Photosynth Res (2008) 96:37–50
DOI 10.1007/s11120-007-9281-y

REGULAR PAPER

Proteomic analysis of highly purified prolamellar bodies reveals their significance in chloroplast development

Lisa A. Blomqvist · Margareta Ryberg · Christer Sundqvist

Received: 10 October 2007 / Accepted: 29 November 2007 / Published online: 11 December 2007

Abstract The prolamellar body (PLB) proteome of dark-grown wheat leaves was characterized. PLBs are formed not only in etioplasts but also in chloroplasts in young developing leaves during the night, yet their function is not fully understood. Highly purified PLBs were prepared from 7-day-old dark-grown leaves and identified by their spectral properties as revealed by low-temperature fluorescence spectroscopy. The PLB preparation had no contamination of extra-plastidal proteins, and only two envelope proteins were found. The PLB proteome was analysed by a combination of 1-D SDS-PAGE and nano-LC FTICR MS. The identification of chlorophyll synthase in the PLB fraction is the first time this enzyme protein was found in extracts of dark-grown plants. This finding is in agreement with its previous localization to PLBs using activity studies. NADPH:protochlorophyllide oxidoreductase A (PORA), which catalyses the reduction of protochlorophyllide to chlorophyllide, dominates the proteome of PLBs. Besides the identification of the PORA protein, the PORB protein was identified for the first time in dark-grown wheat. Altogether 64 unique proteins, representing pigment biosynthesis, photosynthetic light reaction, Calvin cycle proteins, chaperones and protein synthesis, were identified. The in number of proteins' largest group was the one involved in photosynthetic light reactions. This fact strengthens the assumption that the PLB membranes are precursors to the thylakoids and used for the formation of the photosynthetic membranes during greening. The present work is important to enhance our understanding of the significance of PLBs in chloroplast development.

L. A. Blomqvist (✉) · M. Ryberg · C. Sundqvist
Department of Plant and Environmental Sciences, Göteborg University, P.O. Box 461, 405 30 Göteborg, Sweden
e-mail: Lisa.Blomqvist@dpes.gu.se

Keywords Chlorophyll synthase · Chloroplast development · Etioplast · NADPH:protochlorophyllide oxidoreductase · Prolamellar body

Abbreviations

1-D	One-dimensional
2-D	Two-dimensional
comp-PLB	Comparative-PLB
EPIM	Etioplast inner membrane
nano-LC	Nanoliquid chromatography
FTICR MS	Fourier transform-ion cyclotron resonance mass spectrometry
Pchlide	Protochlorophyllide
PLB	Prolamellar body
POR	NADPH:protochlorophyllide oxidoreductase
PT	Prothylakoid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Introduction

Chlorophyll biosynthesis and chloroplast development is favourably studied during the transition of dark-grown plastids, etioplasts, to chloroplasts. The etioplast inner membranes (EPIMs) are distinguished as two structurally different, but in several respects similar, systems, namely the tubular prolamellar bodies (PLBs) and the flat membranes, the prothylakoids (PTs). PLBs of dark-grown plants have been studied extensively (for review, see e.g. Ryberg and Sundqvist 1991; Sundqvist and Dahlin 1997), though their exact role during etioplast-to-chloroplast transition is

still not known. PLBs are believed indeed to play a role in the chloroplast differentiation process, e.g. in young cells and in cotyledons (Rascio et al. 1980; Rebeiz and Rebeiz 1986; Solymosi et al. 2007). The significance of PLBs for a rapid onset of photosynthetic activity is obvious, as PLBs host a pool of protochlorophyllide (Pchlide) that is rapidly reduced and esterified to chlorophyll upon illumination (Rüdiger et al. 1980; Ryberg and Sundqvist 1988, 1991). Thus, PLBs have been proposed to enhance photomorphogenesis (Sundqvist and Dahlin 1997).

The dominating protein of PLBs is the enzyme NADPH:protochlorophyllide oxidoreductase (POR), which catalyses the light-dependent reduction of Pchlide to chlorophyllide (Chlide) in higher plants (Ikeuchi and Murakami 1982; Ryberg and Sundqvist 1982; Lindsten et al. 1988). Several isoforms of this enzyme, named PORA, PORB and PORC, have been detected, and their presence or role in various species or tissues may vary (e.g. Armstrong et al. 1995; Holtorf et al. 1995; Masuda et al. 2003; Donnelly et al. 2005; von Zychlinski et al. 2005). Upon illumination, most of the accumulated Pchlide of dark-grown plants is rapidly reduced to Chlide. Pchlide is present in different spectral forms. The low-temperature (77 K) fluorescence emission spectra of Pchlide in leaves show typical peaks at about 633 ($Pchlide_{F633}$) and 657 ($Pchlide_{F657}$) nm. The main reason for the formation of different spectral forms of Pchlide is differences in the association between Pchlide, NADPH and the POR protein and the aggregation of this pigment-protein complex (Ryberg and Sundqvist 1991; Schoefs and Franck 2003). $Pchlide_{F633}$ can only to a limited amount be phototransformed to Chlide (Kósa et al. 2005), while $Pchlide_{F657}$ is readily transformed with a short (ms) flash of light. This phototransformable form of Pchlide is present in ternary complexes of POR, Pchlide and NADPH and is by far the most abundant Pchlide form in PLBs (e.g. Ryberg and Sundqvist 1982, 1988, 1991). The aggregation of the complexes in PLBs is believed to be associated with the highly regular structure of PLBs in dark-grown leaves (Ryberg and Sundqvist 1988; Böddi et al. 1989; Selstam 1998).

The localization of chlorophyll synthase, which catalyses the esterification of Chlide to chlorophyll, has not been successful up to now. The activity of this enzyme has been studied extensively (for review, see Schmid et al. 2001). Esterification of Chlide was possible in both PLB- and PT-enriched fractions, but enzyme activity was latent in PLBs as long as the PLB structure was well preserved (Lindsten et al. 1990). Later experiments indicated a translocation of chlorophyll synthase from transforming PLBs to developing thylakoids (Lindsten et al. 1993). The gene has been cloned and the protein characterized for *Arabidopsis* (Gaubier et al. 1995; Oster and Rüdiger 1997), *Avena* (Schmid et al. 2001) and *Oryza* (The International Rice Genome Sequencing Project).

The aim of the present work was to analyse the PLB proteome, including tentative hydrophobic proteins lost in our previous study (Blomqvist et al. 2006), to enhance our understanding of the significance of PLB membranes in etioplasts and their transition to chloroplasts. As an informative subetioplast proteomic study requires highly purified organelle subfractions, the procedure for PLB purification was optimized. Screening of the subetioplast location of enzymes active in pigment biosynthesis, as well as of other proteins, will provide valuable information for the understanding of plastid development. The protein compositions of EPIMs (Blomqvist et al. 2006) and whole etioplasts (von Zychlinski et al. 2005; Kleffmann et al. 2007) have been analysed, though no previous study has focused on the PLB proteome. We adopted a relatively new combination of analytical methods, with high potential in proteomic analysis—the one-dimensional (1-D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) combined with nanoliquid chromatography Fourier transform-ion cyclotron resonance mass spectrometry (nano-LC FTICR MS), an approach used with great success (Olsen et al. 2004; Carlsohn et al. 2006). By this method, proteins are separated by SDS-PAGE, and gel pieces from an entire sample lane are excised and subjected to mass spectrometry analysis. The use of SDS-PAGE allows separation of proteins within a high dynamic range, compared to the two-dimensional (2-D) PAGE approach, which is limited by e.g. discrimination against hydrophobic proteins and proteins with high or low molecular masses. However, it should be mentioned that the 1-D SDS-PAGE separation of proteins does not discriminate between different isoelectric isoforms of proteins. Nano-LC coupled to FTICR MS is currently the most powerful proteomic approach in terms of overall separation power and sensitivity and is thus compatible with the analysis of mixtures of many proteins (Zimmer et al. 2006). Using this method we determined the proteome of PLBs from etioplasts of dark-grown wheat and managed to identify 64 proteins including chlorophyll synthase, PORB and 20 proteins known to participate in the photosynthetic light reactions.

Materials and methods

Plant material and isolation of PLBs

Wheat grains (*Triticum aestivum* L. cv. Kosack, Weibull, Landskrona, Sweden) were grown in darkness for 7 days at 23°C. The isolation procedure for PLBs was based on a method described by Ryberg and Sundqvist (1982), but

with several modifications. Figure 1 shows a simplified scheme of the method used here.

