from two separate effects: one dependent on the activity of phytochromes A and B1 and one phytochrome-independent effect that is attributed to a feedback inhibition of the tetrapyrrole biosynthesis pathway. Cotyledons only show phytochrome-independent inhibition of Pchlide synthesis. Analysis of NADPH:protochlorophyllide oxidoreductase levels by western blotting showed that the reduction in Pchlide in *au* and *yg-2* is accompanied by a correlative, but less substantial, decrease in NADPH:protochlorophyllide oxidoreductase. Consistent with this result, *in vivo* fluorescence spectra demonstrate that both mutants are primarily deficient in non-phototransformable Pchlide. Analysis of etioplast structure indicates that plastid development in *au* and *yg-2* is retarded in hypocotyls and partially impaired in cotyledons, again correlating with the reduction in Pchlide. Since Pchlide synthesis is also reduced in chromophore-deficient mutants of pea (*Pisum sativum* L.) and *Arabidopsis thaliana* (L.) Heynh. (Landsberg *erecta*) these results may be significant for explaining aspects of the phenotype of this mutant class that are independent of the loss of phytochrome.

Keywords Chloroplast development · NADPH:protochlorophyllide oxidoreductase · Phytochromobilin · Prolamellar body · *Solanum* (chloroplast development) · Tetrapyrrole synthesis

Abbreviations AC: Ailsa Craig genetic background · ALA: 5-aminolevulinic acid · *au*: *aurea* mutant · BV: biliverdin · *CAB*: gene encoding chlorophyll *a*/*b*-binding protein · Chl: chlorophyll · MM: Moneymaker genetic background · Pchlide: protochlorophyllide · PΦB: phytochromobilin · POR: NADPH:protochlorophyllide oxidoreductase · WT: wild type · *yg-2*: *yellow-green-2* mutant

Introduction

Phytochrome chromophore-deficient mutants have been used extensively for understanding the role of different photoreceptors in photomorphogenesis (Terry 1997). These mutants, which include *hy1* and *hy2* of arabidopsis (Koornneef et al. 1980; Chory et al. 1989), *aurea* (*au*) and *yellow-green-2* (*yg-2*) of tomato (Koornneef et al. 1985) and *pcd1* and *pcd2* of pea (Weller et al. 1996, 1997), are impaired in their ability to synthesize the linear tetrapyrrole chromophore of phytochrome and are deficient in all members of the phytochrome family of photoreceptors. This phytochrome deficiency is most apparent at the seedling stage when these mutants are almost completely blind to both red and far-red light and have a pale, elongated phenotype under white light (Terry 1997). A pale phenotype is also characteristic of mature mutant plants grown in white light, which remain yellow-green throughout development (Chory et al. 1989; van Tuinen et al. 1996; Weller et al. 1996) due to

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lower chlorophyll (Chl) levels and reduced chloroplast development (Koornneef et al. 1985; Chory et al. 1989). In contrast, other phytochrome-mediated responses in plants of this developmental stage have largely recovered (López-Juez et al. 1990; Weller et al. 1996), leading to the hypothesis that other effects of these mutations, independent of the loss of phytochrome, might contribute to the phenotype of mutants deficient in phytochrome chromophore synthesis (Terry and Kendrick 1996, 1999).

The phytochrome chromophore is synthesized in the plastid from heme in two committed steps (Terry et al. 1993, 1995; Weller et al. 1996; see Fig. 1). In the first, the macrocyclic ring of heme is cleaved by the enzyme heme oxygenase to give the linear tetrapyrrole biliverdin (BV) IXα. This is followed by the reduction of BV IXα to 3(*Z*)-phytochromobilin (PΦB) by the enzyme PΦB synthase (Terry and Lagarias 1991; Terry et al. 1995). 3(*Z*)-PΦB is then isomerized to 3(*E*)-PΦB, the immediate precursor of the bound chromophore, by an, as yet, unidentified PΦB isomerase. However, as the autocatalytic assembly of 3(*E*)-PΦB with apophytochrome (Lagarias and Lagarias 1989) can also occur following incubation with 3(*Z*)-PΦB (Terry et al. 1995; Weller et al. 1996) it is perhaps not surprising that only chromophore-deficient mutants in the first two committed and essential steps in this pathway have been identified. Biochemical analyses have demonstrated that *yg-2* and *pcd1* lack heme oxygenase activity (Terry and Kendrick 1996; Weller et al. 1996) while *au* and *pcd2* are deficient in PΦB synthase activity (Terry and Kendrick 1996; Weller et al. 1997; Fig. 1). Recently, the *HY1* (Davis et al. 1999; Muramoto et al. 1999) and *HY2* (Kohchi et al. 2001) genes have been isolated, and analysis of the encoded proteins indicates that *hy1* and *hy2* also lack heme oxygenase and PΦB synthase, respectively (Muramoto et al. 1999; Kohchi et al. 2001). Further analysis has indicated that both *yg-2* and *pcd1* lack a protein detected by an antibody raised against HY1 (M.J. Terry and T. Kohchi, unpublished results), confirming that heme oxygenase is the primary target of these mutations.

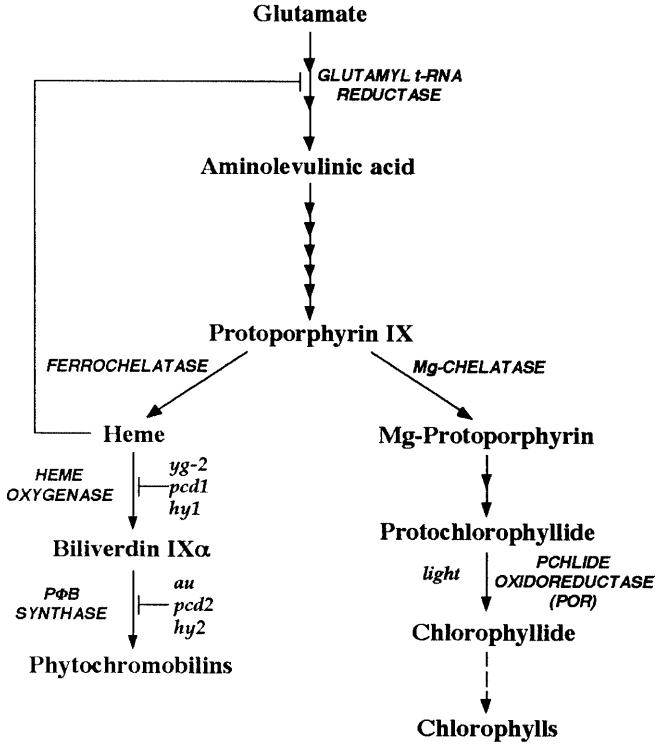

Fig. 1 The tetrapyrrole biosynthesis pathway. The steps inhibited in the phytochrome chromophore-deficient mutants used in this study are indicated

