1. INTRODUCTION

Proteins participate in every process in every cell of every organism. To understand the function, ontogenesis and biosynthetic pathways of a certain cell or organelle it is crucial to characterize the proteome of the compartment of interest. The protein composition of a compartment is not static but varies with developmental stage and environmental condition. Proteomics is the term used for studies of the proteome, i.e. the protein composition of a certain cell or organelle, during a defined condition. Proteomic studies have increased immensely during the last ten to fifteen years. The development within this field has been driven by the technical advancement of mass spectrometry (MS) analyses as well as by the explosive increase in the number of proteins with known sequence (for review, see Chen and Harmon 2006; Sadowski et al. 2008).

Photosynthesis is a process of essential importance for almost all life on earth. During this process, the energy of sunlight is used to convert carbon dioxide into organic compounds and oxygen. In plants, the photosynthesis process takes place in the organelles called chloroplasts. Chloroplasts contain large amounts of the green pigment chlorophyll, which captures the light energy. In angiosperms, chlorophyll biosynthesis is a strictly light-dependent process. In the absence of light the chlorophyll biosynthesis is arrested at the stage of protochlorophyllide (Pchlide). Plants grown in the absence of light accumulate significant amounts of Pchlide and the enzyme NADPH:protochlorophyllide oxidoreductase (POR), which catalyses the reduction of Pchlide into chlorophyllide (Chlide). The absence of chlorophyll makes these plants appear yellow (Figure 1a). Dark-grown plants do not contain chloroplasts, but another type of plastids called etioplasts. Chloroplasts and etioplasts are structurally very dissimilar. The etioplast inner membranes (EPIMs) are continuous membrane systems laterally separated into two structurally different, but in several respects similar, systems, namely the prolamellar bodies (PLBs) and the prothylakoids (PTs). The PLBs are highly regular three-dimensional (3-D) lattices of tubular membranes from which the flat perforated PTs radiate (Figure 1b).

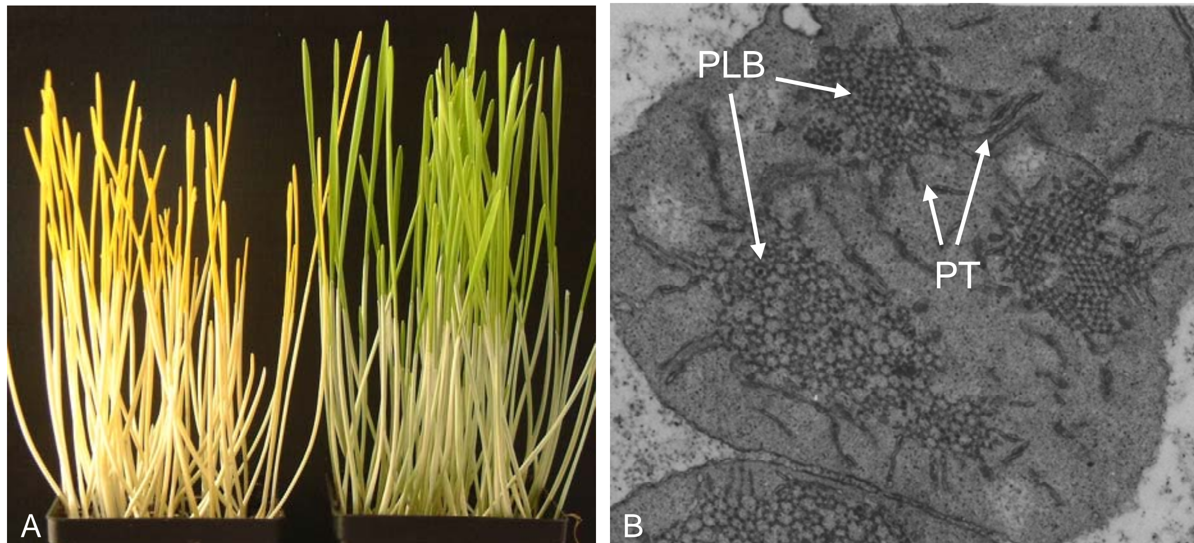


Figure 1. Dark-grown wheat leaves, from the outside and the inside. A. Dark-grown wheat seedlings, non-illumianted and illuminated, respectively. **B.** Electron micrograph of an etioplast with prolamellar bodies (PLBs) and prothylakoids (PTs).

