**LHPP, the light-harvesting NADPH:protochlorophyllide (Pchlide) oxidoreductase:Protochlorophyllide complex of etiolated plants, is developmentally expressed across the barley leaf gradient**

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**Abstract**

NADPH:protochlorophyllide oxidoreductase is a key enzyme for the light-induced greening of etiolated angiosperm plants. In barley, two POR proteins exist termed PORA and PORB that have previously been proposed to structurally and functionally cooperate in terms of a higher molecular mass light-harvesting complex named LHPP, in the prolamellar body of etioplasts [Nature 397 (1999) 80]. In this study we examined the expression pattern of LHPP during seedling etiolation and de-etiolation under different experimental conditions. Our results show that LHPP is developmentally expressed across the barley leaf gradient. We further provide evidence that LHPP operates both in plants that etiolate completely before being exposed to white light and in plants that etiolate only partially and begin light-harvesting as soon as traces of light become available in the uppermost parts of the soil. As a result of light absorption, in either case LHPP converts Pchlide\(^a\) to chlorophyllide (Chlide)\(^a\) and in turn disintegrates. The released Chlide\(^a\), as well as Chlide\(^b\) produced upon LHPP’s light-dependent dissociation, which leads to the activation of the POR as a Pchlide\(^b\)-reducing enzyme, then bind to homologs of water-soluble chlorophyll proteins of Brassicaceae. We propose that these proteins transfer Chlide\(^a\) and Chlide\(^b\) to the thylakoids, where their esterification with phytol and assembly into the photosynthetic membrane complexes ultimately takes place. Presumably due to the tight coupling of LHPP synthesis and degradation, as well as WSCP formation and photosynthetic membrane assembly, efficient photo-protection is conferred onto the plant.

**Keywords:** Chloroplast development; Chlorophyll biosynthesis; Greening; NADPH:protochlorophyllide oxidoreductases (POR) A and B; Light-harvesting POR:protochlorophyllide complex (LHPP)

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**1. Introduction**

NADPH:protochlorophyllide (Pchlide) oxidoreductase (POR) (EC 1.3.3.1) is a key enzyme for the light-induced greening of etiolated angiosperm plants [1–5]. Both POR

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and its pigment substrate Pchlide accumulate to high levels in the leaves of dark-grown plants [6]. Dark-stable Pchlide–NADPH–POR ternary complexes are poised such that absorption of a photon by the pigment itself leads to its immediate reduction (see Ref. [7], for review). As a result, Chlide\(^a\) is produced which is subsequently esterified and further modified to produce Chls\(^a\) and\(^b\) in conjunction with the formation of functional photosynthetic membrane complexes (reviewed in Ref. [8]).

Two different species of Pchlide have been identified by low temperature in situ-fluorescence measurements in etiolated plants: Pchlide-628/632 (the first number indicates the absorption maximum, the second the respective fluorescence
emission maximum at the chosen excitation wavelength) and Pchlide-650/657 [9-12]. Protochlorophyllide-650/657 is also called photoactive Pchlide, because it can be converted to Chlide-684/690 with a single, 1 ms flash of white light [10,13]. It had thus far been believed that Pchlide-650/657 is constituted by photoactive Pchlide–NADPH–POR ternary complexes accumulating in the prolamellar body of etioplasts. Protochlorophyllide-628/632, by contrast, is non-photoconvertible and was supposed to be due to the presence of Pchlide aggregates not bound to POR that would transfer their excitation energy onto photoactive Pchlide (10,13), see also Ref. [7] for review). Energy transfer is known for many decades to take place from photoinactive Pchlide to phototoxic Pchlide and from photoinactive Pchlide to Chlide [10-14].

The existence of Pchlide aggregates has recently been disproven by work of Apel and co-workers. Meskauskiene et al. [19] isolated an Arabidopsis mutant called flu (fluorescent) that is unable to block excess Pchlide accumulation in darkness. In dark-grown angiosperm plants, Pchlide accumulation is normally restricted by a negative feedback loop executed by heme, Pchlide, and the flu protein at the step of 5-aminolevulinic acid formation. The flu protein interacts with glutamyl-tRNA reductase [20] which is one of three enzymes involved in 5-aminolevulinic acid biosynthesis (see Refs. [21,22], for review). In dark-grown flu plants and in light-grown flu plants transferred to darkness, free excited Pchlide molecules interact with O2 and, by triplet–triplet exchange, give rise to highly toxic singlet oxygen [23]. Singlet oxygen in turn triggers membrane lipid peroxidation and has deleterious effects on membrane integrity, plastid ultrastructure, and subsequent plant development. As a result, cell death occurs [23].