As shown in Fig. 1, 5-aminolevulinic acid (ALA) is the common precursor of both heme and Chl synthesis. ALA synthesis is the rate-limiting step for tetrapyrrole synthesis and is therefore the primary target for regulating the flux through this pathway (see Beale and Weinstein 1991 for a discussion of the early literature). A number of different factors have been proposed as feedback inhibitors of this pathway. Numerous studies using seedlings have demonstrated a role for both heme and a phototransformable protochlorophyllide (Pchlide) complex in regulating ALA synthesis (Beale and Weinstein 1991). More recently, experiments in which the levels of Mg-chelatase subunits were manipulated by anti-sense technology have led to the proposal that Mg-chelatase activity is a regulator of ALA synthesis in mature tobacco plants (Papenbrock et al. 2000). Analysis of cotyledons from dark-grown *au* and *yg-2* mutants demonstrated that mutant seedlings had reduced rates of Pchlide synthesis compared with wild-type (WT) seedlings (Terry and Kendrick 1999). As these mutants are blocked in phytochrome chromophore synthesis (Terry and Kendrick 1996), this indicates that inhibition of the heme branch of the tetrapyrrole pathway is clearly exerting a major effect on Pchlide accumulation under these conditions and that this inhibition cannot be overcome by feedback effects from the Mg-porphyrin branch of the pathway. Experiments in which the level of physiologically active heme was manipulated by chelators supported the proposal that inhibition of Pchlide synthesis resulted from feedback inhibition by heme, the precursor of the chromophore biosynthesis pathway (Terry and Kendrick 1999). Direct measurement of this heme pool will be needed to confirm this hypothesis, though this will require the development of new methodology.

Feedback inhibition, independent of the loss of phytochrome, might help explain other phenotypic features of this class of mutants such as the pale yellow-green pigmentation of light-grown plants and the reduced accumulation of transcripts encoding Chl *a*/*b*-binding (CAB) proteins in the dark that have been observed in both *au* (Sharrock et al. 1988; Ken-Dror and Horwitz 1990) and *hy1* (López-Juez et al. 1996). In order to examine this hypothesis, the present investigation focuses on determining the consequences

of feedback inhibition of the tetrapyrrole pathway on the accumulation of NADPH:protochlorophyllide oxidoreductase (POR) protein and etioplast development. In addition, since microinjection of *au* hypocotyl cells has been extensively used for studying phytochrome signal transduction, including the regulation of plastid development (Bowler and Chua 1994), we have examined whether hypocotyl cells of *au* and *yg-2* also show this phytochrome-independent phenotype.

Materials and methods

Plant material and growth conditions

Seeds of the tomato (*Solanum lycopersicum* L.) genotypes used in this study were a generous gift from Dr. R.E. Kendrick (Wageningen University, The Netherlands). The origins of all the *au* and *yg-2* alleles have been described in detail previously (Terry and Kendrick 1999). The phytochrome mutants used, *phyA* and *phyB1*, are the *fri*^{2} (van Tuinen et al. 1995a) and *tri*^{4} (van Tuinen et al. 1995b) alleles, respectively. These are both in the Moneymaker (MM) background, as is the corresponding *phyAphyB1* double mutant (Kerckhoffs et al. 1997). The *phyAphyB1phyB2* triple mutant (designated line 70F; Kerckhoffs et al. 1999) was derived by mutation of the *fri*^{1},*tri*^{1} double mutant and is in a mixed MM/GT breeding line background. Seeds of the pea (*Pisum sativum* L.) mutants *pcd1* and *pcd2* (Weller et al. 1997) were provided by Dr. J.L. Weller (University of Tasmania, Australia). The *Arabidopsis thaliana* (L.) Heynh. (Landsberg *erecta*) mutants *hy1-1* and *hy2-1* (Koornneef et al. 1980) were a gift from Professor M. Koornneef (Wageningen University, The Netherlands).

Tomato seeds were treated with 1% (v/v) bleach, washed thoroughly, and sown on 0.7% (w/v) agar containing 0.46 g/l of Murashige-Skoog salts (Life Technologies, Rockville, Md., USA) in plant tissue culture containers (Flow Laboratories, McLean, Va., USA). Seedlings were grown in the dark for 5 days at 25 °C. All mutants were sown 12 h prior to WT seeds to synchronize germination. Pea seeds were sown in water-saturated vermiculite and grown in complete darkness for 7 days at 25 °C. *Arabidopsis* seeds were treated with sterilizing solution (28 ml H_2O, 2 ml bleach, one drop Triton X-100), washed thoroughly, and sown on 0.6% (w/v) agar containing 0.46 g/l of Murashige-Skoog salts. Following a 24-h white-light treatment they were grown in complete darkness for 7 days (mutants) or 6 days (WT) at 25 °C.

Pchlide measurement

To extract Pchlide, tomato hypocotyl segments (the central 2 cm; 0.5 g) or cotyledons (0.25 g) were harvested under dim green safelight, homogenized in cold acetone/0.1 M NH_4OH (90/10, v/v) and the extract washed with hexane exactly as described previously (Terry and Kendrick 1999). Pea seedlings were harvested between 14 and 22 cm long (to ensure that they were of an approximately equivalent developmental stage) and separated into apex (apical leaf tissue; 0.15 g) and stem (top 1 cm of stem tissue; 0.3 g) samples. These were then extracted in 1.75 ml and 1.25 ml of solvent, respectively. For *Arabidopsis*, 0.1 g of whole seedlings was homogenized in 1.5 ml of solvent. In all cases, subsequent washes and second extractions were performed using the same proportionate solvent volumes as described previously (Terry and Kendrick 1999).

Pchlide was measured either by absorption spectroscopy (for pea apex samples) using a Hitachi U-3410 spectrophotometer (Hitachi, Tokyo, Japan) or by relative fluorescence following excitation at 440 nm using a Hitachi F-3010 fluorescence spectrophotometer.

Immunoblotting

Ten cotyledon pairs (with hooks still attached) or 1-cm hypocotyl segments (0.4 g) were ground in liquid N_2 and heated at 65 °C for 20 min in 400 µl SDS sample buffer [80 mM Tris-HCl (pH 8.8) containing 10% (w/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue]. Samples were then centrifuged at top speed for 10 min at 4 °C in a bench-top microcentrifuge and either diluted 4-fold in sample buffer (cotyledon samples) or loaded directly (hypocotyl samples) onto 12% or 15% SDS-PAGE gels, respectively. A 4% stacking gel was used. Samples were run for approximately 45 min at 150 V using a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, Calif., USA) in a running buffer consisting of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. Proteins were blotted onto polyvinylidenedifluoride (PVDF) membranes (Immobilon-P; Sigma Chemical Co., St. Louis, Mo., USA) in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol for 1 h at 100 V using the Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories). Membranes were blocked at room temperature for 2 h in 20 mM Tris-HCl (pH 7.5) containing 167 mM NaCl and 0.05% (v/v) Tween 20 (TBST) with the addition of 5% (w/v) fat-free milk powder, washed with TBST and incubated overnight at 4 °C in TBST containing 1 µg/ml (1:10,000 dilution) of a polyclonal antibody raised to POR from wheat (Rowe and Griffiths 1995). After washing, membranes were incubated at room temperature for 80 min in a 1:7,500 dilution in TBST of anti-rabbit IgG-alkaline phosphatase conjugate (Protoblot; Promega, Madison, Wis., USA or Sigma Chemical Co.). Colour development was performed according to the manufacturer's instructions.