When dark-grown plants are transferred into light etioplasts are transformed into photosynthetically functioning chloroplasts. This process is occurring naturally for instance during bud break or when seedlings break through the soil surface. The transformation into chloroplasts is initiated by the light-dependent reduction of Pchlide catalysed by POR. Chlide is measurable within milliseconds after onset of light. Chlide is esterified to chlorophyll and the PLBs are dispersed and reorganized together with PTs into thylakoids (for review, see Ryberg and Sundqvist 1991; Sundqvist and Dahlin 1997).

The processes of chlorophyll biosynthesis and chloroplast development have been studied widely during the transition of etioplasts to chloroplasts. The unique structure and function of PLBs has attracted researchers' interest for ages. Tightly connected to this is the intriguing function of POR, being one of only two known proteins that require light for its function, the other one being DNA photolyase. Despite immense investigations the role of PLBs and the precise characters of POR remain obscure (for review, see Masuda et al. 2004; Solymosi and Schoefs 2008).

The aim of this thesis is to elucidate the significance of protein composition and protein compartmentalization between PLBs and PTs to understand their roles in etioplasts as well as during etioplast to chloroplast transition. For this purpose proteomic studies of isolated etioplast membrane fractions were performed. EPIMs were analysed with two-dimensional (2-D) gel electrophoresis and MS as a novel mapping of the proteome of PLBs and PTs (**I**). The specific roles of PLBs and PTs were addressed by fractionation of EPIMs (**II**, **III**). The procedure for PLB isolation was optimised to meet with the high demands of purity of modern MS techniques (**II**). PLB proteins were analysed by a combination of one-dimensional (1-D) gel electrophoresis and highly sensitive MS. The significance and the role of the lateral heterogeneity in protein compositions of PLBs and PTs were examined by comparisons of the proteomes of PLBs and PTs (**II**, **III**). Since POR is the most abundant protein of EPIMs and has been suggested to be important for the generation of PLBs, important characters of POR were investigated by isoform separation (**I**), transmembrane (TM) helix predictions and phylogenetic studies (**IV**).

2. PROTEOMICS

The term "proteomics" was first coined in 1997 (James 1997) to make an analogy with the term genomics, the study of genes. Proteomics is defined as the systematic analysis of the proteome, i.e. the entire set of proteins expressed at a given time under defined conditions. Proteomics is one of the fastest growing areas of biological research and aims to identify and characterise expression pattern, subcellular localization, activity, protein-protein interactions, structures, post-translational modifications and functions of proteins in a biological system (Chen and Harmon 2006). The first review of plant proteomics was published by Thiellement et al. (1999). However, most of these reviewed studies were based on expression level comparisons without actual identification of the proteins. At that time proteomic analyses were lagged behind mainly because of the low number of sequenced plant proteins. The completed sequence mapping of whole plant genomes such as the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative 2000) as well as development of commercial and user-friendly MS techniques have facilitated more comprehensive plant proteomic studies. One example of the immense development in this area is that there are at least 80 small- to large-scale MS-based proteomic datasets published for Arabidopsis (Sun et al. 2009).

2.1. Experimental design

Proteomic studies are much more complex than genomic studies mainly because the genome of a cell or tissue is more or less constant while the proteome may vary from cell to cell and from time to time. The proteome is also much larger than the genome due to alternative splicing of genes and post-translational modifications like glycosylation and phosphorylation. The dynamic range of protein concentrations could be many orders of magnitude from the most abundant to the least abundant protein within a cell or tissue. Thus, the extreme complexity and dynamic nature of proteomes makes proteomic analysis an enormous challenge. Common for all proteomic studies is the importance of a sound and efficient sample preparation often including protein fractionation. The mixture of proteins is then to be separated and identified by MS (for review, see van Wijk 2004; Chen and Harmon 2006). A brief summary of these technically important matters, with emphasis on methods used for systematic identification of proteomes, is given below.