A solution for the apparent paradox as to how to explain the presence of photoactive and photoinactive Pchlide species has been provided by previous work. We discovered that in etiolated barley plants two POR proteins exist, termed PORA and PORB [24], that structurally and functionally cooperate in terms of a novel light-harvesting complex termed LHPP [25]. If expressed from corresponding cDNA clones and reconstituted to POR–pigment complexes, using Pchlide a and Pchlide b or their Zn analogs, Zn protoporphororphobilinogen (ZnP4) a and ZnP4b, respectively, plus NADPH as substrates, PORA and PORB were found to form oligomers in vitro [25-27]. In these oligomeric complexes, 5 PORA–Pchlide b-NADPH complexes interacted with just 1 PORB–Pchlide a-NADPH ternary complex. The resulting supra-complex additionally bound galacto- and sulpholipids [25,26]. We observed that light which was absorbed by PORB–bound Pchlide b was transferred onto PORB–bound Pchlide a [25,27]. Similar energy transfer reactions were observed in planta [25]. Moreover, a Pchlide a- and Pchlide b-containing PORA/PORB protein complex could be resolved from isolated prolamellar bodies that displayed the same POR, pigment and lipid stoichiometries and interactions as the in vitro-reconstituted complex [26]. Both the in vitro-reconstituted and authentic complex dissociated upon white light illumination [26,37], consistent with previous findings on the light-induced disintegration of the prolamellar body [10,13]. Based on these findings we put forth that the reconstituted and authentic complexes are identical [26].

Little information has thus far been available on the regulated expression of LHPP during plant development. In this study, we analyzed the expression pattern and assembly of PORA and PORB, formation of photoactive Pchlide-F650/657, and accumulation of porphyrins and chlorins in barley plants during their etiolation and subsequent de-etiolation.

2. Materials and methods

2.1. Plant growth

Seeds of barley (Hordeum vulgare cv. Carina) were germinated either in the dark or underneath a 4 cm layer of vermiculite at 25 °C in the presence of 5 μE·m–2·s−1. Light quality and quantity measurements were made as described in [28].

2.2. Plastid Isolation

Seedlings were cut into consecutive 1 cm sections and plastids isolated by differential centrifugation and Percoll density gradient centrifugation, as described [29].

2.3. Pigment analyses

Either extraction with 100% (v/v) acetone containing 0.1% diethyl pyrocarbonate (DEP) or non-aqueous extraction with heptane–carbon tetrachloride [C7H16/CCI4 66:34 (v/v), density 1.28 g/cm3] were used [26]. Pigment extraction with acetone and hexane used to quantify esterified and non-esterified pigments was carried out according to Helfrich et al. [30], using the following modifications. For in vivo analyses, leaf tissues were extracted with 100% (v/v) acetone containing 0.5% (v/v) DEP. In turn the assays were centrifuged to sediment protein. After a step of re-extraction of pigments, the withdrawn acetone phases were pooled and supplemented with one third volume of reaction buffer containing 10 mM MgCl2 and 3 mM MnCl2 [30]. To the resulting mixture, 50 mg diethylamino-ethyl-cellulose (DEAE2, Whatman) and one half volume of hexane were added. After phase separation, the amount of esterified and non-esterified pigments was determined in the upper and lower solvent phases, respectively [30]. While esterified pigments are retained in the upper, hexane phase, non-esterified pigments partition into the lower, aqueous acetone phase [30]. For in vitro analyses, the same protocol was used except for the fact that each sample was diluted two-fold with doubly concentrated reaction buffer containing 20 mM MgCl2 and...
6 mM MnCl₂ before 50 mg diethylamino-ethyl-cellulose (DEAE2, Whatman) and one half volume of hexane were added. HPLC was performed on either C18 reverse phase silica gel columns (Macherey-Nagel Co., 250 mm x 4.6 mm, Nucleosil ODS 5 μm) [31] or C30 reverse phase columns (YMC Inc., Willmington, NC, USA, 250 mm x 4.6 mm, 5 μm) [32], using established procedures and a Varian ProStar model 410 apparatus, ProStar model 240 pump and ProStar 330 photodiode array detector. In some experiments, Chlides a and b were separated on RP18 Gromsil columns (Grom, Herrenberg, Germany), using a gradient of 100% acetone, applied for 3 min, and 60% acetone/40% acetic acid–supplemented water, pH 6.5, reached within 20 min [33]. Coupled POR-chlorophyll synthetase reactions were carried out according to Helfrich et al. [30]. Low-temperature luminescence spectroscopy was performed at 77 K at excitation wavelengths of either 440 or 470 nm in a spectrometer LS50 (Perkin Elmer Corp., Norwalk, CT) [34].

2.4. Protein analyses

Denaturing 10–20% (w/v) polyacrylamide gradients containing SDS were prepared according to Laemmli [35], as modified by Scharf and Nover [36]. Non-denaturing separations were as described by Krauspe et al. [37]. Western blotting was carried out according to Towbin et al. [38], exploiting either an anti-rabbit, anti-goat, alkaline phosphatase system or an ECL system (Amersham Pharmacia Biotech, http://www.apbiotech.com), and the antisera indicated in the text. Sequencing of electrophoretically resolved, protease-digested proteins was performed as described by Chang [39] and Chang et al. [40]. WSCP oligomerization was analyzed according to Satoh et al. [41]. Immunoprecipitations were performed according to Wiedmann et al. [42] using an anti-WSCP antiserum of L. virginicum.