In vivo fluorescence spectroscopy

One cotyledon pair or two hypocotyl sections (1 cm each) were frozen in liquid N_2 before or directly after one flash of white light (Braun F800 photo-flash equipment) to phototransform Pchlide. Fluorescence emission spectra were recorded at –196 °C using an SLM Aminco 8100C spectrofluorometer (SLM Instruments, Urbana, Ill., USA) following excitation at 440 nm. The spectra were smoothed and corrected for the sensitivity of the photomultiplier and comparisons of the various pigment forms were facilitated by normalization of the spectra. Representative spectra are shown.

Electron microscopy

Cotyledon and hypocotyl samples were dissected out under green safelight and placed in a fixative comprising 3% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M Pipes buffer (pH 7.2). This primary fixation was carried out at room temperature for 2 h in total darkness. Specimens were then rinsed in 0.1 M Pipes buffer, postfixed in 1% (w/v) buffered osmium tetroxide for 1 h, rinsed in buffer, dehydrated and embedded in Spurr resin following standard procedures. Silver sections were cut on a Leica OMU 3 ultramicrotome, stained with uranyl acetate followed by Reynold's lead citrate stain and viewed on a Hitachi H7000 transmission electron microscope. Three independent experiments were performed with samples of cotyledon and hypocotyl tissue of each genotype processed and photographed in each case. All of the specimens were cut in the same orientation: along the median longitudinal axis of whole cotyledons and 1.5-mm lengths of hypocotyl tissue. This enabled us to select plastids from a single section containing a large number of cells without the risk of sampling the same plastid twice. Electron micrographs used for the analysis of etioplast ultrastructure were taken at the same magnification (×17,000) and were chosen on the basis of large plastid size and the most "well-structured" prolamellar body in order to ensure as far as possible that those selected were from the middle of the plastid. The total number of photographs taken for each tissue sample from each

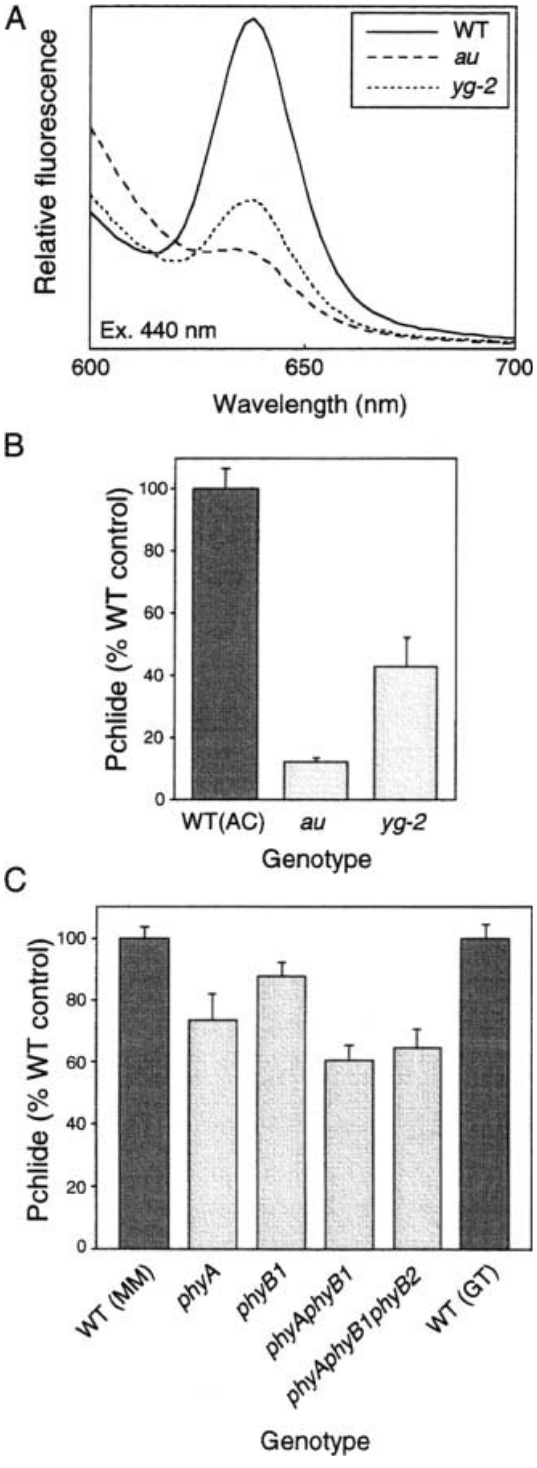

Fig. 2A–C Analysis of Pchlide levels in phytochrome-deficient tomato (*Solanum lycopersicum*) mutants. **A** Room-temperature fluorescence spectra (excitation 440 nm) of hexane-washed acetone extracts from hypocotyls of 5-day-old, dark-grown wild-type (*WT*; Ailsa Craig, *AC*), *au* and *yg-2* seedlings. **B, C** Quantitation of relative Pchlide levels from multiple fluorescence spectra in *au* and *yg-2* (**B**) and phytochrome apoprotein-deficient mutants (**C**). The *phyA*, *phyB1* and *phyAphyB1* mutants are in the Moneymaker (MM) background and the *phyAphyB1phyB2* mutant is in a mixed MM/GT breeding line background. Relative values were calculated on a fresh-weight basis and represent the mean ± SE ($n \geq 3$)

genotype ranged from 11 to 20 (see *Results* for details), each one representing a different cell.

Results

Hypocotyls of dark-grown *au* and *yg-2* seedlings are severely deficient in Pchlide

To determine if hypocotyl cells show feedback inhibition of Pchlide synthesis, as had been observed in cotyledons (Terry and Kendrick 1999), we investigated Pchlide levels in hypocotyl segments from 5-day-old dark-grown WT, *au* and *yg-2* seedlings. The low levels of Pchlide in hypocotyls precluded absolute quantitation by absorption spectroscopy. Instead, hexane washed-acetone extracts were analyzed by fluorescence spectroscopy following excitation at 440 nm (Rebeiz et al. 1975). Representative spectra are shown in Fig. 2a. Both *au* and *yg-2* contained very reduced levels of Pchlide compared with WT. Relative quantitation from multiple experiments showed that *au* hypocotyls had 12% of WT Pchlide on a fresh-weight basis while *yg-2* contained 43% (Fig. 2b). This compares with 68% and 84% of WT Pchlide in cotyledons of *au* and *yg-2* respectively (Terry and Kendrick 1999).