Purification of etioplasts

Leaf tissue from 3-cm sections taken 2 cm from the leaf tips was used for the isolation of etioplasts in order to obtain a homogenous etioplast population. Around 30 g leaf material was homogenized in 250 ml sorbitol medium (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 20 mM HEPES/KOH pH 8.0) (Aronsson and Jarvis 2002). The homogenate was filtered through two layers of gauze and four layers of nylon cloth. The leaf residue was rehomogenized in 100 ml sorbitol buffer. The filtered homogenates were combined and centrifuged at 270*g* for 6 min and the pellet, likely of nuclei, was discarded. Etioplasts were collected by centrifugation at 1,100*g* for 10 min. This centrifugation step was repeated once after resuspension of the pellet in sorbitol medium. Crude etioplasts were resuspended in 2 ml sorbitol medium and subjected to Percoll gradient centrifugation. The Percoll gradient [50% (v/v) Percoll (Amersham Biosciences, Uppsala, Sweden) in sorbitol buffer with a trace of gluthathion] was performed by centrifugation at 28,000*g* for 30 min. The etioplast suspension was layered on top of the gradient and centrifuged at 5,300*g* for 10 min. Two yellowish bands were obtained and checked for plastid intactness by phase-contrast microscopy (Olympus BX50). The lower band, containing intact etioplasts, was collected and washed twice in sorbitol medium by centrifugation at 1,400*g* for 10 min. The pellet was resuspended in sucrose medium (0.5 M sucrose, 1 mM $MgCl_2$, 20 mM TES, 10 mM HEPES and 1 mM Na_2–EDTA, pH 7.2).

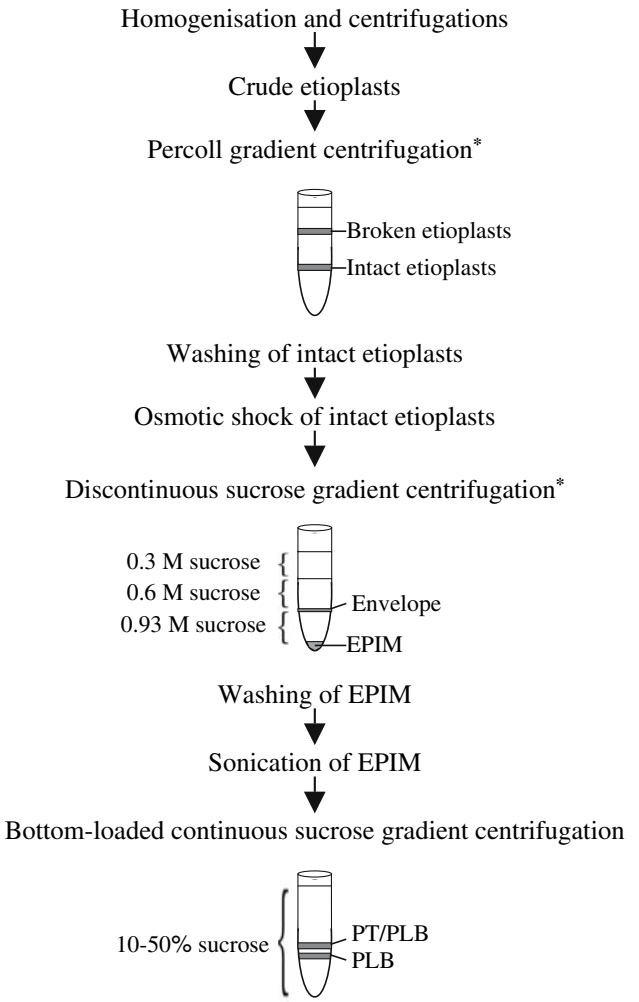


Fig. 1 The flow scheme for the isolation of highly purified prolamellar bodies (PLBs). *Two purification steps added to the protocol by Ryberg and Sundqvist (1982). EPIM, etioplast inner membrane; PT, prothylakoid. For further details, see 'Materials and methods'

Purification of EPIMs

Purified intact etioplasts were lysed in shock medium (1 mM $MgCl_2$, 20 mM TES, 10 mM HEPES and 1 mM Na_2–EDTA, pH 7.2). The lysed suspension was layered on top of a discontinuous sucrose gradient (0.93, 0.6, 0.3 M sucrose in shock medium). Envelope membranes were separated from the EPIMs by centrifugation at 73,000*g* for 1 h. The envelope fraction was collected at the 0.93/0.6 M interface (Bahl et al. 1976; Ferro et al. 2003). The pellet, containing EPIMs, was resuspended in a few ml of sucrose medium, diluted with shock medium, and centrifuged at 10,000*g* for 15 min. This washing step was repeated once. EPIMs were resuspended in 50% (w/v) sucrose in shock medium containing 3 mM NADPH.

Purification of PLBs

The EPIM suspension was sonicated and subjected to centrifugation in bottom-loaded continuous sucrose gradients [10–50% (w/v) sucrose in shock medium, Ryberg and Sundqvist 1982]. The gradients were centrifuged at 93,000*g* for 14.5 h. The centrifugation resulted in two bands. Samples from the two bands were withdrawn for fluorescence spectra. All steps up to this point were performed under weak green light. The remains of the two bands were collected under white light for maximum yield and to avoid mixing. The PLB fraction, collected from the lower band, was diluted with seven volumes of shock medium and centrifuged at 19,000*g* for 1 h. The resulting pellet was resuspended in sodium dodecyl sulphate (SDS) sample buffer [2.5% (w/v) SDS, 60 mM dithiothreitol and 62.5 mM Tris–Cl, pH 6.8] and proteins were denatured according to Blomqvist et al. (2006). The protein content of

an SDS-solubilized PLB fraction without dithiothreitol was determined according to the method of Lowry et al. (1951).

Pigment analyses

The various fractions were analysed for presence of Pchlide spectral forms. Samples of intact leaves, different etioplast fractions (crude, intact and broken) as well as of all other membrane fractions were frozen in liquid nitrogen and fluorescence emission spectra were recorded. The very same samples were then irradiated for 10 min at 255 K, and new spectra were recorded to show the phototransformability of Pchlide. Spectra were recorded at 77 K using a Fluorolog 3 spectrofluorimeter (Spex instruments S.A. Inc., New Jersey, USA). The excitation was set at 440 nm. The spectra were smoothed and corrected for the spectral sensitivity of the photomultiplier and then normalized to the highest peak. The presented spectra are the averages of at least two recordings.

1-D gel electrophoresis and in-gel protein digestion

A sample of the PLB fraction, containing 50 μg of protein, was precipitated with aceton (80%) and resuspended in SDS-PAGE sample buffer [2% (w/v) SDS, 30% (w/w) glycerol, 50 mM dithiothreitol, 0.01% (v/w) bromophenol blue, 62.5 mM Tris, pH 7.2] and loaded on a Criterion gradient gel, 10.5–14% (Bio-Rad technologies, Hercules, USA). After staining the gel with Coomassie brilliant blue R-250 (Bio-Rad), a 2 mm broad gel slice was cut along the entire lane and divided into six pieces. Similarly, and for comparison, a sample (comp-PLB) was excised between 27 and 37 kD from a gel of separated proteins from PLBs isolated according to the protocol by Ryberg and Sundqvist (1982).

The method for in-gel protein digestion with trypsin, described by Shevchenko et al. (1996), was applied with some minor modifications. Briefly, the gel pieces were destained by washing three times in 25 mM NH_4HCO_3 in 50% CH_3CN and once in 25 mM NH_4HCO_3 in 50% CH_3OH. Gel pieces were dried in a vacuum centrifuge and incubated with digestion buffer (50 mM NH_4HCO_3, 10 ng/μl trypsin) at 37°C overnight. Peptides were extracted in 50% CH_3CN/1% CH_3COOH and the supernatant was evaporated to dryness in a vacuum centrifuge. Prior to MS analysis, the peptides were reconstituted in 0.2% HCOOH.

Nanoflow-LC FTICR MS

Two-microlitre sample injections were made with an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) equipped with a Cheminert valve (0.25 mm bore, C2V-1006D-CTC, Valco Instruments Co, Schenkon, Switzerland), connected to an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). The peptides were trapped on a precolumn (45 × 0.075 mm i.d.) packed with 3 μm C_{18}-bonded particles and separated on a reversed-phase column, 200 × 0.050 mm. Both columns were packed in-house with 3 μm Reprosil-Pur C_{18}-AQ particles (Dr Maisch, GmbH, Germany). The flow through the analytical column was reduced by a split to approximately 100 nl/min. A 40 min gradient of 10–50% CH_3CN in 0.2% COOH was used for separation of the peptides.