3. Results

3.1. The experimental design

Plastid and leaf development are coupled in monocotyledonous plant species such as barley. Leaf development is determined by basipetal cell growth in the lower, youngest parts of the leaf (the leaf meristem) and maturation of tissues in the older parts. Plastid development follows a similar course: proplastids present in the leaf base continuously differentiate into mature forms. Depending on whether leaf development proceeds in darkness or light (processes termed skotomorphogenesis and photomorphogenesis, respectively, Ref. [43]) etioplasts and chloroplasts are produced [44]. In a typical germinating barley seedling a developmental gradient thus is established, comprising proplastids in the leaf base, developing etioplasts or chloroplasts in the middle, and mature plastids in the leaf tip [45]. Taking into account this pattern, 5-day-old etiolated barley seedlings were cut under a dim green light into consecutive 1 cm segments designated I–V in Fig. 1A. Subsequently, each of the different leaf samples was divided laterally into two halves. One half was used for in situ-fluorescence spectroscopy, whereas the other half was immediately used for protein extraction and analysis of POR-related proteins by SDS-PAGE.

Fig. 1. Developmental expression of POR in a typical etiolated barley seedling. (A) Depiction of the experimental design. (B) Western blot analysis of POR-related proteins in the various leaf sectors indicated in (A). (C) Quantification of PORA (grey columns) and PORB protein levels relative to the total amount of POR in the indicated leaf segments. (D) Partial amino acid sequences of the pPOR, PORA and PORB. In addition to part of pPOR’s transit peptide, two regions in the mature PORA and PORB are shown in which the obtained peptide sequences are most divergent. Dots highlight fingerprint amino acids. Staphylococcus aureus V8 protease-derived and endoproteinase Lys C-derived amino acid sequences are overlined and underlined, respectively. Amino acid sequences obtained after cyanogen bromide cleavage are in bold face.
3.2. **POR A and POR B are developmentally expressed across the barley leaf gradient**

Total protein was recovered from sonicated leaf material by precipitation with trichloroacetic acid, washed with ethanol and ether, and electrophoresed in denaturing 10–20% polyacrylamide gradients containing SDS. Nitrocellulose filters containing protein from the above defined leaf fractions (see Fig. 1A) were then probed with a POR-specific antiserum (see Section 2).

Fig. 1B demonstrates that three POR-related proteins could be detected, displaying molecular masses of 44, 38 and 36 kDa, respectively. According to our previous studies [24,46], these POR proteins were likely to represent the cytosolic precursor of the POR A, pPOR A (44 kDa), the mature POR B (38 kDa) and the mature POR A (36 kDa). Large-scale isolation from multiple gels of the 44, 38 and 36 kDa bands plus subsequent sequencing of proteolytic fragments generated with cyanogen bromide or different proteases confirmed that the 44 kDa protein indeed represents the pPOR A and its mature, 36 kDa form, whereas the partial sequence information for the 38 kDa band demonstrated that it is identical with POR B (Fig. 1D).

The total amount of POR was lowest in the youngest parts of the leaf and increased with leaf age, but the abundances of the POR A and POR B changed differentially in the various leaf sections (Fig. 1B and C). POR A seemed confined to the uppermost, oldest parts of the etiolated leaf. By contrast, POR B accumulated to similar levels throughout the entire leaf gradient (Fig. 1B and C). Remarkably, the 44 kDa pPOR A was most abundant in the youngest parts of the leaf, containing proplastids, while its level declined in the older parts, such that the protein was barely detectable in the leaf tip (Fig. 1B).

3.3. **Coordinated expression of both POR A and POR B correlates with the appearance of Pchlide F657 and higher molecular mass complexes indicative of LHPP**

Low temperature (77 K) in situ-fluorescence spectroscopy was performed at an excitation wavelength of 440 nm [34] of replicate samples corresponding to those used for protein extraction. When the spectra were compared, Pchlide-628/632 and Pchlide-650/657 were readily detectable in the leaf tip (sector I) and middle leaf sector (sector III) (Fig. 2A, solid and dotted lines, respectively). In the leaf base, only Pchlide-628/632 appeared (Fig. 2A, dashed line). At an excitation wavelength of 470 nm, in leaf sectors I and III only Pchlide-650/657 was observed (Fig. 2B, solid and dotted lines, respectively). In the leaf base, no fluorescence was detectable (Fig. 2B, dashed line). After illumination with a single, 1 ms flash of white light, Pchlide-650/657 was converted to Chlide-684/690 (Fig. 2C and D). By contrast, Pchlide-628/632 remained unchanged (Fig. 2C and D).

![Fig. 2. In situ-fluorescence analysis at 77 K of pigments in a typical etiolated barley leaf before and after exposure to a single, 1 ms flash of white light. Leaf material was prepared as described in Fig. 1A and probed by low temperature in situ-fluorescence spectroscopy at 77 K (A and B). Then the samples were warmed to about ~25 °C and subjected to a 1 ms flash of white light before being measured a second time at 77 K (C and D). (A) Fluorescence emission analysis at an excitation wavelength of 440 nm of etiolated leaf samples. (B) as (A), but at an excitation wavelength of 470 nm. (C) Fluorescence emission analysis at an excitation wavelength of 440 nm of flashed leaf samples. (D) as (C), but at an excitation wavelength of 470 nm. Note the almost quantitative conversion of Pchlide-650/657 to Chlide F690 in the leaf tip (sector I, solid line) and the middle leaf sector (sector III, dotted line), but the lack of Chlide-684/690 and other long-wavelength pigments in the leaf base (sector V, dashed line).](image-url)
HPLC analyses were performed to identify and quantify pigments present in the different leaf samples before and after flash light-illumination. As shown previously, Pchlide $b$ and Pchlide $a$ as well as the presumed intermediate in Pchlide $b$ to Pchlide $a$ conversion, 7-OH-Pchlide $a$, can be resolved on C18 RP material [27,31]. Also the product of light-dependent pigment conversion, Chlide $a$, is separable on this column material [27].