The reduction of Pchlide in cotyledons of *au* and *yg-2* was independent of the loss of phytochrome (Terry and Kendrick 1999). To determine whether this was also the case for hypocotyl tissue we measured Pchlide levels in a series of phytochrome apoprotein-deficient mutants. Figure 2c shows that hypocotyls of both the *phyAphyB1* double mutant (Kerckhoffs et al. 1997) and the *phyAphyB1phyB2* triple mutant (Kerckhoffs et al. 1999) contained less Pchlide than WT indicating a role for phytochrome in Pchlide accumulation in this tissue. Experiments with individual apophytochrome mutants showed that both phytochrome A and phytochrome B1 contribute to this effect (Fig. 2c). Mutant seeds were sown 12 h prior to WT seeds to negate any possible effect of phytochrome deficiency on the rate of germination and consequently the developmental stage of the seedlings. To confirm that the loss of phytochrome did not substantially affect the developmental stage of the dark-grown seedlings we measured the hypocotyl lengths of the same seedlings used for Pchlide determinations. Mutant seedlings were very similar in height to WT seedlings with the largest difference recorded in any individual experiment being 11.6%, and the mean heights of the *phyAphyB1* double mutant and the *phyAphyB1phyB2* triple mutant over all the experiments measured being 93.3% and 99.5% of WT, respectively (data not shown). It is therefore unlikely that the reduction in Pchlide results from slower growth of the mutants. The reduction in Pchlide in the phytochrome apoprotein-deficient mutants is not sufficient to account for the very low levels of Pchlide in *au* and *yg-2*, although it may contribute to the more severe effect of the *au* and *yg-2* mutations in this tissue. We also examined

Pchlide accumulation in cotyledons of the *phyA-phyB1phyB2* triple mutant. Triple-mutant seedlings accumulated 52.0 ± 4.6 pmol Pchlide/seedling which was not appreciably different from that measured previously for WT (MM) of 52.6 ± 1.2 pmol/seedling (Terry and Kendrick 1999), indicating, as noted previously, that phytochrome deficiency has very little effect on Pchlide accumulation in cotyledon tissue.

POR accumulation is reduced in dark-grown chromophore-deficient mutants

In order to determine whether feedback inhibition of Pchlide synthesis could account for the light-independent phenotype of chromophore-deficient mutants we wanted to determine the immediate consequences of reduced Pchlide synthesis. As Pchlide pigment was reported to be required for the normal import and stabilization of PORA (Reinbothe et al. 1995; Lebedev and Timko 1998) it was hypothesized that a reduction in Pchlide might lead to a reduced accumulation of POR protein and we therefore examined POR protein levels in the mutants.

Figure 3a shows an immunoblot of POR protein in cotyledons of dark-grown WT and mutant tomato seedlings. The antibody, which was raised against POR from wheat seedlings, specifically recognizes a doublet of equal intensity at approximately 37 kDa. This molecular mass is consistent with that previously reported for mature POR proteins in other species (Lebedev and Timko 1998). To confirm that the antibody was recognizing POR we also analyzed WT seedlings that had been irradiated with white light. White-light irradiation for 4 h resulted in the disappearance of both bands (Fig. 3a), and measurement of band intensity from multiple experiments using NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/) showed that POR levels declined to 14 ± 5% (mean ± SE; $n = 3$) of dark controls. Such a rapid disappearance following white-light irradiation is characteristic of PORA proteins (Reinbothe et al. 1996) and therefore confirms the identity of the bands detected by the antibody. However, higher plants generally contain two POR species, PORA and PORB (Reinbothe et al. 1996), and we cannot exclude the possibility that the antibody can also recognize PORB from tomato.

Analysis of POR levels in cotyledons from mutant seedlings showed that they were generally reduced compared to WT, but not in all cases (Fig. 3a). POR levels in the *au* allele were reduced to 80 ± 2% ($n = 3$) of WT levels while the au^w allele had similar POR levels to WT. However, in this case it was noticeable that POR accumulation was lower in the MM background compared to the Ailsa Craig (AC) background, and an effect of the au^w mutation on POR accumulation was seen in the *yg-2*^{aud} mutant background as the au^w*yg-2*^{aud} double mutant (27% of WT Pchlide; Terry and Kendrick 1999) contained the least POR (33 ± 6%; $n = 2$). The *yg-2* allele