The nanoflow LC-MS/MS was performed on a hybrid linear ion trap-FTICR mass spectrometer equipped with a 7T ICR magnet (LTQ-FT, Thermo Electron, Bremen, Germany) equipped with a nanospray source modified in-house. The spectrometer was operated in a data-dependent mode, automatically switching to MS/MS mode. MS spectra were acquired in the FTICR, while MS/MS spectra were acquired in the LTQ trap. For each scan of FTICR, the three most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision-induced dissociation. All the tandem mass spectra were searched by MASCOT (Matrix Science, London) against the NCBInr database, restricted to plants. The search parameters were set to: MS accuracy of 5 ppm, MS/MS accuracy of 0.5 Da, one missed cleavage by trypsin allowed, fixed propionamide modification of cysteine and variable modification of oxidized methionine. For protein identification, the minimum criteria were at least one peptide matched at, or above, the 99% level of confidence and one at the 95% level.

Bioinformatic analyses

The TargetP program (http://www.cbs.dtu.dk/services/TargetP/) was used for the prediction of transit peptides of the identified proteins not annotated with a location in the NCBI database (http://www.ncbi.nlm.nih.gov/). The unassigned identified proteins were searched against the Swiss-Prot database for homologous proteins using the Blast program (http://www.expasy.org/tools/blast/). A function was assigned for proteins sharing at least one of the matched peptides with the homologue.

Results

The novel systematic analysis of the PLB fraction from dark-grown wheat identified 64 unique proteins (Table 1). The modified protocol for PLB isolation (Fig. 1) developed in the present study resulted in highly purified PLBs as confirmed by pigment and proteomic analyses.

Table 1 Proteins identified in prolamellar bodies (PLBs) from dark-grown wheat

Protein name[a]	Mass (kDa)	Accession number[b]	Cover MS[c]	%	Protein source[d]	Genome[e]	Additional bioinformatic analysis[f]
Pigment biosynthesis							
PORA (NADPH:protochlorophyllide oxidoreductase A)	41.4	gil510677	75		*Triticum aestivum*	N	
PORB (NADPH:protochlorophyllide oxidoreductase B)	42.4	gil683476	63.5		*Hordeum vulgare*	N	
Protoporphyrinogen IX oxidase	57.2	gil6715441	3.2		*Zea mays*	N	
Mg-protoporphyrin IX ester cyclase	48.4	gil58617796	32.1		*Hordeum vulgare*	N	
Chlorophyll synthase	41.1	gil7378659	8.5		*Avena sativa*	N	
GGH (Geranylgeranyl hydrogenase)	51.1	gil72256525	38.1		*Triticum aestivum*	N	
Phytonene desaturase	46.4	gil19879445	6.4		*Hordeum vulgare*	N	
Photosynthetic light reaction							
CF1α (ATP synthase α-chain, AtpA)	55.3	gil14017569	50.8		*Triticum aestivum*	C	
CF1β (ATP synthase β-chain, AtpB)	53.9	gil14017579	61.2		*Triticum aestivum*	C	
CF1ε (ATP synthase ε-chain, AtpE)	15.3	gil67870	43.8		*Hordeum vulgare*	C	
CF1γ (ATP synthase γ-chain, AtpC)	40.2	gil110278822	12.5		*Zea mays*	N	
CF0 b (ATP synthase B-chain, AtpF)	21.0	gil224550	36.1		*Triticum aestivum*	C	
OEC16 (Oxygen-evolving complex 16 kDa, PsbQ)	20.1	gil32400800	27.7		*Triticum aestivum*	N	
OEC23 (Oxygen-evolving complex 23 kDa, PsbP)	27.5	gil21837	26.4		*Triticum aestivum*	N	
OEC33 (Oxygen-evolving complex 33 kDa, PsbO)	35.0	gil21844	33.5		*Triticum aestivum*	N	
PC (Plastocyanin)	15.8	gil130269	20.6		*Hordeum vulgare*	N	
Cyt *b*559 (Cytochrome *b*559 alpha chain, PsbE)	9.5	gil14017588	22.9		*Triticum aestivum*	C	
Cyt *b*6 (Cytochrome *b*6, PetB)	26.2	gil11596	14.2		*Hordeum vulgare*	C	
Cyt *f* (Cytochrome *f*, PetA)	35.5	gil758344	48.8		*Triticum aestivum*	C	
10 kD phosphoprotein (PsbH)	7.8	gil11595	41.1		*Hordeum vulgare*	C	
Rieske Fe-S protein (PetC)	24.2	gil32394644	25.7		*Triticum aestivum*	N	
FNRI (Ferredoxin-NADPH oxidoreductase)	39.3	gil20302471	38.8		*Triticum aestivum*	N	
FNRII (Ferredoxin-NADPH oxidoreductase)	40.6	gil20302473	51.5		*Triticum aestivum*	N	
PsbS (PSII subunit, 22 KDa, PSII-S)	27.9	gil34908652	21.6		*Oryza sativa*	N	
Hcf136 (PSII stability/assembly factor)	45.5	gil54291349	23.3		*Oryza sativa*	N	
PS1 assembly protein (Stress-inducible protein)	14.6	gil50944589	11.6		*Oryza sativa*	N	
PSII lumenal subunit	31.8	gil50878369	12.7		*Oryza sativa*	N	
Calvin cycle							
RbcS (Ribulose-1,5-bisphosphate carboxylase small subunit)	19.8	gil1167948	35.1		*Hordeum vulgare*	N	
RbcL (Ribulose-1,5-bisphosphate carboxylase large subunit)	53.5	gil61378609	31.2		*Australopyrum retrofractum*	C	
RcaB (Ribulose 1,5-bisphosphate carboxylase activase)	47.5	gil167095	29.2		*Hordeum vulgare*	N	
GAPA (Glyceraldehyde-3-phosphatase A)	43.1	gil50924788	15.4		*Oryza sativa*	N	TargetP:C2
FBP aldolase (Fructose 1,6-bisphosphate aldolase)	42.2	gil8272480	5.4		*Avena sativa*	N	
Chaperones/proteases							
FtsH	72.6	gil52075838	21.9		*Oryza sativa*	N	
FtsH	76.2	gil1483215	26.8		*Arabidopsis thaliana*	N	
ClpC	98.6	gil50923949	7.3		*Oryza sativa*	N	

Table 1 continued

Protein name[a]	Mass (kDa)	Accession number[b]	Cover % MS[c]	Protein source[d]	Genome[e]	Additional bioinformatic analysis[f]
FKBP-type PPIase	27.7	gil50948109	10.3	*Oryza sativa*	N	TargetP:C1
Cyclophilin-type PPIase (CYP38)	48.2	gil45680876	8.5	*Arabidopsis thaliana*	N	TargetP:C1
Cyclophilin-type PPIase (CYP20)	26.1	gil37788308	23.7	*Triticum aestivum*	N	TargetP:C1
Protein synthesis						
Poly A polymerase	50.5	gil2623246	10.1	*Pisum sativum*	N	TargetP:C4
RpoA (RNA polymerase alpha subunit)	39.2	gil12372	8.8	*Triticum aestivum*	C	
RpoB (RNA polymerase beta subunit)	122.1	gil902212	4.9	*Zea mays*	C	
CL9 (Plastid ribosomal protein CL9)	21.6	gil22204126	13.6	*Triticum aestivum*	N	
L11 (Ribosomal protein L11)	23.4	gil50916344	10.9	*Oryza sativa*	N	
L12 (Ribosomal protein L12)	18.4	gil288584	19	*Secale cereale*	N	
L14 (Ribosomal protein L14)	13.7	gil14017608	47.2	*Triticum aestivum*	C	
L19 (Ribosomal protein L19)	25.5	gil14140122	7.7	*Oryza sativa*	C	
Other/unknown functions						
PrxQ (Peroxiredoxin Q)	23.8	gil55833012	38.7	*Triticum aestivum*	N	
APX (Thylakoid-bound ascorbate peroxidase)	41.5	gil25992557	22.2	*Triticum aestivum*	N	
Oxidase (IM1)	26.6	gil9837152	7.6	*Triticum aestivum*	N	
DNAJ homologue	47.5	gil16326131	5.3	*Oryza sativa*	N	
HAD-superfamily hydrolase	117.8	gil108707786	1.8	*Oryza sativa*	N	
NADH dehydrogenase J	18.8	gil14017575	13.8	*Triticum aestivum*	C	
Ferritin	28.4	gil49615739	7.8	*Triticum monococcum*	N	
Thylakoid lumenal 20 kDa protein-like	29.0	gil55296826	8.5	*Oryza sativa*	N	
Unknown (Similar to thylakoid lumenal protein)	34.7	gil54290425	6.7	*Oryza sativa*	N	BLAST (7E-78)
Unknown	28.9	gil50944807	20.7	*Oryza sativa*	N	TargetP:C2
Unknown	20.7	gil34895746	20.2	*Oryza sativa*	N	TargetP:C3
Unknown	19.4	gil77556201	10.0	*Oryza sativa*	N	TargetP:C4
Unknown	27.8	gil34905300	12.7	*Oryza sativa*	N	TargetP:C1
Unknown	38.0	gil50928489	7.5	*Oryza sativa*	N	TargetP:C3
Unknown	42.0	gil50917939	10.6	*Oryza sativa*	N	TargetP:C2
Envelope proteins						
Chloroplast inner envelope protein	108.3	gil37535626	2.8	*Oryza sativa*	N	
Fatty acid hydroperoxide lyase	53.9	gil22265999	5.5	*Hordeum vulgare*	N	