Fig. 3A–C shows chromatograms of pigments extracted with 100% acetone containing 0.1% (v/v) diethyl pyrocarbonate from lyophilized leaf samples corresponding to those designated I, III and V in Fig. 1A. At an absorption wavelength of 455 nm, which corresponds to the Soret band of Pchlide $b$ [31], three main pigment species were detectable, eluting at 11 min (peak 1), 12.5 min (peak 2) and 15 min (peak 3), respectively. These peaks had previously been identified as 7-OH-Pchlide $a$ (peak 1), Pchlide $b$ (peak 2) and Pchlide $a$ (peak 3) [27].

To provide further evidence for the identity of these compounds, the following experiment was performed. Pigments were recovered from the various HPLC fractions, concentrated, and used as substrates for the POR reaction in vitro. PORA and PORB polypeptides were expressed from corresponding cDNAs, purified and reconstituted into POR–pigment–NADPH complexes. PORA was mixed with pigments recovered from peak 2 eluting at 12.5 min, whereas PORB was incubated with pigments recovered from peak 3 eluting at 15 min during HPLC. All incubations were performed in the presence of NADPH. Reconstituted POR–pigment–NADPH complexes were separated from the bulk of non-bound pigment by gel filtration on Sephadex G15 [29]. POR–pigment–NADPH complexes eluting with the flow-through in turn were exposed to a saturating flash of white light to induce enzymatic Chlide formation. After extraction with acetone, the released Chlide was used as substrate for the chlorophyll synthetase reaction [30]. Esterified pigments (i.e., Chl $a$ and Chl $b$) were separated from non-esterified pigments by acetone/hexane extraction [30] and analyzed by HPLC. Pigments were identified by their characteristic absorbance spectra and mass spectroscopy.

Fig. 4 shows absorption spectra of pigments produced by the coupled POR-chlorophyll synthetase reaction. It turned out that the absorption spectra of pigments produced with PORB were identical to those of Chl $a$, whereas those obtained with PORA were identical to those of Chl $b$. Mass spectrometry confirmed the identity of these compounds (Table 1) and thus ultimately proved that the pigments eluting at 12.5 and 15 min on the C18 material represent Pchlide $b$ and Pchlide $a$, respectively. For the pigment eluting at 11 min, both with PORA and PORB 7-OH-Chl $a$ was produced by the coupled POR-chlorophyll synthetase reaction. PORA and PORB also converted Pchlide $a$ and Pchlide $b$ present in the different HPLC fraction into their respective products, but their binding affinities for these compounds were 10-fold lower in either case than those measured for their natural substrates (data not shown).

Quantification of 7-OH-Pchlide $a$, Pchlide $b$ and Pchlide $a$ is summarized in Table 2. It revealed that the total amount
of Pchlide was highest in the leaf tip and gradually declined in the younger parts of the plant. In the leaf base, low but statistically significant levels of Pchlide a were detected, whereas the leaf tip and middle leaf sector contained an excess of Pchlide b relative to Pchlide a (Table 2).

### Table 1
Identification of pigments by liquid secondary-ion mass spectrometry

<table>
<thead>
<tr>
<th>Pigment</th>
<th>m/z</th>
<th>Natural compound</th>
<th>Standard</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OH-Chl a</td>
<td>909.3</td>
<td>909.5</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Chl b</td>
<td>907.2</td>
<td>907.25</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Chl a</td>
<td>893.5</td>
<td>893.53</td>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

Leaf samples were prepared as described in Fig. 1A and pigments extracted with acetone. After HPLC on C18 RP material, 7-OH-Pchlide a, Pchlide b and Pchlide a were converted to 7-OH-Chl a, Chl b and Chl a by coupled POR-chlorophyll synthetase reactions. The esterified pigments were then subjected to mass spectrometry. m/z-values were obtained from mass spectra of the molecular-ion regions of chemically pure compounds. References refer to either synthetic (7-OH-Chl a) or commercially available (Chl a and Chl b) standards and are the followings: (A) [78]; (B) http://www.sigma-aldrich.com.