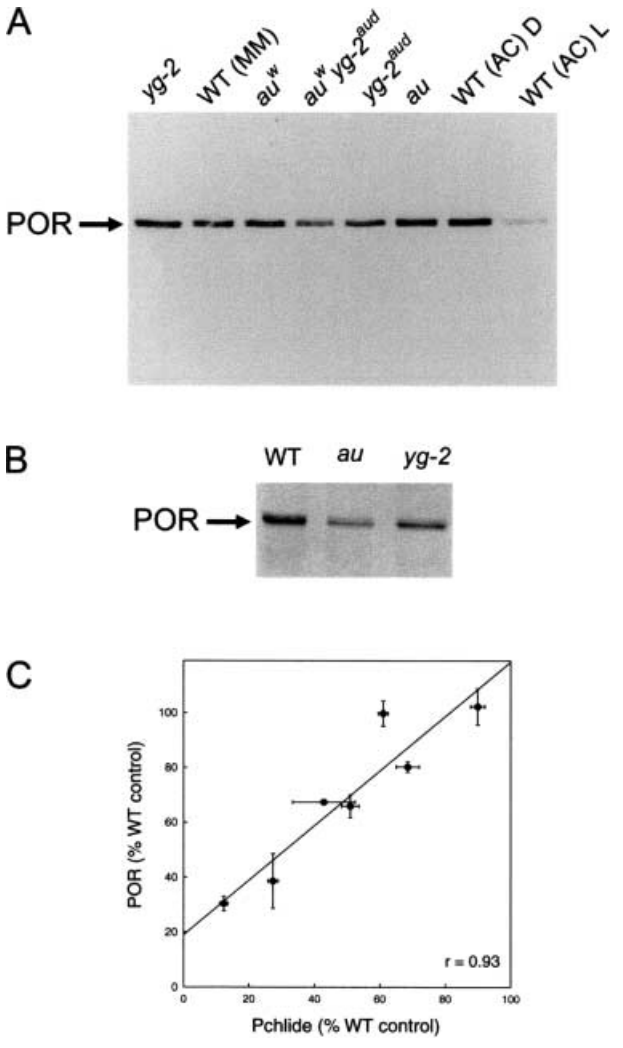

Fig. 3A–C Analysis of NADPH:protochlorophyllide oxidoreductase (POR) protein accumulation in *au* and *yg-2* mutants of tomato. **A, B** Immunoblots showing the accumulation of POR in cotyledons (**A**) and hypocotyls (**B**) of 5-day-old, dark-grown wild-type (WT), *au* and *yg-2* seedlings. Samples were loaded on an equivalent-seedling (cotyledon) or fresh-weight (hypocotyl) basis. The *au* and *yg-2*^{aud} alleles are in the WT Ailsa Craig (AC) background and au^w is in the Moneymaker (MM) background. WT(AC) seedlings were also treated with 4 h white light (L) prior to harvest. **C** Plot of relative Pchlide levels against relative POR levels for all mutant samples shown in **A** and **B**. POR was quantified from multiple blots using NIH Image software (National Institutes for Health;http://rsb.info.nih.gov/nih-image/). Pchlide data for hypocotyl samples were taken from Fig. 2 and for cotyledon samples from Terry and Kendrick (1999). Values shown are the mean ± SE ($n \geq 2$ for POR; $n \geq 3$ for Pchlide)

(84% of WT Pchlide) did not appear to affect the accumulation of POR at all, although a second *yg-2* allele, *yg-2*^{aud}, that contained only 51% of WT Pchlide had much lower POR levels than WT (66 ± 4%; $n = 2$). No evidence was seen for the accumulation of the precursor form of POR in any of the mutants.

POR protein levels were more severely affected in hypocotyls of the mutants (Fig. 3b). In extracts from this tissue the relative intensity of the lower band of the POR doublet was much greater. Quantitation of blots

demonstrated that *au* contained 30.4 ± 2.6% ($n = 2$) of WT POR while *yg-2* contained 67.4 ± 0.6% ($n = 2$). Therefore, in nearly all cases POR protein levels in the mutants appeared to be closely correlated to the levels of Pchlide pigment, although they were reduced to a lesser extent. Figure 3c shows a plot of Pchlide against POR levels for all mutants examined and demonstrates that there is a very strong linear correlation between the two with a correlation coefficient of $r = 0.93$.

The *au* and *yg-2* mutants lack free Pchlide *in vivo*

In most dark-grown higher plants, Pchlide exists in a number of forms *in vivo* (Böddi et al. 1992). While the majority of Pchlide is bound to POR as part of a POR:Pchlide:NADPH photoactive complex, some non-phototransformable Pchlide is also present. From the data shown in Fig. 3c we would predict that the *au* and *yg-2* mutants would contain very little non-phototransformable Pchlide *in vivo*. To test this hypothesis directly we examined the properties of Pchlide *in vivo* using low-temperature fluorescence spectroscopy. Following excitation at 440 nm, cotyledons from dark-grown WT seedlings showed two emission maxima at 630 and 655 nm that corresponded to non-phototransformable Pchlide (and also esterified protochlorophyll) and Pchlide bound to the active site of POR, respectively (Fig. 4a). One light flash converted the bound Pchlide to chlorophyllide with an emission maximum at 691 nm in samples frozen immediately while the non-phototransformable Pchlide peak remained unchanged (Fig. 4b). Spectra from cotyledons of *au* and *yg-2* indicated that these mutants did indeed contain very low levels of free Pchlide while retaining the bound form with the same emission maximum of 655 nm (Fig. 4a). The ability to phototransform Pchlide to chlorophyllide in the mutants was unaffected (Fig. 4b). Control experiments with the *phyAphyB1phyB2* triple mutant showed that non-phototransformable Pchlide was still readily detectable, indicating that the absence of this Pchlide form is unrelated to phytochrome deficiency (data not shown). The spectra in Fig. 4a, b have been normalized, and estimation of total fluorescence emission shows that there is less phototransformable Pchlide in *au* and *yg-2* compared with WT (Fig. 4a, insert) consistent with the quantitative data on total Pchlide previously published (Terry and Kendrick 1999).

Total Pchlide fluorescence in hypocotyls of WT seedlings was much lower than observed in cotyledon tissue (Fig. 4c, insert). Both Pchlide forms appeared to have slightly shorter wavelength emission maxima (Fig. 4c) and the long-wavelength Pchlide was phototransformable to chlorophyllide by a single light flash (Fig. 4d) as had been observed for cotyledon samples. However, for hypocotyl samples the proportion of phototransformable Pchlide relative to non-phototransformable Pchlide was much lower. Consistent with our *in vitro* analyses, both non-phototransformable and phototransformable Pchlide were severely reduced in *au* and *yg-2* compared to WT hypocotyls (Fig. 4c, d).

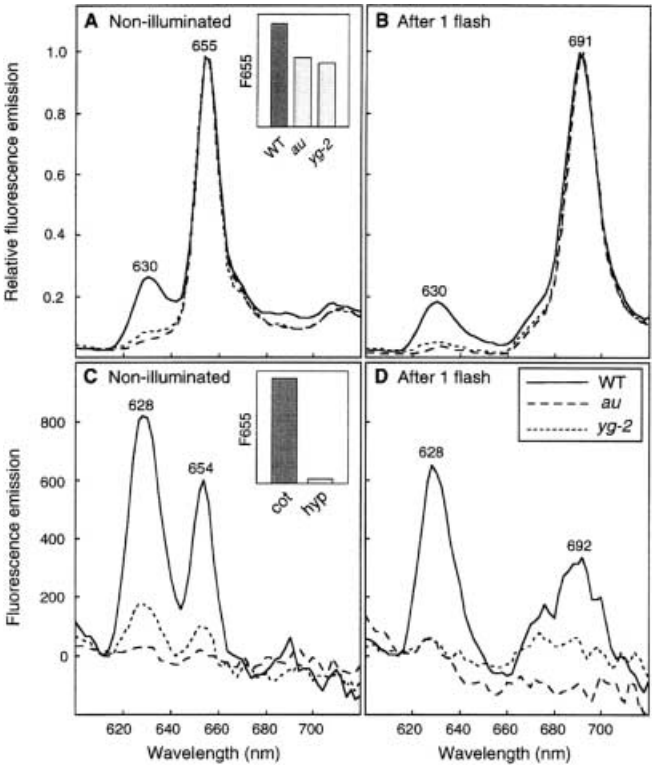

Fig. 4A–D Low-temperature *in vivo* fluorescence spectroscopy of Pchlide in *au* and *yg-2* mutants of tomato. Representative fluorescence spectra (excitation 440 nm) from 5-day-old dark-grown wild-type (WT), *au* and *yg-2* cotyledons (**A, B**) and hypocotyls (**C, D**) taken in complete darkness (**A, C**) or following one white light flash (**B, D**). The spectra in **A** and **B** are normalised to 1 at 655 nm and 691 nm, respectively. The relative fluorescence at 655 nm (F655) of the samples shown is indicated by the bar chart in **A**. The spectra in **C** and **D** are presented with the troughs at approximately 610 nm set to zero. Hypocotyls gave much lower fluorescence emission than cotyledons and the relative fluorescence at 655 nm (F655) of WT cotyledon and hypocotyl samples is shown by the bar chart in **C**