2.1.1. Sample preparation

Despite the technical advances in detection of extremely small amounts of proteins, some kind of protein fractionation is normally required for proteomic studies aiming at the systematic identification of the proteome of a certain compartment. Reducing the complexity of a sample will increase the proteome coverage and may be a prerequisite for studying of low-abundant proteins. Proteins may be fractionated according to biochemical, biophysical or cellular properties (for review, see Chen and Harmon 2006). Subcellular fractionation, in which only the proteome of a small compartment of a cell is studied, is often beneficial. Knowing the exact subcellular location is also important to establish the functional role of a certain protein. However, it must be taken into consideration that subcellular fractionation always implies a risk of contamination between different cellular compartments. Even after an apparently “pure” preparation, free of contamination, the question about which proteins that can be assigned as “true” constituents of a certain compartment still remains. The limit between e.g. an integral thylakoid membrane protein and a stromal protein loosely associated to the thylakoid may in some cases be hard to outline and is further complicated by a possible dual localization (Rolland et al. 2008).

2.1.2. Protein separation and mass spectrometry (MS)

Proteomic techniques involve the combined application of advanced separation techniques, MS and bioinformatic tools to characterize complex protein mixtures. The classical method for separation of protein mixtures is 2-D gel electrophoresis (O´Farrel 1975) where proteins are separated based on intrinsic charge and mass. The technique offers the visualization of protein isoforms, potential post-translational modifications and degradation products. Traditional 2-D gel electrophoresis is limited in terms of quantitative gel-to-gel comparisons mainly due to variations and difficulties in automation. These problems can be overcome by differential in-gel electrophoresis (DIGE, Ünlü et al. 1997), which allows comparison of proteins from two samples in the same gel. Despite considerable progress of 2-D gel electrophoresis separation this technique is limited by discrimination against hydrophobic proteins and proteins with very high or low molecular masses. Membrane proteomes are preferably separated by 1-D gel electrophoresis or by gel-free, liquid chromatography (LC)-based, separation (for review, see Chen and Harmon 2006; Sadowski et al. 2008). The combination of 1-D gel electrophoresis and nano-LC coupled to high resolution MS has been used with great success for analyses of membrane proteomes (e.g. Carlsohn et al. 2006).

Proteomics has been driven by the advances in various MS methods together with the increased amount of sequence data from a great number of organisms. The high

sequence similarity generally shared between proteins of different plant species enables identification of proteins in organisms with a not yet sequenced genome. The immense technological revolution of MS has resulted in highly sensitive techniques capable of identifying extremely small amounts of proteins. The basic principle of MS is to ionize a sample, separate the ions based on mass to charge ratios in an analyzer and detect the ions in a detector (for recent review, see El-Aneed et al. 2009). The two primary methods for ionization used in proteomic analyses are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). There are a number of different mass analyzers, e.g. time-of-flight (ToF), quadrapole (Q), quadrapole ion trap (QIT) and Fourier transform ion cyclotrone resonance (FTICR). Tandem MS (MS/MS) is usually performed by coupling of two or more mass analyzers, e.g. coupling of Q and ToF resulted in high-resolution mass spectrometers named Q-ToF. MS/MS may be used for *de novo* sequencing of peptides.

MS analyses may be used to characterize a protein with respect to post-translational modifications, especially phosphorylation (for review, see Collins et al. 2007). Phosphorylated peptides are detected by a mass increment of 80 Da per phosphate. However, due to the ionization problems and the generally low stoichiometry of phosphorylated peptides, this type of MS analysis is far more difficult than the analysis of non-phosphorylated peptides. To increase the stoichiometry of phosphorylated peptides, these peptides may be enriched by immobilized metal affinity chromatography (IMAC) or metal oxide affinity chromatography (MOAC). Nonetheless, the selectivity of both methods is compromised by the detection of non-phosphorylated peptides containing multiple acidic residues.