Proteins were separated by SDS-PAGE and identified by nano-LC FTICR MS. All proteins were identified with at least one peptide at, or above, the 99% level and one peptide at the 95% level

[a] Protein name/protein name abbreviation

[b] Accession number in the NCBI database of the identified protein sequence

[c] The percentage of the identified protein's sequence covered at 5 ppm for the MS/MS peptides

[d] The source organism of the identified protein

[e] The genome coding for the protein. C, chloroplast genome; N, nuclear genome

[f] One of the unknown proteins could be assigned by homology using the Blast program (with the corresponding *E*-value). The TargetP was used for the prediction of location for unknown proteins and for proteins not annotated with a location. The sequence contains a predicted chloroplast (C) transit peptide; reliability class 1–5, where 1 indicates the strongest prediction

Purification of PLBs

PLBs were isolated from the part of dark-grown wheat leaves that contains well-developed etioplasts, which rapidly turn into chloroplasts upon illumination (Gunning and Steer 1996; Wellburn et al. 1986, see 'Materials and methods'). The PLB isolation procedure was based on the method described by Ryberg and Sundqvist (1982) but with certain modifications in order to optimize the purity (Fig. 1). The first modification was etioplast purification by

Percoll gradient centrifugation, which resulted in a significant reduction of broken etioplasts (Aronsson and Jarvis 2002). At the same time the intact etioplasts were purified from contaminants from nuclei and mitochondria. Two yellowish bands were obtained as well as a brown-white pellet not further analysed. Intact etioplasts were concentrated to the lower band, as verified by phase-contrast microscopy.

Using of the protocol from Ryberg and Sundqvist (1982) might lead to a comigration of envelope membranes and EPIMs. To diminish the comigration intact etioplasts were lysed and subjected to discontinuous sucrose gradient centrifugation. EPIMs were concentrated in the pellet while envelope membranes were trapped at the 0.93/0.6 M interface (Bahl et al. 1976; Ferro et al. 2003). The envelope fraction contained known envelope proteins such as the triose phosphate translocator and Toc34, as proven by proteomic analyses (data not shown). The pigment dominating the envelope fraction was the non-phototransformable $Pchlide_{F633}$, while phototransformable $Pchlide_{F657}$ was the dominating pigment of the EPIM fraction (Fig. 2a). The Pchlide present in the envelope fraction was not transformed to Chlide during irradiation. To separate PLBs from PTs, EPIMs were sonicated in 50% sucrose medium in the presence of 3 mM NADPH to increase the preservation of native Pchlide-POR complexes (cf. Ryberg and Sundqvist 1988). The membranes were then subjected to bottom-loaded continuous sucrose gradient centrifugation giving a separation of the membranes into two bands.

The lower band contained PLBs and the upper band contained PTs and fragments of PLBs. This conclusion is based on studies where PLBs and PTs were characterized by their spectral properties in combination with electron microscopy (Ryberg and Sundqvist 1982, 1988, 1991; Lindsten et al.1988). The position of the PLB band in the gradient was approximately at the same density as in Ryberg and Sundqvist (1982) and showed spectral properties of PLBs. This band was used for the analysis of the PLB proteome. NADPH preserved the PLB structure intact through the final collection and centrifugation steps, despite collection in light, as indicated by the preservation of the $Chlide_{F694}$ (Fig. 2c, cf. Ryberg and Sundqvist 1988). The upper band contained $Pchlide_{F657}$ as well as $Pchlide_{F633}$ (Fig. 2b). This band is enriched in PTs, but contains also fragments of PLBs and is here referred to as the PT/PLB fraction.

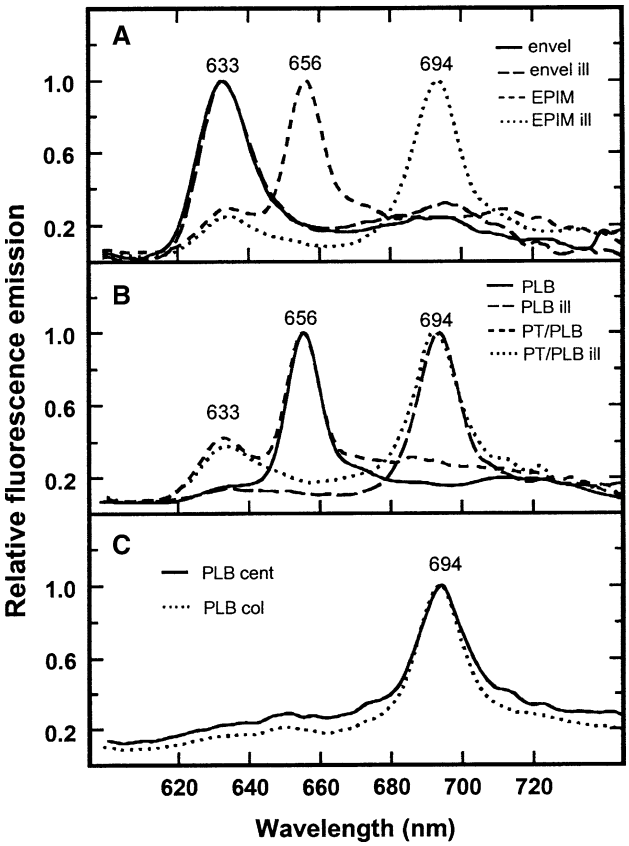
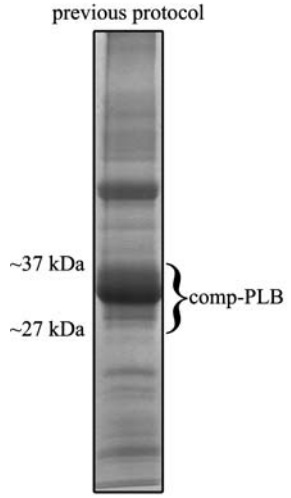


Fig. 2 Fluorescence emission spectra of membrane fractions isolated from wheat etioplasts. Samples were frozen in liquid nitrogen and spectra recorded at 77 K. The excitation wavelength was 440 nm. (**a**) Fractions of envelope (envel) and etioplast inner membrane (EPIM) separated by a discontinuous sucrose gradient before and after illumination (ill) at 255 K for 10 min; (**b**) Prolamellar body (PLB) fraction and a mixed membrane fraction (PT/PLB) before and after illumination (ill) at 255 K for 10 min; (**c**) Sample of PLB fraction collected (PLB col) in white light and sample of the PLB fraction after pelleting and resuspension (PLB cent) for solubilization in SDS buffer. The preservation of $Chlide_{F694}$ during centrifugation indicates the conservation of the regular PLB structure

Separation and identification of PLB proteins

PLB proteins were analysed by combining SDS-PAGE with nano-LC FTICR MS, an extremely sensitive and accurate proteomic approach (Olsen et al. 2004; Carlsohn et al. 2006). The SDS-PAGE allows the separation of proteins with various hydrophobicity in a wide molecular mass range. All proteins presented in Table 1 and Fig. 3 were unambiguously identified with a high confidence level (see 'Materials and methods'). This proteomic approach, SDS-PAGE combined with mass spectrometry, however, does not reveal any quantitative differences in the expression rates of different proteins. Consequently, this approach allows only a mapping of which proteins are present in the gel at a detectable level. It should also be mentioned that a prerequisite for a separated protein to be identified is that it is either sequenced in the organism being examined, or that it shares a high sequence similarity