Reduced POR levels are correlated to altered etioplast ultrastructure

POR is the major protein component of the prolamellar body in etioplasts (Ryberg and Sundqvist 1982; Ikeuchi and Murakami 1983; Dehesh and Ryberg 1985; Ryberg and Dehesh 1986) and reduced POR levels might therefore be expected to affect etioplast development. Electron micrographs of etioplasts from both cotyledons and hypocotyls of WT, *au*, and *yg-2* seedlings are shown in Fig. 5. Etioplasts from WT cotyledons contained highly regular prolamellar bodies with a narrow-type structure (Fig. 5a) in 59% of those examined ($n = 17$, see *Materials and methods*) with a wide-type structure generally observed in the remaining etioplasts (see Henningsen and Boynton 1969 for a definition of these plastid types). In

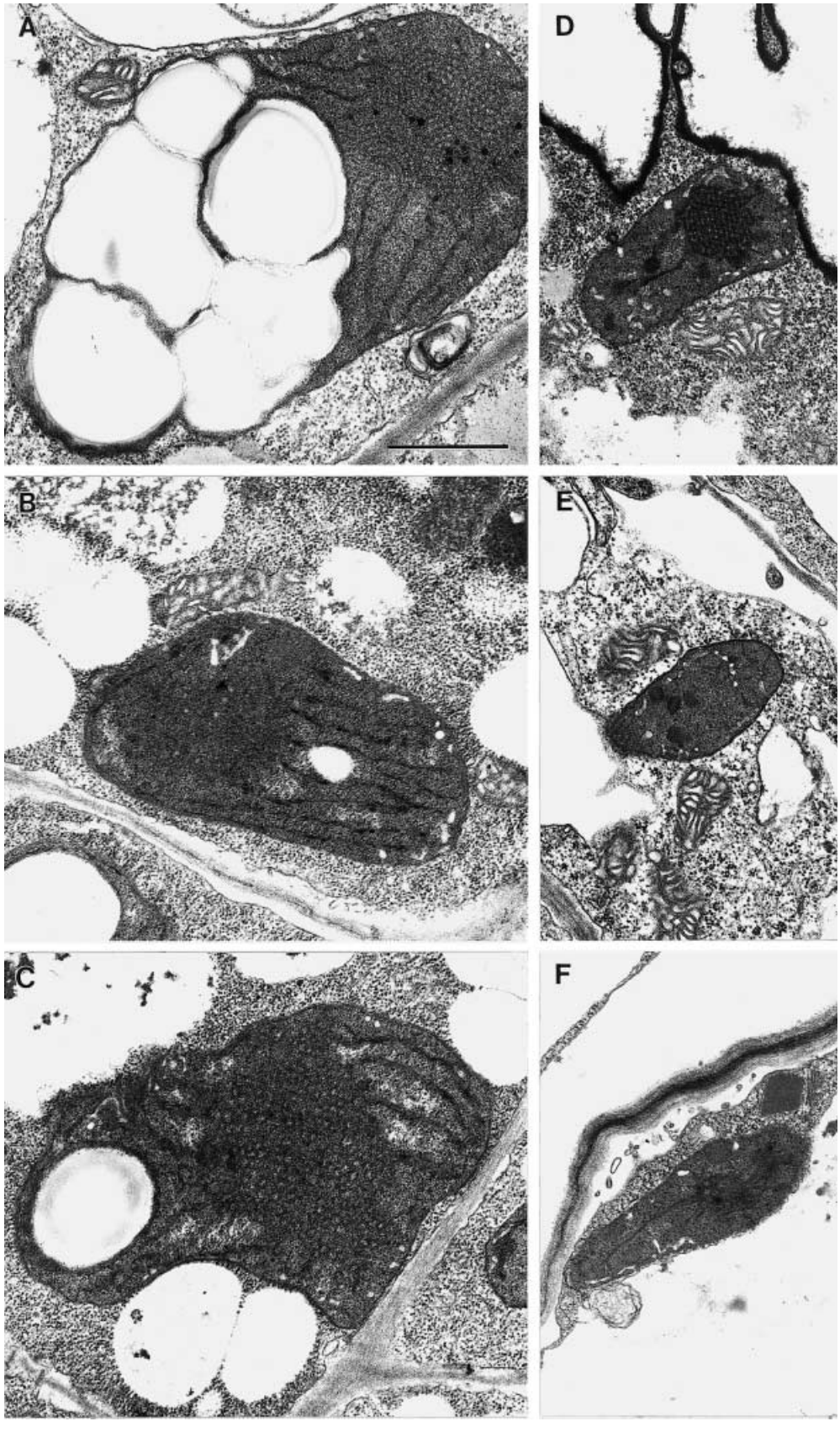

Fig. 5A–F Ultrastructure of etioplasts in *au* and *yg-2* mutants of tomato. Electron micrographs showing representative etioplasts from 5-day-old dark-grown wild-type (**A, D**), *au* (**B, E**) and *yg-2* (**C, F**) cotyledons (**A–C**) and hypocotyls (**D–F**). Bar = 1 μm

contrast, etioplasts from *au* seedlings usually contained an electron-dense, prolamellar body-like structure rather than a true prolamellar body (Fig. 5b) and wide-type prolamellar bodies were observed only occasionally in *au* etioplasts (3 out of 14). Only 15% of *yg-2* etioplasts contained a narrow-type prolamellar body ($n = 13$) with most of the remaining prolamellar bodies having a wide-type structure (Fig. 5c). In addition to the degree of membrane development, mutant etioplasts were noticeably smaller than WT etioplasts with relative areas of 100.0 ± 8.0 for WT, and 72.5 ± 5.0 and 86.0 ± 5.5 for *au* and *yg-2*, respectively. Control experiments using the *phyAphyB1* double mutant showed that etioplasts of this mutant were identical in size to those from WT(GT) and also contained narrow-type prolamellar bodies (data not shown).

In hypocotyls the difference between WT and mutant plastids was more obvious (Fig. 5d–f). WT etioplasts from hypocotyl cells were much smaller than cotyledon etioplasts, but still contained a prolamellar body in most of those examined (87%; Fig. 5d). *au* hypocotyl cells contained mostly proplastid-like structures (Fig. 5e) with no clear evidence for the presence of a prolamellar body ($n = 11$) while *yg-2* contained a rudimentary prolamellar body in just 3 of the 13 etioplasts examined (see Fig. 5f for an example). The absence of prolamellar bodies thus correlates well with the observation that POR and phototransformable Pchlide are reduced in these tissues.