2.2. Proteomics of chloroplasts

The chloroplast possesses one of the most thoroughly studied plant subcellular proteomes (Chen and Harmon 2006). Chloroplast proteomes and subproteomes have been subjected to comprehensive proteomic mapping using a variety of approaches and species, e.g. analyses of whole chloroplasts (Kleffman et al. 2004, 2007; Baginsky et al. 2005), envelope membranes (Ferro et al. 2000, 2002, 2003; Froehlish et al. 2003; Friso et al. 2004), stroma (Peltier et al. 2006), thylakoid membranes (Peltier et al. 2000, 2002, Hippler et al. 2001; Gomez et al. 2002) and thylakoid lumen (Peltier et al. 2000, 2002; Schubert et al. 2002). Many of these studies have demonstrated the use of subfractionation of samples before identification. However, it also raises the question about the interpretation of localization when cross-contamination between different parts of the chloroplasts during isolation is inevitable (van Wijk 2004).

The exact number of chloroplast localized proteins is not known. The plastid genome codes for only 70 to130 proteins, depending on species. Most proteins are nucleus encoded and thus imported into the plastid. Estimated values of the number of nucleus encoded chloroplast proteins have been obtained by different transit peptide-based predictions and varies immensely, from 1707 to 4800, depending on tool and species (Richly and Leister 2004; van Wijk 2004). In Arabidopsis, 1240 proteins were confirmed to be localized to the chloroplast by cross-referencing published experimental data of reported subcellular location (Heazlewood et al. 2005). The number of proteins targeted to the plastid is most likely dependent on the developmental stage of the plastid (Dahlin and Cline 1991).

2.3. Proteomics of etioplasts

Compared to the chloroplast proteome, the knowledge of the etioplast proteome is rather limited. Six years ago the presence of only 10 to 20 different proteins had been verified in etioplasts, even though electrophoresis had revealed the presence of many more but

unidentified proteins. Today, hundreds of etioplast proteins have been identified. Analyses of the proteome of rice (*Oryza sativa*) etioplasts have generated the identification of totally 477 proteins (von Zychlinski et al. 2005; Kleffman et al. 2007). Recently, the expression of protein complexes of etioplasts and etio-chloroplasts in pea (*Pisum sativum*) were presented (Kanervo et al. 2008a).

Subfractionation of etioplasts has resulted in systematic identification of the proteome of wheat (*Triticum aestivum*) EPIMs (**I**, **II**, **III**). Proteins of isolated EPIMs were subjected to 2-D gel electrophoresis (Figure 2) and identified by either MALDI-TOF MS, ESI-MS/MS or LC-MS/MS (**I**). Additional subfractionation was performed in order to study the proteomes of PLBs (**II, III**) and PTs (**III**). The proteomic approach was altered to incorporate proteins possibly discriminated by 2-D gel electrophoresis. The method for PLB isolation was optimized and highly purified PLBs were separated by 1-D gel electrophoresis followed by nano-LC FTICR MS (**II**). The same approach was used for a comparative study of the proteomes of PLBs and PTs (**III**). The results from my studies of the EPIM proteome are presented and discussed in the following chapters.

The inner membranes of etioplasts and chloroplasts are structurally very different. For characterization of novel EPIM proteins we are directed to references of proteomics of chloroplast suborganells (for review, see van Wijk et al. 2004; Kleffman et al. 2006). It must be taken into consideration that e.g. the physical character and function of a certain protein in the etioplast may not be the same as in the chloroplast. Proteins identified in etioplasts with no counterpart in chloroplasts may be plastid targeted proteins specifically directed to etioplasts (von Zychlinski et al. 2005).