A



B Proteins identified in comp-PLB

Protein name[a]	Mass (kDa)	Accession number[b]	Cover% MS[c]	Additional bioinformatic analysis[f]
Plastid proteins identified also in the PLB-fraction (Table 1)				
PORA (Protochlorophyllide oxidoreductase A)	41.4	gi|10720235	51,8	
PORB (Protochlorophyllide oxidoreductase B)	42.4	gi|10720236	21,8	
Chlorophyll synthase	41.2	gi|7378659	10,6	
CF1γ (ATP synthase γ-chain, AtpC)	40.2	gi|110278822	12,5	
Cyt f (Cytochrome f, PetA)	35.5	gi|14017584	38,8	
OEC33 (Oxygen evolving complex 33 kDa, PsbO)	26.6	gi|739292	46,6	
FNRI (Ferredoxin-NADPH oxidoreductase)	39.3	gi|20302471	10,5	
FNRII (Ferredoxin-NADPH oxidoreductase)	40.6	gi|20302473	22,3	
PsbS (PSII subunit, 22KDa, PSII-S)	27.9	gi|115441299	15,8	
Hcf136 (PSII stability/assembly factor)	45.5	gi|75252730	15,4	
FBP aldolase (Fructose 1,6-bisphosphate aldolase)	42.4	gi|218155	12,9	
Unknown (Similar to thylakoid lumenal protein)	34.5	gi|54290426	6,7	Blast: 3E-76
Plastid proteins not identified in the PLB-fraction (Table 1)				
Envelope triose phosphate translocator	43.5	gi|13195734	7,2	
Harpin binding protein 1	29.5	gi|38679331	33,2	
Mg-protoporphyrin IX methyltransferase	35.1	gi|6006382	8,9	
Ribosomal protein L3	23.0	gi|42409125	21,9	
Cyclophilin PPIase	36.3	gi|115475740	6,7	TargetP: C2
Unknown	32.8	gi|115461160	5,7	TargetP: C1
Unknown	38.0	gi|38344143	9,5	TargetP: C3
Unknown	31.9	gi|115440489	5,2	TargetP: C1
Unknown	37.6	gi|115459540	6,4	TargetP: C1
Mitochondrial proteins not identified in the PLB-fraction (Table 1)				
ADP/ATP carrier protein	36.0	gi|944842	17,8	
Prohibitin-like protein	16.2	gi|63080996	12,4	
Malate dehydrogenase	36.5	gi|21388550	9,3	
2-oxoglutarate	32.5	gi|1100739	6,6	
Cytochrome c oxidase	29.9	gi|14321	6,5	
Nuclease	35.1	gi|58531976	10,5	TargetP: M5
Unknown	29.5	gi|115441101	5,6	TargetP: M5

Fig. 3 (**a**) SDS-PAGE of prolamellar bodies (PLBs) isolated according to (Ryberg and Sundqvist 1982). The sample was denatured in SDS buffer, separated in a 10.5–14% gradient gel and stained with Coomassie brilliant blue R-250. A sample of proteins, comp-PLB, was excised from the lane as indicated and analysed by nano-LC FTICR MS for comparison with the PLB fraction using the new protocol. (**b**) Of the 28 proteins identified by nano-LC FTICR MS analysis, 12 proteins were identified in both the comp-PLB and in the PLB fraction. Nine plastid proteins as well as seven mitochondrial proteins were identified in the comp-PLB sample but not in the PLB fraction. ([a–c,f]see footnotes in Table 1; M, mitochondrial transit peptide)

with a sequenced protein from another organism. It is thus likely that the proteome of PLBs consists of more proteins than the ones identified in the present analysis.

The modified protocol for PLB isolation used in this study resulted in an improved purity of the PLB fraction. For a comparison a sample of PLB proteins, here referred to as comp-PLB (Fig. 3), was isolated using the protocol of Ryberg and Sundqvist (1982). The comp-PLB contained several proteins, which were not found in the present PLB fraction. Of the 28 identified proteins in the comp-PLB, seven are mitochondrial proteins (25%) and one is the most abundant protein of envelopes, the triose phosphate translocator. Nine true plastid proteins (see below for criteria of accepted true plastid proteins) were identified in the comp-PLB but not in the PLB fraction.

Proteome of the PLB fraction

The lack of contaminants in the PLB fraction isolated in this novel study was confirmed by proteomic analyses (Fig. 4, Table 1). Proteins from the mitochondrion, an organelle known to be a classical contaminant of purified plastids (Ferro et al. 2003), were totally excluded from the PLB fraction. Neither were any nucleus proteins found. All 64 identified proteins can be considered as genuine plastid proteins. Proteins were accepted as true plastid proteins when they either were annotated as plastid proteins in the NCBI/TrEMBL databases, were predicted to have a plastid transit peptide or were highly homologous to a known plastid protein. The presence of some stromal proteins probably reflects the trapping of these proteins within the three-dimensional network of the PLBs (Wellburn et al. 1977, 1982). The identification of merely two envelope proteins raises the question on how the inner envelope participates in the formation of EPIMs, which is discussed later.

For most of the identified proteins the possible functions were previously described or assessed here by homology searches using the bioinformatic tool Blast. Many of the proteins are directly or indirectly involved in the photosynthetic machinery. Seven different enzymes involved in pigment biosynthesis were identified. Interestingly, the chlorophyll synthase protein was identified for the first time in plant extracts, and PORB was identified for the first time in dark-grown wheat.

A considerable number of proteins participating in the photosynthetic light reactions were identified in the PLB fraction, though only one known chlorophyll *a*/*b*-binding protein (PsbS) was identified. Fourteen of the 64 identified proteins are encoded by the chloroplast genome in wheat. Six proteins, with predicted plastid transit peptides but with

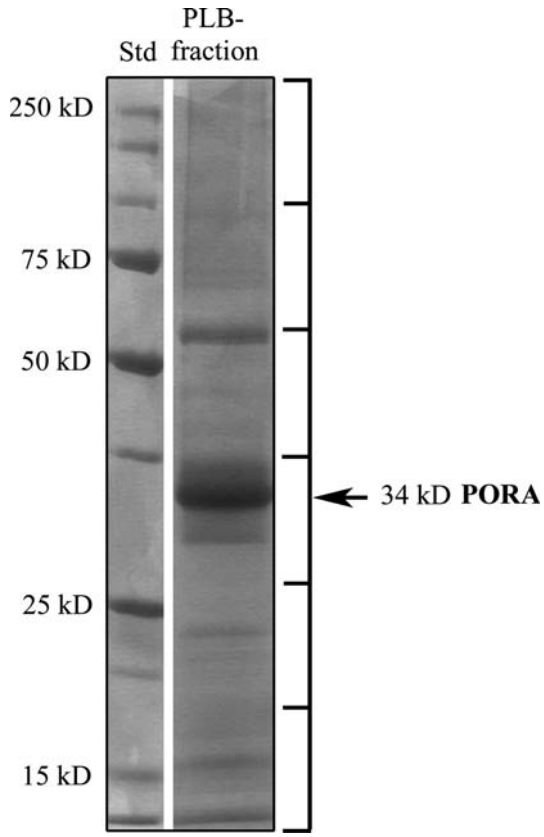


Fig. 4 SDS-PAGE separation of proteins from purified prolamellar bodies (PLBs). The sample was denatured in SDS buffer, separated in a 10.5–14% gradient gel and stained with Coomassie brilliant blue R-250. A gel slice of the entire lane was excised and divided into six pieces as indicated in the Figure. The mass spectra produced by nano-LC FTICR MS of the six gel pieces were merged before MASCOT searches. The heavy band at 34 kDa corresponds to NADPH:protochlorophyllide oxidoreductase A (PORA)

unknown functions, could not be assessed a function by homology searches. These proteins represent proteins that potentially comprise a novel etioplast function.

Discussion

The number of previously described PLB proteins is rather few. Rough estimations of the protein composition of PLBs has been based mostly on SDS-PAGE gel patterns and concluded to be rather simple (for review, see Bruce et al. 1998). The dominating protein of PLBs is POR (Ikeuchi and Murakami 1982; Ryberg and Sundqvist 1982). Presence of a number of other proteins have been observed, though shown only for the large subunit of Ribulose-1,5-bisphosphate carboxylase (RbcL) and cytochrome *f* (e.g. Lütz et al. 1981; Shaw et al. 1985; Lindsten et al. 1988). During the past decades the refinements of sensitivity and accuracy of mass spectrometry and bioinformatic data analysis have highly improved the identification of complex protein samples from a number of biological sources. Subcellular proteomics allows a more detailed localization and has improved the proteome coverage (for review, see Chen et al. 2006; Rossignol et al. 2006). Not only the proteome of whole plastids has been described (Kleffman et al. 2004; Baginsky et al. 2005) but more important the proteome of sub-organelle fractions such as plastid envelope (Ferro et al. 2003; Froehlich et al. 2003), thylakoids (Friso et al. 2004; Peltier et al. 2004), thylakoid lumen (Peltier et al. 2002; Schubert et al. 2002) and plastoglobuli (Ytterberg et al. 2006). Inevitably, the increased sensitivity of proteomic techniques sets higher demands on the purity of the samples analysed. Our identification of 64 proteins in highly purified PLBs has thus proven the PLB proteome to be more complex than previously described.

The present study resulted in three major findings. The first, and most interesting finding, is the novel detection of the chlorophyll synthase protein. The second finding, and not too surprising one, is the detection of PORB in dark-grown wheat. The third finding, and perhaps the most challenging one to discuss, is the detection of many thylakoid proteins in the PLBs of a plant that was grown in darkness. These findings are discussed in the light of the improved isolation method, which resulted in a PLB fraction devoid of detectable contamination, as well as the improved protein analysis that allowed detection of both hydrophilic and hydrophobic proteins. We discuss the impact of our results on the significance of PLBs for a rapid ontogenesis of photosynthetically functioning chloroplasts.