In addition to the HPLC analyses, non-denaturing PAGE was performed which has previously allowed the identification of higher molecular mass POR-pigment complexes from lyophilized prolamellar bodies [26]. Fig. 5A (darkness) shows a non-denaturing PAGE of POR-pigment complexes recovered from the same type of leaf samples as those used before for pigment extraction and HPLC. Due to the red light-induced auto-fluorescence of Pchlide-650/657, higher molecular mass complexes indicative of LHPP could be detected. Immunoprecipitations using the anti-POR antisemur described previously revealed that these complexes contained POR (Fig. 5B). Upon flash light-illumination, they rapidly dissociated into the PORA and PORB (Fig. 5A). Protein identification and quantification made as described [26] showed that the non-dissociated complex present in the non-flashed leaf tips contained ca. 8.5-fold higher levels of PORA than PORB and that the amount of PORA relative to PORB thus exceeded previously reported levels [25,27]. However, in leaf sector I only a five-fold higher level of PORA than PORB was found (Fig. 5A, flash light), consistent with previous results in which the leaf tip had been disregarded for analysis. Similar ~8:5:1 and ~5:1 stoichiometries as those measured for PORA and PORB, respectively, were determined for Pchlide b and Pchlide a. These results were in agreement with the results obtained for the total leaf samples (see Table 2). Leaf sector V did not seem to contain detectable PORA protein levels and only traces of Pchlide b were found, which confirmed the results shown in Figs. 1 and 3, respectively.

In flash light-illuminated samples, which no longer contained higher molecular mass complexes indicative of LHPP (Fig. 5A, Flash light), only Chlide a was produced (Table 2). This Chlide a co-migrated with the
Fig. 5. Non-denaturing, analytical PAGE of POR-pigment complexes recovered from lyophilized leaf tissues. Higher molecular mass POR-pigment complexes were extracted from leaf sections corresponding to those in Fig. 1, and aliquots kept in darkness or exposed to a 1 ms flash of white light. After solubilization with 0.2% Triton X100, a fraction of POR-pigment complexes recovered from the dark samples was subjected to immunoprecipitation with POR antiserum. (A) Detection of POR-pigment complexes in non-flashed (darkness) and flashed (flash light) plastid samples by their pigment auto-fluorescence at an excitation wavelength at 455 nm. (B) Immunoprecipitation of POR-pigment complexes from an aliquot of the extracts of non-flashed samples shown in (A). (C) Size fractionation of POR-pigment complexes recovered from leaf sector II of non-flashed plants. Migration of size markers is indicated on top.

PORB protein band (data not shown, but see [26]). Pchlide \( b \) present in similar amounts both before and after flash light-illumination (Table 2) co-fractionated with the PORA protein band (data not shown, but see [26]). These results confirmed that only Pchlide \( a \) had been photoreduced.

Fractionation on Superose 6 was used to determine the size of the higher molecular mass complex recovered from leaf sector II of the non-flashed sample. Fig. 5C shows that the resolved complex had an apparent size of ca. 660 kDa.

In addition, PORB dimers and trace amounts of PORA monomers were detected (Fig. 5C).

3.4. Greening under natural conditions

The phenomenon of etiolation has sometimes been disregarded as a natural aspect of seedling development, because most plants green without a prolonged period of dark growth. However, plants which display a hypogeic type of germination partially etiolate while reaching the uppermost parts of
3.5. Light-induced disintegration of LHPP during natural greening

An explanation for these findings could be that the formation of LHPP correlates with the establishment of Pchlide-650/657 and that upon light absorption LHPP would dissociate. As shown previously, the prolamellar body which contains LHPP rapidly disintegrates upon illumination [10,47–49]. We hypothesized that if LHPP’s light-dependent dissociation was to occur, PORA may be activated and could convert its bound Pchlide \( b \) to Chlide \( b \). As a result, PORA would be destabilized and turned over. As shown previously, PORA complexed with Chlide is rapidly degraded by a light-, ATP- and metal ion-dependent stromal protease [50,51].

We hypothesized that Chlide \( b \) produced during the degradation of the PORA could bind to other proteins. A water-soluble chlorophyll protein (WSCP) was recently discovered for barley etiochloroplasts [52] that displays similarity to WSCPs of Brassicaceae [41]. Interestingly, in cauliflower WSCPs bind both Chl \( a \) and Chl \( b \) but exhibited pigment fluorescence at 680 nm only when present in a homo-oligomeric state [41]. We assumed that if similar homo-oligomeric WSCP complexes would be present in barley etiochloroplasts, their accumulation could explain the appearance of the fluorescence peak at \( \sim \)684 nm (Fig. 6B).

Alternatively, this fluorescence peak could be due to formation of either free Chlide \( a \) or Chl \( a \)-complexed type II reaction centers (RCII) [34].

Protein analyses and pigment measurements were performed to gain insight into these different possibilities. When we probed western blots with the POR antiserum, both PORA and PORB were detectable in sectors II and III, but only a small amount of PORA was present in the leaf tip (sector I) (Fig. 7A). In leaf sector IV, only PORB was found. The leaf base did not contain either PORA or PORB, but expressed significant levels of the 44 kDa pPORA (Fig. 7A). The level of this pPORA declined in leaf sectors IV and III, and it was below the limit of detection in sectors II and I.