Pchlide synthesis is inhibited in chromophore-deficient mutants of pea and *Arabidopsis*

To understand the significance of our results for interpreting the general phenotype of phytochrome chromophore-deficient mutants we determined Pchlide levels in pairs of mutants from two additional species. Figure 6 shows that the *pcd1* and *pcd2* mutants of pea (*Pisum sativum* L.) and the *hy1* and *hy2* mutants of *Arabidopsis thaliana* Landsberg *erecta* all contained less Pchlide than their respective WTs, as measured by relative fluorescence spectroscopy of hexane-washed acetone extracts. In addition, the levels of Pchlide were more severely reduced in pea stems (Fig. 6b) than in the apex (Fig. 6a), paralleling the results obtained in tomato.

Discussion

A role for red-light-absorbing (Pr) forms of phytochrome in regulating Pchlide accumulation?

The results presented here demonstrate that Pchlide levels are severely reduced in dark-grown hypocotyls of *au* and *yg-2*, and that this inhibition appears to have two components: phytochrome dependent and phytochrome independent (Fig. 2). Experiments with phytochrome apoprotein-deficient mutants showed a clear effect of phytochrome deficiency in the dark, with the *phyAphyB1* double mutant and the *phyAphyB1phyB2* triple mutant having approximately 60–65% of WT Pchlide. Both phytochrome A and phytochrome B1 contributed to this result, and measurement of seedling hypocotyl lengths confirmed that this effect was not due to a slower rate of development caused by inhibition of phytochrome-mediated germination. However, both *au* and *yg-2* were more severely affected than the triple mutant, suggesting that phytochrome-independent inhibition also contributes to the reduced Pchlide levels in this tissue. This dual effect on Pchlide accumulation in hypocotyls is in contrast to the situation in cotyledons. In this tissue, the *phyAphyB1phyB2* triple mutant had no effect on Pchlide accumulation, a result that extended our previous observations that inhibition of Pchlide synthesis was independent of the loss of phytochrome (Terry and Kendrick 1999). It is likely that the mechanism of the phytochrome-independent reduction of Pchlide levels is the same in both tissues. The proposed explanation

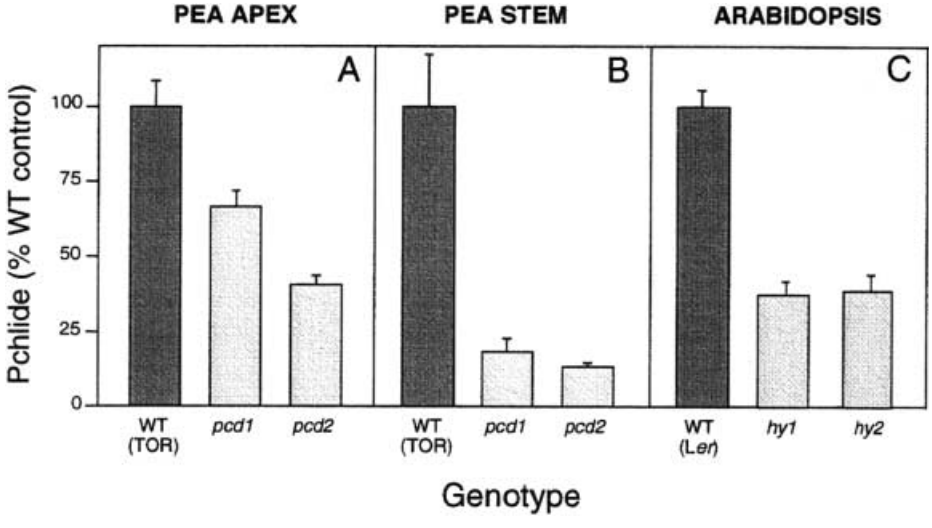

Fig. 6A–C Analysis of Pchlide levels in phytochrome chromophore-deficient mutants. Relative Pchlide levels were determined from multiple absorption (pea apex) or fluorescence spectra (pea stem, *Arabidopsis*) of hexane-washed acetone extracts from 7-day-old, dark-grown wild-type (WT) and mutant seedlings. *pcd1* and *pcd2* are in the Torsdag (TOR) background and *hy1* and *hy2* are in the Landsberg *erecta* (L*er*) background. Relative values were calculated on a fresh-weight basis and represent the mean ± SE ($n \geq 3$)

is that inhibition of the conversion of heme to BV IXα and PΦB results in an accumulation of a regulatory heme pool that inhibits ALA synthesis and consequently Pchlide synthesis (see Fig. 1; Terry and Kendrick 1999). The mechanism for this inhibition is currently unknown, but may be related to the observation that heme has been shown to inhibit the first committed step of ALA synthesis, glutamyl-tRNA reductase, *in vitro* (Vothknecht et al. 1996).

The reduction in Pchlide synthesis in phytochrome apoprotein-deficient mutants that were completely dark-grown suggests a role for the inactive Pr form of phytochrome in controlling etioplast development. There are precedents for Pr having a role in hypocotyl development in dark-grown seedlings. The inhibition of hypocotyl elongation in phytochrome B-deficient *lh* mutants of cucumber grown in the dark is one such example (Saefkow et al. 1995). However, an effect of Pr on etioplast development has not previously been reported. Further experiments will be required in order to address the possibility that the inhibition of Pchlide synthesis results from a reduction in Pfr, the far-red-absorbing form of phytochrome, inherited from the parent plant. Such a Pfr pool may have effects during seed maturation that could persist into early seedling development.

Tetrapyrroles have an important role in etioplast development

Inhibition of Pchlide accumulation showed a strong correlation with the loss of POR protein (Figs. 3, 4) and retardation of etioplast development, particularly in hypocotyls (Fig. 5). Given the strong evidence that the primary effect of the mutations results in the inhibition of Pchlide synthesis we hypothesize that the inhibition of POR accumulation and etioplast development is a secondary consequence of inhibiting Pchlide synthesis. It is well established that inhibition of tetrapyrrole synthesis leads to profound effects on plastid development (Sommerville 1986). Inhibition of Pchlide synthesis by treatment with the tetrapyrrole synthesis inhibitor, gabaculine, slowed the rate of prolamellar body formation and resulted in abnormal etioplast structures (Younis et al. 1995). Similarly, mutations in maize (Mascia and Robertson 1978), barley (von Wettstein et al. 1971) and *Arabidopsis* (Runge et al. 1995) that affect tetrapyrrole synthesis also resulted in a reduction (or complete absence) of prolamellar bodies, with the severity of the effects dependent on the degree of leakiness of the mutations.