The isolation of PLBs and PTs

The protocol for PLB isolation used in this study resulted in an altered migration pattern of PLBs and PTs in the continuous sucrose gradient (Fig. 1), as compared to the protocol from Ryberg and Sundqvist (1982). The lower band, the PLB band, was recognized by its density and spectral properties similar to those of previous PLB preparations (Ryberg and Sundqvist 1982, 1988, 1991; Lindsten et al. 1988) characterized also with electron microscopy. The high content of $Pchlide_{F657}$ and the lack of contaminating proteins from other organelles as shown by the proteomic analyses (Table 1) underline the purity of the PLB fraction. The PT-containing band (described here as PT/PLB), is commonly contaminated by fragments of PLBs (Ryberg and Sundqvist 1988, 1991) and often referred to just as the "PT-enriched" band.

In previous studies, the "PT-enriched" band was found at higher density, below the PLB band in the gradient, and the dominating pigment was $Pchlide_{F633}$ (e.g. Ryberg and Sundqvist 1982; Lindsten et al. 1988). The high portion of $Pchlide_{F657}$ in the PT/PLB band in this study (Fig. 2b)

suggests that PLBs constitute a relatively higher part of the membrane content of the PT-containing band than in previous results. The reason for the changed migration pattern of the PT-containing band could be an effect of the increased exposure of the membranes to different sucrose solutions, i.e. during centrifugation on the additional discontinuous sucrose gradient and/or the increased centrifugation time of the continuous sucrose gradient. During isolation, PTs form vesicles entrapping fluids which could result in a different density. A part of the PT membranes might comigrate with the envelope in the discontinuous sucrose gradient and the $Pchlide_{F633}$ dominating the pigment content of the envelope fraction could in part originate from PTs, though it has been shown that envelope membranes of chloroplasts contain $Pchlide_{F633}$ (Pineau et al. 1986; Joyard et al. 1990). The possible loss of some PTs in the discontinuous sucrose gradient could explain the increased proportion of $Pchlide_{F657}$/$Pchlide_{F633}$ in the PT/PLB fraction compared to previous results (Ryberg and Sundqvist 1982; Lindsten et al. 1988).

The novel detection of the chlorophyll synthase protein

The presence of the chlorophyll synthase protein was established for the first time in higher plants. We conclude that it is a true component of PLBs, since it was found in an etioplast membrane fraction with no contaminating proteins known to originate from other organelles and with a density and spectral properties typical of PLBs. Chlorophyll synthase activity was previously localized in both PLB- and PT-enriched fractions (Lindsten et al. 1990), but many attempts to confirm the localization of the enzyme protein have failed. We do not exclude presence of chlorophyll synthase also in PTs. However, neither was the proteome of PTs the topic of this study, nor were PTs isolated to purity. In this context it may be of interest to note that protochlorophyll, i.e. esterified Pchlide, is present, albeit in low amounts, in PTs (Böddi et al. 1989). However, it is not known whether the esterification of Pchlide is catalysed by chlorophyll synthase.

Another strong indication that chlorophyll synthase is a true component of PLBs is that the light-induced relocalization of POR from transforming PLBs to developing thylakoids was accompanied by an increased chlorophyll synthase activity in the PT fraction and a concomitant decrease of activity in the PLB fraction (Lindsten et al. 1993). It was hypothesized that POR and chlorophyll synthase form a complex in PLBs (Domanskii et al. 2003), though the possibility of simultaneous binding of the two enzymes to Chlide was excluded (Rüdiger et al. 2005). This conclusion is strengthened by the observation that no chlorophyll synthase activity took place in isolated PLBs as long as Chlide was still in complex with POR (Lindsten et al. 1990). A detailed review of the esterification of chlorophyll, catalysed by chlorophyll synthase, and its implication for thylakoid development was given by Rüdiger (1993).

The chlorophyll synthase proteins that have been cloned were described as highly hydrophobic with as many as nine transmembrane helices, as predicted by HMMTOP (Gaubier et al. 1995; Oster and Rüdiger et al. 1997; Schmid et al. 2001). Proteins with such high hydrophobicity can hardly be separated by 2-D gel electrophoresis; thus, it is not surprising that chlorophyll synthase was not identified in our previous proteomic study (Blomqvist et al. 2006). With the present identification and localization of chlorophyll synthase, also the final major step in chlorophyll biosynthesis in greening leaves was shown to be connected with PLBs.

The detection of PORB in wheat

PORB, another enzyme in chlorophyll biosynthesis that has been the subject of extensive research and discussion, was identified here for the first time in dark-grown wheat. This protein was not observed among the proteins separated by 2-D gel electrophoresis in our previous study on wheat, only the PORA isoform was found (Blomqvist et al. 2006). It is well known that POR is the dominating protein of PLBs (e.g. Ryberg and Sundqvist 1982; Ryberg and Dehesh 1986; Lindsten et al. 1988), but it has been questioned whether wheat has a second POR gene or just one, like pea (Spano et al. 1992) and cucumber (Kuroda et al. 1996). Several different POR isoforms have been reported in a number of angiosperms. PORA and PORB, two structurally similar but differently regulated isozymes, were identified for the first time in barley (Holtorf et al. 1995) and *Arabidopsis* (Armstrong et al. 1995). While PORB is present and active throughout the life of the plant, PORA appears to be present and functional only during the first few hours of greening. A third isozyme, PORC, was characterized in *Arabidopis* and found to be induced by high light irradiation (Masuda et al. 2003).

We used the transmembrane prediction tool HMMTOP to analyse the PORA (from wheat) and the PORB sequences (from barley, since it is not sequenced from wheat). PORB was predicted to have three transmembrane helices, while PORA was predicted to have only one transmembrane helix. This could explain why we were able to identify only PORA and not the more hydrophobic PORB in our previous study (Blomqvist et al. 2006). The use of a combination of 1-D SDS-PAGE and nano-LC FTICR MS was thus more efficient in identifying membrane proteins than the method involving 2-D SDS-PAGE.

It is known that POR has a large number of hydrophobic amino acids. It was found tightly bound to the membrane (Grevby et al. 1989; Widell-Wigge and Selstam 1990), even though POR was described as a non-transmembrane protein (Benli et al. 1991; Timko 1993; Birve et al. 1996). The possibility of POR and its isoforms as being integral membrane proteins invites to new discussions concerning their membrane connection and role in PLB formation as well as their aggregation and interaction with pigments and other proteins, e.g. chlorophyll synthase.

Thylakoid proteins as components of PLBs

The rich content of photosynthetic proteins found in the PLBs (Table 1) strengthens the idea that not only PTs but also PLBs must be regarded as thylakoid precursors. Many proteins involved in, or connected to, the electron transport chain were identified in the PLBs. Some of the proteins in the photosynthetic machinery, e.g. Hcf136 and the oxygen-evolving complex, are of importance already at an early stage of chloroplast development, because of their roles in the correct assembly of the photosystems (Ryrie et al. 1984; Hashimoto et al. 1993; Müller and Eichacker 1999; Plücken et al. 2002). The presence of chlorophyll is a prerequisite for the accumulation of most chlorophyll-binding proteins, e.g. those of the light-harvesting complex II (for review, see Paulsen 2001). One chlorophyll-binding protein, PsbS, was identified in the present study. However, this protein was suggested not to have a light-harvesting function but rather to play a key role in the H^+-dependent thermal deactivation in PSII, the process termed non-photochemical quenching (Funk et al. 1995; Horton and Ruban 2004).

On the significance of PLBs in chloroplast development

Light induces a rapid loss of the regular structure of PLBs. This is followed by a relocation of POR from the PLBs to the PTs, an event which coincides in time with the Shibata shift of newly formed Chlide (Ryberg and Dehesh 1986). Likewise, chlorophyll synthase activity was found to be relocated from transforming PLBs to developing thylakoids during irradiation (Lindsten et al. 1993). Vesicle flow from the envelope to the thylakoids has been proposed as a way of transporting lipids for the building of thylakoid membranes as well as a transport system for proteins involved in photosynthesis (Morré et al. 1991; Kroll et al. 2001; Sherameti et al. 2004). The low number of envelope proteins identified in PLBs in our study, together with the absence of the triose phosphate translocator which is the dominating envelope protein, indicates that there is some protein sorting in the envelope before vesicle formation, as is the case for membrane flow through ER and golgi (Hinz et al. 1999; Jürgens 2004; Pfeffer 2007). Protein sorting can also occur when PLBs are formed from PTs as more and more Pchlide and proteins, especially POR, are incorporated.