Pigment measurements were made by HPLC in order to quantify Pchlide \( a \) and Pchlide \( b \) as well as Chlide \( a \) and Chlide \( b \) levels in the various leaf tissues. In addition to the described C18 RP material (see above), separations were performed on C30 columns that have previously been shown to permit simultaneous separation of Chlide \( a \) and Chlide \( b \) and their esterified products, i.e., Chl \( a \) and Chl \( b \), re-
Fig. 7. POR expression in relation to Pchlide and Chlide levels during natural greening. Plants were grown as described in Fig. 6 and proteins and pigments extracted and quantified. (A) Western blot of POR-related proteins. (B) Quantification of Pchlide b (grey columns) and Pchlide a (white columns) levels relative to the total amount of Pchlide in the indicated leaf segments. (C) Quantification of Chlide a and Chlide b levels relative to the total amount of Chlide in the indicated leaf samples. Bars indicate standard deviations determined from five independent experiments.

Collectively the results shown in Figs. 6–8 strengthened the hypothesis that LHPP would progressively disintegrate in leaf sectors III, II and I, and that Chlide a and Chlide b released from the POR A and POR B upon their dissociation and subsequent degradation would bind to WSCPs. The
barley WSCP is a nucleus-encoded plastid protein which is synthesized as a 27 kDa precursor. Import into the plastid occurs under conditions when Chloride is produced, and the 27 kDa precursor is processed to mature size of 22 kDa [52].

Immunological studies were performed with a heterologous antiserum against Lepidium virginicum WSCP to which the barley homolog is most related [52]. Fig. 9A demonstrates that both the 27 kDa precursor and its mature, 22 kDa WSCP could be detected on Western blots of proteins from leaf sections as used for protein and pigment extraction. While highest 27 kDa pWSCP levels were present in leaf sector V, maximal amounts of the mature 22 kDa WSCP were found in leaf sector I.

To identify, quantify and study the functional state of WSCP-bound pigments, plastids were isolated from the various leaf sectors described in Fig. 6A, lysed, and stromal proteins subjected to immuno-precipitation with the anti-Lepidium-WSCP antiserum described before. The resulting immunocomplexes in turn were analyzed further in two different ways. One part of the immunocomplexes was directly extracted with 100% acetone containing 0.1% diethyl pyrocarbonate, and pigments resolved by HPLC and quantified by absorbance and fluorescence measurements as specified above. The other part was analyzed by non-denaturing PAGE.

Fig. 9B demonstrates that both Chloride a and Chloride b could be extracted with acetone from the stromal immunoprecipitates of sectors I and II. In the leaf tip, approximately equimolar levels of Chloride a and Chloride b were detected (Fig. 9B). In leaf sector II, Chloride a levels exceeded those of Chloride b (Fig. 9B). The relative ratio of Chloride a to Chloride b was even further pronounced in favor of Chloride a in leaf sector III (Fig. 9B).

The second half of the recovered immunocomplexes was subjected to a brief pH shift to deplete them of the bound IgG and subsequently electrophoresed under non-denaturing conditions (see Section 2). WSCP oligomers presumably present in the different leaf sectors were detected under UV light by their pigment auto-fluorescence (Fig. 9C, left panel). Then, the gel was blotted onto nitrocellulose and the filter probed with anti-WSCP antiserum (Fig. 9C, right panel). This type of analysis revealed that part of the

Fig. 9. Expression of WSCP-related proteins in relation to Chloride a and Chloride b levels during natural greening. Plants were grown as described in Fig. 6 and proteins and pigments extracted and quantified. In parallel, stromal proteins were prepared from isolated chloroplasts and subjected to immuno-precipitation using anti-WSCP antiserum of L. virginicum. After a brief pH shift in order to release the bound IgG, aliquots of the resulting immunocomplexes were extracted with acetone or administered to non-denaturing PAGE. (A) Western blot of WSCP-related proteins in barley. (B) Quantification of WSCP-bound Chloride a and Chloride b after their extraction with acetone. Bars indicate standard deviations determined from five independent experiments. (C) Detection of WSCP oligomers by non-denaturing PAGE and subsequent inspection of the gels under UV light or by western blotting.

Fig. 10. CP47, CP43, and D1 protein, as well as CFo/CF1-ATPase subunit expression in relation to Chl levels during natural greening. Leaf protein extracts were prepared as described in Fig. 9. After immunoprecipitation using the indicated anti-CP47, anti-CP43, anti-D1 and anti-52 kDa CFo/CF1-ATPase subunit antisera, proteins and pigments were recovered from acetone extracts and quantified. (A) Western blot of CP47, CP43, D1 and 52 kDa CFo/CF1-ATPase subunit. (B) Quantification of Chl a and Chl b levels relative to the total amount of Chl in the various leaf tissues. Bars indicate standard deviations determined from five independent experiments.
Chlide-complexed WSCP in leaf sectors I and II is present in terms of auto-fluorescing oligomers (Fig. 9C, upper band). In addition, also non-assembled, but non-fluorescing WSCP (Fig. 9C, lower band) was detectable in these leaf sectors and leaf sector III. In leaf sectors IV and V, WSCP levels remained below the limit of detection (data not shown, but see Fig. 8A).