The mechanism by which inhibition of tetrapyrrole synthesis results in impaired etioplast development is most probably a sequential process in which reduced Pchlide leads to reduced POR accumulation, which in turn results in reduced prolamellar body formation. We see two possible explanations for the reduced accumulation of POR. In an etioplast, POR forms a complex with its substrates Pchlide and NADPH (Griffiths 1978; Apel et al. 1980) and it is therefore likely that the reduction in POR is a result of increased intra-plastidic degradation of mature protein that is unable to assemble into this stable ternary complex. A second possibility comes from the observation that transport of POR into etioplasts has been reported to be dependent on the presence of Pchlide (Reinbothe et al. 1995). A reduced Pchlide concentration could therefore inhibit POR import and this might also be expected to lead to increased POR turnover as excess extra-plastidic POR may be degraded. However, this second explanation is contradicted by more recent results showing that POR can be imported equally well in the presence or absence of Pchlide (Dahlin et al. 2000) and we therefore favour a role for plastid proteases in the reduced accumulation of plastid-localized POR.

The suggestion that the limitation of Pchlide determines POR accumulation is supported by the strong correlation between Pchlide and POR accumulation (Fig. 3) and the observation that there is little non-phototransformable Pchlide *in vivo* in cotyledons of *au* and *yg-2* (Fig. 4). Pchlide pigment in cotyledons of the mutants is therefore almost exclusively present bound to POR and is fully phototransformable, indicating that it is correctly assembled. The severe reduction in non-phototransformable Pchlide is particularly intriguing as this is usually only observed in isolated prolamellar bodies (Ryberg and Sundqvist 1988). The loss of non-phototransformable Pchlide, however, is not strictly correlated with reduced Pchlide accumulation as hypocotyls that contain less total Pchlide have a higher proportion of non-phototransformable Pchlide. This observed difference between Pchlide forms in cotyledons and hypocotyls may be related to our observation that only hypocotyls have a phytochrome-dependent component to the inhibition of Pchlide accumulation.

Reduced POR accumulation in tetrapyrrole-deficient mutants has been noted previously. The barley mutant *xantha-l* that is blocked between Mg-protoporphyrin IX and Pchlide (von Wettstein et al. 1971) accumulates less POR protein (Dehesh et al. 1986). Similarly, another barley mutant, *albostrians*, that lacks plastid ribosomes and $tRNA^{Glu}$ required for ALA synthesis, has greatly reduced levels of both Pchlide and POR (Hess et al. 1992). In addition to the strong correlation between Pchlide and POR levels in the *au* and *yg-2* alleles of tomato, an inhibition of POR accumulation has also been reported for *hy1* (López-Juez et al. 1998), which also has lower levels of Pchlide (this study; Montgomery et al. 1999).

Since POR is the major protein component of the prolamellar body (Ryberg and Sundqvist 1982; Ikeuchi and Murakami 1983; Dehesh and Ryberg 1985) and, in well-developed etioplasts, POR is localized almost exclusively to prolamellar bodies (Ryberg and Dehesh 1986) it is not surprising that POR levels are a major determinant of etioplast structure. Indeed it has been

shown that the regular ultrastructure of isolated prolamellar bodies requires the presence of the POR:Pchlide:NADPH ternary complex (Ryberg and Sundqvist 1988) and more recently that overexpression of either PORA or PORB correlates strongly with prolamellar body formation (Sperling et al. 1998). In *au* and *yg-2* cotyledons the impaired etioplast development manifested itself as a reduced percentage of etioplasts with prolamellar bodies, an increased proportion of wide-type prolamellar bodies compared to narrow type and a reduction in etioplast size (Fig. 5). Since the inhibition of Pchlide in mutant cotyledons is not very severe and *yg-2*, for example, has no apparent reduction in POR levels, these effects indicate that there is a very tight correlation between synthesis of the POR:Pchlide:NADPH ternary complex, the formation of prolamellar bodies and etioplast development, and that even relatively moderate changes in tetrapyrrole synthesis can affect this process.

In hypocotyl plastids a much more dramatic effect was seen, with *au* plastids resembling proplastids with little internal membrane structure. A similar result for au^{w} was obtained by Neuhaus et al. (1993). Again the reduction in POR levels is the most likely explanation for this phenotype although it is difficult to exclude the possibility that there are other factors that also affect etioplast development in the mutants. These could include the loss of phytochrome (Pr or Pfr), the reduction in bilin synthesis, or the accumulation of heme.

Why are chromophore-deficient mutants impaired in plastid signalling?

One of the interesting features of phytochrome chromophore-deficient mutants is that these mutations affect the ability of the plastid to signal the nucleus. Dark-grown *au* seedlings have reduced *CAB* gene expression (Sharrock et al. 1988; Ken-Dror and Horwitz 1990) and the *hy1* mutation has also been reported to reduce *CAB* expression in the dark (López-Juez et al. 1996). In addition, both *hy1* and *hy2* were isolated in a screen for *gun* (*g*enomes *un*coupled) mutants that retained *CAB* expression after the loss of signals from the plastid (Susek et al. 1993; Mochizuki et al. 2001). It is therefore interesting that inhibition of Pchlide synthesis is a consistent feature of phytochrome chromophore-deficient mutants, including both *hy1* and *hy2* (Fig. 6).

Based on our current results, the simplest explanation for the reduction of *CAB* expression in dark-grown *au* seedlings is that the inhibition of plastid development resulting from feedback inhibition of Pchlide synthesis leads to the loss of a promotive plastid signal. This type of phenomenon is common in light-grown plants and many conditions that impair chloroplast development also lead to a loss of a plastid signal (Taylor 1989; Susek and Chory 1992). Whether such a general effect can also explain the *gun* phenotype of chromophore-deficient mutants is less clear. An alternative explanation is that perturbation of the tetrapyrrole pathway itself directly affects the plastid signal or signals. Such a mechanism could also account for the reduction in *CAB* expression in the dark and might involve the loss of bilins, an increase in heme or protoporphyrin IX (Terry and Kendrick 1999), or a reduction in the accumulation of Mg-porphyrins. There is considerable evidence for tetrapyrroles having signalling functions in other organisms. Heme is a regulator of gene expression in both yeast and mammalian systems (Padmanaban et al. 1989) while Mg-protoporphyrin IX has been shown to regulate the expression of two heat-shock genes in *Chlamydomonas* (Kropat et al. 1997). In angiosperms, the situation may be more complicated and it has been proposed that *CAB* expression might respond to the ratio of two tetrapyrroles: one an inhibitor and one an activator (Vinti et al. 2000). In order to understand the role of tetrapyrroles in plastid signalling it will be important to develop a much more complete understanding of what regulates the flux through this vital biosynthetic pathway.

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