It can be discussed whether dispersed 'intact' PLB vesicles re-organize into thylakoids in light, or whether PLB components migrate from the transforming PLBs to the PTs, which then develop to thylakoids. Previous studies of PLBs in vitro showed that light caused a vesicle formation on the surface of PLBs (Ryberg and Sundqvist 1988), an indication favouring the former idea, while the light-induced relocalization of POR from transforming PLBs to PTs (Ryberg and Dehesh 1986) may indicate a migration of individual components or complexes. The fact that Pchlide and POR are present as complexes in the PLBs makes these membranes a 'safe' storage site preventing proteolytic breakdown of these components. It is not far-fetched to assume that also other PLB components, which are of fundamental importance for a rapid onset of photosynthesis, e.g. chlorophyll synthase, are protected from proteolytic breakdown as long as the PLB membrane structure is intact. By this means, these proteins are readily available for the onset of thylakoid formation and photosynthesis at the time when the leaves are exposed to light. Besides proteins involved in pigment biosynthesis and photosynthesis, proteins active in proteolysis and protein synthesis, to mention some other functions, were identified as PLB components in our study. Whether these are inactive like chlorophyll synthase, as long as the PLB structure remains highly regular, remains to be shown. No doubt, presence of PLBs favours a rapid onset of chloroplast development, as compared to dark-grown tissues devoid of PLBs (Virgin 1993; Böddi et al. 1996). Almost 80% of the identified proteins are coded for by nuclear genes and thus imported already at the etioplast stage. The presence of photosynthesis proteins, as well as a pool of Pchlide that is rapidly transformed to chlorophyll, in PLBs likely facilitates a rapid formation of photosynthetically functional membranes during the emergence of the seedling from growth underground to a life in light.

Acknowledgements We thank Elisabet Carlsohn and Sjoerd van der Post at the Proteomics Core Facility, Göteborg University, for performing the MS analyses. This work was supported in part by the Royal Society of Arts and Sciences in Göteborg and the Swedish Research Council.

References

Armstrong GA, Runge S, Frick G, Sperling U, Apel K (1995) Identification of NADPH:protochlorophyllide oxidoreductases A and B: a branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol 108:1505–1517

Aronsson H, Jarvis P (2002) A simple method for isolating import-competent *Arabidopsis* chloroplasts. FEBS Lett 529:215–220

Baginsky S, Kleffmann T, von Zychlinski A, Gruissem W (2005) Analysis of shotgun proteomics and RNA profiling data from *Arabidopsis thaliana* chloroplasts. J Proteome Res 4:637–640

Bahl J, Francke B, Monéger R (1976) Lipid composition of envelopes, prolamellar bodies and outer plastid membranes in etiolated, green and greening wheat leaves. Planta 129:193–201

Benli M, Schulz R, Apel K (1991) Effect of light on the NADPH-protochlorophyllide oxidoreductase of *Arabidopsis thaliana*. Plant Mol Biol 16:615–625

Birve SJ, Selstam E, Johansson LB-Å (1996) Secondary structure of NADPH:protochlorophyllide oxidoreductase examined by circular dichroism and prediction methods. Biochem J 317:549–555

Blomqvist LA, Ryberg M, Sundqvist C (2006) Proteomic analysis of the etioplast inner membranes of wheat (*Triticum aestivum*) by two-dimensional electrophoresis and mass spectrometry. Physiol Plant 128:368–381

Bruce BD (1998) The role of lipids in plastid protein import. Plant Mol Biol 38:223–246

Böddi B, Lindsten A, Ryberg M, Sundqvist C (1989) On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. Physiol Plant 76:135–143

Böddi B, Evertsson I, Ryberg M, Sundqvist C (1996) Protochlorophyllide transformation and chlorophyll accumulation in epicotyls of pea (*Pisum sativum*). Physiol Plant 96:706–713

Carlsohn E, Nyström J, Karlsson H, Svennerholm A-M, Nilsson CL (2006) Characterization of the outer membrane protein profile from disease-related *Helicobacter pylori* isolates by subcellular fractionation and nano-LC FT-ICR MS analysis. J Proteome Res 5:3197–3204

Chen S, Harmon AC (2006) Advances in plant proteomics. Proteomics 6:5504–5516

Domanskii V, Rassadina V, Gus-Mayer S, Wanner G, Schoch S, Rüdiger W (2003) Characterization of two phases of chlorophyll formation during greening of etiolated barley leaves. Planta 216:475–483

Donnelly BE, Madden RD, Ayoubi P, Porter DR, Dillwith JW (2005) The wheat (*Triticum aestivium* L.) leaf proteome. Proteomics 5:1624–1633

Ferro M, Salvi D, Brugière S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. Mol Cell Proteomics 2:325–345

Friso G, Giacomelli L, Ytterberg J, Peltier J-B, Rudella A, Sun Q, van Wijk KJ (2004) In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. Plant Cell 16:478–499

Froelich JE, Wilkerson CG, Ray WK, McAndrew RS, Osteryoung KW, Gage DA, Phinney BS (2003) Proteomic study of the *Arabidopsis thaliana* chloroplastic envelope membrane utilizing alternatives to traditional two-dimensional electrophoresis. J Proteome Res 2:413–425

Funk C, Schröder WP, Napiwotzki A, Tjus SE, Renger G, Andersson B (1995) The PSII-S protein of higher plants: a new type of pigment-binding protein. Biochemistry 34:11133–11141

Gaubier P, Wu H-J, Laudié M, Delseny M, Grellet F (1995) A chlorophyll synthetase gene from *Arabidopsis thaliana*. Mol Gen Genet 249:58–64

Grevby C, Engdahl S, Ryberg M, Sundqvist C (1989) Binding properties of NADPH-protochlorophyllide oxidoreductase as revealed by detergent and ion treatments of isolated and immobilized prolamellar bodies. Physiol Plant 77:493–503

Gunning BES, Steer MW (1996) Plant cell biology structure and function. Jones and Bartlett Publishers International, London

Hashimoto A, Akasaka T, Yamamoto Y (1993) Characteristics of the assembly of the 33 kDa oxygen-evolving complex protein in the etioplasts and the developing chloroplasts of barley seedlings. Biochim Biophys Acta 1183:397–407

Hinz G, Hillmer S, Bäumer M, Hohl I (1999) Vacuolar storage proteins and the putative vacuolar sorting receptor BP-80 exit the golgi apparatus of developing pea cotyledons in different transport vesicles. Plant Cell 11:1509–1524

Holtorf H, Reinbothe S, Reinbothe C, Bereza B, Apel K (1995) Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). Proc Natl Acad Sci USA 92:3254–3258

Horton P, Ruban A (2004) Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. J Exp Bot 56:365–373

Ikeuchi M, Murakami S (1982) Measurement and identification of NADPH:protochlorophyllide oxidoreductase solubilised with Triton X-100 from etioplast membranes of squash cotyledons. Plant Cell Physiol 23:1089–1099

Joyard J, Block M, Pineau B, Albrieux C, Douce R (1990) Envelope membranes from mature spinach chloroplasts contain a NADPH:protochlorophyllide reductase on the cytosolic side of the outer membrane. J Biol Chem 265:21820–21827

Jürgens G (2004) Membrane trafficking in plants. Annu Rev Cell Dev Biol 20:481–504

Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjolander K, Gruissem W, Baginsky S (2004) The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol 14:354–362

Kleffmann T, von Zychlinski A, Russenberger D, Hirsch-Hoffmann M, Gehrig P, Gruissem W, Baginsky S (2007) Proteome dynamics during plastid differentiation in rice. Plant Physiol 143:912–923

Kósa A, Márton Z, Böddi B (2005) Fast phototransformation of the 636 nm-emitting protochlorophyllide form in epicotyls of dark-grown pea (*Pisum sativum*). Physiol Plant 124:132–142

Kroll D, Meierhoff K, Bechtold N, Kinoshita M, Westphal S, Votchknecht UC, Soll J, Westhoff P (2001) VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. Proc Natl Acad Sci USA 98:4238–4242

Kuroda H, Masuda T, Ohta H, Shioi Y, Takamiya K (1996) Effects of light, developmental age and phytohormones on the expression of the gene encoding NADPH-protochlorophyllide oxidoreductase in *Cucumis sativus*. Plant Physiol Biochem 34:17–22

Lindsten A, Ryberg M, Sundqvist C (1988) The polypeptide composition of highly purified prolamellar bodies and prothylakoids from wheat (*Triticum aestivium*) as revealed by silver staining. Physiol Plant 72:167–176

Lindsten A, Welch CJ, Schoch S, Ryberg M, Rüdiger W, Sundqvist C (1990) Chlorophyll synthetase is latent in well preserved prolamellar bodies of etiolated wheat. Physiol Plant 80:277–285