Immunoprecipitations with heterologous antisera were performed to examine pigment binding to the CP47, CP43 and D1 proteins of photosystem II in the various leaf sectors. Fig. 10A demonstrates that the level of all three proteins continuously increased from sectors III to I, reflecting leaf development and proplastid-to-chloroplast differentiation. In the youngest parts of the plants (sectors IV and V) no immune signals were obtained (Fig. 10A). These leaf tissues expressed significant amounts of the 52 kDa subunit of the CF0/CF1-ATPase, however (Fig. 10A). HPLC analyses on C30 RP material and differential solvent extractability with mixtures of hexane and acetone [30] demonstrated that the recovered pigments were present in their esterified forms (data not shown). Pigment identification and quantification by absorbance and fluorescence measurements at different wavelength unveiled binding of Chl a, but not Chl b, to the various PSI proteins (Fig. 10B).

4. Discussion

In the present study, we examined the regulated expression and function of the PORA and PORB of barley during seedling development. Two different experimental approaches were used to this end. In the first case, we grew barley plants in darkness and subsequently exposed the seedlings to a single, saturating 1 ms flash of white light. This experimental regime is referred to as “artificial greening” throughout the rest of the discussion. In the second case, we germinated barley seeds under quasi-native conditions under a layer of vermiculite in the presence of dim white light. This regime is referred to as “natural greening”.

Consistent with previous findings [53,54], POR expression was found to be under developmental control. Highest POR protein levels were detected in the leaf tip containing mature etioplasts, whereas the middle leaf sector and the leaf base contained less POR protein (Fig. 1B). Superimposed on this developmental effect were differences in the actual PORA and PORB protein levels in the various leaf sections. PORA accumulated in the differentiated plastids present in sector I, II and III of the leaf, but not in proplastids found in the leaf base (Fig. 1B). However, in these less differentiated tissues, substantial amounts of the cytosolic PORA precursor, pPORA, were found. Despite the presence of Pchlide-628/632 (Fig. 2), these PORA precursor molecules were obviously not imported into the plastids and processed to mature size (Fig. 1B). An explanation for this finding could be that different pools of Pchlide were present in barley plastids and that Pchlide a accumulating in proplastids (Fig. 3) was insufficient to trigger pPORA import. Consistent with this hypothesis is previous work showing that PORA binds Pchlide b with 10-fold higher affinity than Pchlide a [26]. The lack of Pchlide b in the leaf base would thus be limiting for pPORA import. Alternatively, the import machinery through which the pPORA is imported [55,56], may be under developmental control and thus may not be existent in a functional state in proplastids. Dahlin and Cline [45] observed that protein import is determined by developmental age of the plant.

In contrast to PORA, similar amounts of PORB accumulated across the barley leaf gradient (Fig. 1B). As shown previously, PORB is expressed in etiolated, illuminated and light-adapted plants [24], containing etioplasts, etioplasts and mature chloroplasts, respectively. Import of the cytosolic PORB precursor (pPORB) does not depend on Pchlide [29,50,57–61] and occurs via the Toc machinery [55,60].

4.1. Size modulation of LHPP

PORA and PORB structurally interact with each other, as shown in this (Figs. 5 and 8) and previous work [25–27,62]. In addition to PORA and PORB and their cognate pigment substrates, the resolved supra-complex contains galacto- and sulfolipids that shift the absorption maximum of the reconstituted complex from 630 to 650 nm [25–27]. The resulting higher molecular mass complex displays UV, blue and red light-induced auto-fluorescence indicative of the presence of Pchlide-650/657. This correlative evidence suggests that the resolved supra-complex obeys the criteria previously set for LHPP [25]. Indeed, POR protein and pigment stoichiometries shown in this and a recent paper [26] demonstrated that the supra-complex recovered from leaf sectors II and III of plants kept under artificial and natural greening conditions, respectively, consisted of ~5 PORA–Pchlide b–NADPH and ~1 PORB–Pchlide a–NADPH ternary complexes (Figs. 1 and 7, as well as Table 2). In tissues prepared from the leaf tip of etiolated plants, even higher Pchlide b to Pchlide a levels and a ca. 8.5-fold excess of PORA relative to PORB were detected (Fig. 1 and Table 2). Previous work in which the leaf tips of etiolated plants had been disregarded for protein and pigment extraction [25–27] thus underestimated the actual POR and pigment levels.

Size modulation of LHPP may be due to POR protein phosphorylation and changes in the protein–lipid interface not executed in previous in vitro-reconstitution experiments. Wiktorsson et al. [63] and Kovacheva et al. [64] proposed that ADP/ATP and POR phosphorylation may affect the formation and dispersal, i.e. the size, of the prolamellar body and organization state of POR–Pchlide complexes in etiolated plants. Kőta et al. [65] put forth gross alterations in the secondary structure of membrane proteins; conformation, composition, and dynamics of lipid acyl chains; as well as protein pattern. The authors argued that formation of the prolamellar body and subsequent greening may be accompa-
nied by major reorganization events concerning both membrane protein assemblies and the protein–lipid interface.