Lindsten A, Wiktorsson B, Ryberg M, Sundqvist C (1993) Chlorophyll synthetase activity is relocated from transforming prolamellar bodies to developing thylakoids during irradiation of dark-grown wheat. Physiol Plant 88:29–36

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Lütz C, Röper U, Beer NS, Griffiths T (1981) Sub-etioplast localization of the enzyme NADPH:protochlorophyllide oxidoreductase. Eur J Biochem 118:347–353

Masuda T, Fusada N, Oosawa N, Takamatsu K, Yamamoto YY, Ohto M, Nakamura K, Goto K, Shibata D, Shirano Y, Hayashi H, Kato T, Tabata S, Shimada H, Ohta H, Takamiya K (2003) Functional

analysis of isoforms of NADPH:protochlorophyllide oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana*. Plant Cell Physiol 44:963–974

Morré DJ, Selldén G, Sundqvist C, Sandelius AS (1991) Stromal low temperature compartment derived from the inner membrane of the chloroplast envelope. Plant Physiol 97:1558–1564

Müller B, Eichacker LA (1999) Assembly of the D1 precursor in monomeric photosystem II reaction center precomplexes precedes chlorophyll *a*-triggered accumulation of reaction center II in barley etioplasts. Plant Cell 11:2365–2377

Olsen JV, Ong SE, Mann M (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics 3:608–614

Oster U, Rüdiger W (1997) The G4 gene of *Arabidopsis thaliana* encodes a chlorophyll synthase of etiolated plants. Bot Acta 110:420–423

Paulsen H (2001) Pigment assembly—transport and ligation In: Aro E-M, Andersson B (eds) Regulation of photosynthesis. Kluwer, Dordrecht, pp 219–233

Peltier JB, Emanuelsson O, Kalume DE, Ytterberg J, Friso G, Rudella A, Liberles DA, Soderberg L, Roepstorff P, von Heijne G, van Wijk KJ (2002) Central functions of the lumenal and peripheral thylakoid proteome of *Arabidopsis* determined by experimentation and genome-wide prediction. Plant Cell 14:211–236

Peltier JB, Ytterberg AJ, Sun Q, van Wijk KJ (2004) New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. J Biol Chem 279:49367–49383

Pfeffer SR (2007) Unsolved mysteries in membrane traffic. Annu Rev Biochem 76:629–645

Pineau B, Dubertret G, Joyard J, Douce R (1986) Fluorescence properties of the envelope membranes from spinach chloroplasts. J Biol Chem 261:9210–9215

Plücken H, Müller B, Grohmann D, Westhoff P, Eichacker LA (2002) The HCF136 protein is essential for assembly of the photosystem II reaction center in *Arabidopsis thaliana*. FEBS Lett 532:85–90

Rascio N, Mariani Colombo P, Orsinego M (1980) The ultrastructural development of plastids in leaves of maize plants exposed to continuous illumination. Protoplasma 102:131–139

Rebeiz CC, Rebeiz CA (1986) Chloroplast biogenesis 53: ultrastructural study of chloroplast development during photoperiodic greening. In: Akoyunoglou G, Senger H (eds) Regulation of chloroplast differentiation. AR Liss, New York, pp 389–396

Rossignol M, Peltier J-B, Mock H-P, Matros A, Maldonado AM, Jorrin JV (2006) Plant proteome analysis: a 2004–2006 update. Proteomics 6:5529–5548

Rüdiger W (1993) Esterification of chlorophyllide and its implication for thylakoid development. In: Sundqvist C, Ryberg M (eds) Pigment-protein complexes in plastids: synthesis and assembly. Academic, San Diego, pp 219–240

Rüdiger W, Benz J, Guthoff C (1980) Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur J Biochem 109:193–200

Rüdiger W, Böhm S, Helfrich M, Schulz S, Schoch S (2005) Enzymes of the last steps of chlorophyll biosynthesis: modification of the substrate helps to understand the topology of the active center. Biochemistry 44:10864–10872

Ryberg M, Dehesh K (1986) Localization of NADPH-protochlorophyllide oxidoreductase in dark-grown wheat (*Triticum aestivum*) by immuno-electron microscopy before and after transformation of the prolamellar bodies. Physiol Plant 66:616–624

Ryberg M, Sundqvist C (1982) Characterization of prolamellar bodies and prothylakoids fractionated from wheat etioplasts. Physiol Plant 56:125–132

Ryberg M, Sundqvist C (1988) The regular ultrastructure of isolated prolamellar bodies depends on the presence of membrane-bound NADPH-protochlorophyllide oxidoreductase. Physiol Plant 73:218–226

Ryberg M, Sundqvist C (1991) Structural and functional significance of pigment-protein complexes of chlorophyll precursors. In: Scheer H (ed) Chlorophylls. CRS press, Boca Raton, pp 587–612

Ryrie IJ, Young S, Anderson B (1984) Development of the 33-, 23- and 16-kDa polypeptides of the photosynthetic oxygen-evolving system during greening. FEBS Lett 177:269–273

Schmid HC, Oster U, Kögel J, Lenz S, Rüdiger W (2001) Cloning and characterization of chlorophyll synthase from *Avena sativa*. Biol Chem 382:903–911

Schoefs B, Franck F (2003) Chlorophyll biosynthesis: mechanisms and evolution. Photochem Photobiol 78:543–557

Schubert M, Petersson UA, Haas BJ, Funk C, Schroder WP, Kieselbach T (2002) Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. J Biol Chem 277:8354–8365

Selstam E (1998) Development of thylakoid membranes with respect to lipids. In: Siegenthaler P-A, Murata N (eds) Lipids in photosynthesis: structure, function and genetics. Kluwer, Dordrecht, pp 209–224

Shaw P, Henwood J (1985) Immunogold localisation of protochlorophyllide oxidoreductase in barley etioplasts. Eur Cell Biol 39:50–55

Sheramati I, Shahollari B, Landsberger M, Westermann M, Cherepneva G, Kusnetsov V, Oelmüller R (2004) Cytokinin stimulates polyribosome loading of nuclear-encoded mRNAs for the plastid ATP synthase in etioplasts of *Lupinus luteus*: the complex accumulates in the inner-envelope membrane with the CF_1 moiety located towards the stromal space. Plant J 38:578–593

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Solymosi K, Vitányi B, Hideg É, Böddi B (2007) Etiolation symptoms in sunflower (*Helianthus annuus*) cotyledons partially covered by the pericarp of the achene. Annu Bot 99:857–867

Spano AJ, He Z, Michel H, Hunt DF, Timko MP (1992) Molecular cloning, nuclear gene structure, and developmental expression of NADPH:protochlorophyllide oxidoreductase in pea (*Pisum sativum* L.) Plant Mol Biol 18:967–972

Sundqvist C, Dahlin C (1997) With chlorophyll pigments from prolamellar bodies to light-harvesting complexes. Physiol Plant 100:748–759

Timko MP (1993) Regulation of gene expression involved in chlorophyll and heme formation in higher plants. In: Lodha ML, Mehta SL, Ramagopal S, Srivastava GP (eds) Advances in plant biotechnology and biochemistry. Indian Soc Agril Biochemists, Kanpur, pp 9–19

Virgin HI (1993) Effectiveness of light of different wavelengths to induce chlorophyll biosynthesis in rapidly and slowly greening tissues. Physiol Plant 89:761–766

von Zychlinski A, Kleffman T, Krishnamurthy N, Sjölander K, Baginsky S, Gruissem W (2005) Proteome analysis of the rice etioplast: metabolic and regulatory networks and novel protein functions. Mol Cell Proteomics 4:1072–1084

Wellburn AR (1982) Bioenergetic and ultrastructural changes associated with chloroplast development. Int Rev Cyt 80:133–191

Wellburn AR, Quail PH, Gunning BES (1977) Examination of ribosome-like particles in isolated prolamellar bodies. Planta 134:45–52

Wellburn AR, Gounaris I, Owen JH, Laybourn-Parry EM, Wellburn FAM (1986) Development of bioenergetic function in light-grown seedlings. In: Akoyunoglou G, Senger H (eds) Regulation of chloroplast differentiation. A.R.Liss, New York, pp 371–381

Widell-Wigge A, Selstam E (1990) Effects of salt wash on the structure of the prolamellar body membrane and the membrane binding of NADPH-protochlorophyllide oxidoreductase. Physiol Plant 78:315–323

Ytterberg J, Peltier J-B, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol 140:984–997

Zimmer JSD, Monroe ME, Qian W-J, Smith RD (2006) Advances in proteomics data analysis and display using an accurate mass and time tag approach. Mass Spectrom Rev 25:450–482