The size of LHPP recovered from leaf sector II of fully etiolated plants of approximately 660 kDa (Fig. 5C) at first glance seems to match that of the Pchlide holochrome of bean reported previously [6]. It is not known whether two differentially expressed por genes are present in bean plants. In the closely related pea system only one por gene has been cloned thus far [66]; the identity of which is at variance with the detection of two differentially expressed POR polypeptides bands on western blots ([67] and S. Reinbothe, unpublished results). In cucumber, only one por gene and protein were reported [68,69]. These differences suggest that LHPP complexes similar to those identified in this and previous work for barley [36], wheat and Arabidopsis thaliana [70] may not be present in other oxygenic photosynthesizers. On the other hand, less evolved organisms, such as the liverwort (Marchantia paleacea) [71] and the green alga Chlamydomonas reinhardtii [72], lack multiple, differentially regulated por gene families. Instead they contain a second, highly conserved light-independent system for making chlorophyll in the dark. Collectively, these differences amongst different phyla and taxa in the por gene complement implicate environmental adaptations to the selection pressures encountered during evolution [73].

4.2. Light-induced dissociation of LHPP

In etiolated barley plants, Pchlide-650/657 was converted to Chlide-684/690 upon flash light-illumination (Figs. 2 and 3). At the same time higher molecular mass complexes containing both PORA and PORB dissociated into their subunits (Fig. 5). We previously observed that Chlide a formed as a result of flash light-treatment co-fractionated with PORB, whereas Pchlide b bound to PORA did not undergo light-dependent conversion to Chlide b [26]. On the basis of these observations we reconfirmed that Pchlide b is not photoactive in LHPP. Nielsen [1,2] has shown that regeneration of Pchlide-650/657 after a flash light-pulse requires a certain lag-phase. This could imply that PORB-bound Chlide a needs to be replaced by fresh Pchlide a. In vivo, Pchlide a may either be freshly synthesized from protoporphyrin IX via the Mg branch of the C5-pathway or could alternatively be formed from excess Pchlide b by virtue of 7-formyl reductase present in the prolamellar body. According to previous and recent work, barley etioplasts contain 7-formyl reductase activity which converts a wide range of b-type porphyrins and chlorins to their a-type compounds [27,74–77]. A presumed intermediate in the conversion of Pchlide b to Pchlide a is 7-OH-Pchlide a detected in this (Fig. 3, peak 1) and previous work [77].

4.3. Greening under natural conditions involves WSCPs

Under natural greening conditions, Pchlide-650/657 has also been identified (Fig. 6B; see also Ref. [78]). We observed that leaf tissues that contain Pchlide-650/657 also expressed higher molecular mass POR–pigment complexes containing the PORA and PORB (compare Figs. 6B and 8). The amount of both Pchlide-650/657 and higher molecular mass POR–pigment complexes was highest in leaf sector III, but lower in the adjacent leaf sector II. In leaf sector II, an additional fluorescence peak appeared, emitting at 672 nm. In the leaf tip (sector I), little Pchlide-650/657 was present, and the emission maximum of this Pchlide species was slightly blue-shifted to ~652 nm. At the same time, a major fluorescence peak was detectable in the leaf tip that displayed an emission maximum at 684 nm. According to previous work, the appearance of this peak may be due to the presence of free Chlide a, Chl a-complexed PSII reaction centers [34] or Chl-complexed WSCPs [41]. The accumulation of auto-fluorescing WSCP oligomers in the stroma of barley plastids recovered from leaf sectors I and II (Fig. 8) and of reaction center proteins and core antenna polypeptides, such as D1, CP47 and CP43 (Fig. 10), is compatible with these different views.

4.4. A second route of chlorophyll synthesis in illuminated plants?

The identity of the fluorescence peak emitting at 672 nm in leaf sectors II and III is unknown. These leaf sectors contained both Pchlide-628/632 and Pchlide-650/657. Interestingly, we observed PORB dimers under non-reducing electrophoresis conditions (Fig. 8). Both barley PORB and pea POR expressed as a maltose-binding protein also have been shown to form dimers and to display pigment fluorescence at 672 nm [79,80]. Also in wheat, POR dimers have been identified by chemical cross-linking [81]. In a photomorphogenetic mutant of Arabidopsis designated det340/cop1, which does not express detectable PORA protein levels and contains drastically reduced prolamellar bodies, the same pigment species has been identified [34]. Lebedev et al. [34] proposed that Chl synthesis in det340/cop1 mutant plants may occur via a route not involving Pchlide-650/657 that is driven by PORB. Because Chl a accumulation was only observed under very low light intensities, Lebedev et al. [34] concluded that PORA’s function might be to confer photoprotection onto the PORB. Work of Sperling et al. [82] confirmed such a view and demonstrated that PORA overexpression gave rise to a greater seedling survival rate after depletion of endogenous PORB by growth in far red light, which leads to a shut-down of PorA gene expression [83], than observed in the non-transformed wild-type. Remarkably, PORB overexpression also rescued the PORA deficiency, suggesting that there might be an overlap between PORA and PORB function in Arabidopsis. However, it is not yet clear how the recently discovered PORC [84–86] influenced data analysis and what role this third POR protein may play during Chl biosynthesis.

In summary, the results shown in this study and in previous work strengthen the view that PORA and PORB of barley
accomplish unique functions in etiolated plants as part of a higher molecular mass light-harvesting complex. It remains to be tested whether there is a PORC in barley that may interact with PORB in light-adapted plants.

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