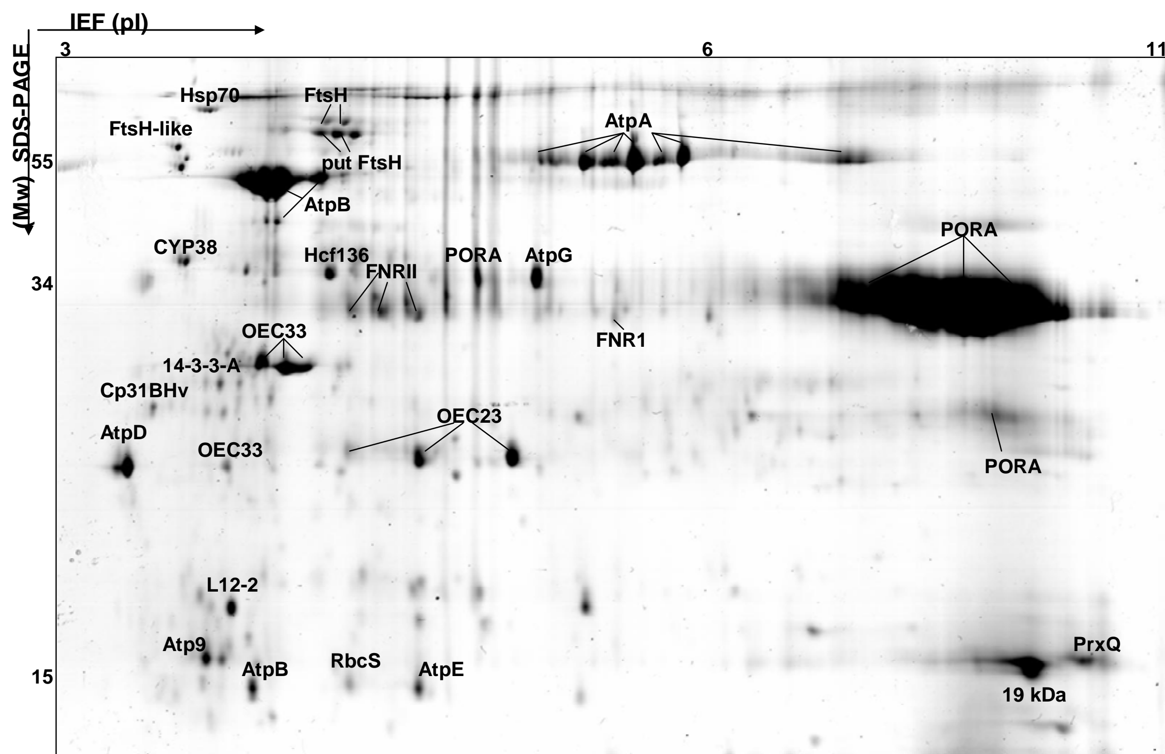


Figure 2. 2-D gel electrophoresis of etioplast inner membrane (EPIM) proteins. EPIM proteins were isolated from dark-grown wheat seedlings. Protein separation and identification were perfomed as described in (**I**).

3. ETIOPLASTS

3.1. The significance of studying etioplasts

Proplastids are undifferentiated plastids mainly found in immature cells. They can develop into several different forms such as chloroplasts or chromoplasts depending on which function they are to play in the mature cell. When plants are grown in the absence of light proplastids develop into etioplasts. Upon illumination etioplasts are transformed into photosynthetically functioning chloroplasts. Etioplasts have been widely used in order to study chlorophyll biosynthesis and chloroplast development. However, the etioplast stage is often considered as an unnatural stage of development and the use of etioplasts as an adequate model for studying of chloroplast development has been questioned. This problem was recently addressed in a comprehensive review article by Solymosi and Schoefs (2008). The review refers to at least 25 different papers that report on the occurrence of PLBs in plants grown under natural or low light conditions, for instance in young leaves before sunrise, during seedling development under the soil surface, before bud break or under shaded plant organs such as the fruit pericarps (e.g. Reibez and Reibez 1986; Solymosi et al. 2004, 2007; Schoefs and Franck 2008). Thus, plastids in the stage of etioplasts or etio-chloroplasts occur frequently in nature and play an important, yet not completely understood, role in plant development. Furthermore, the photoenzymatic cycle of POR proceeds in a similar way during light-dark-cycles as during the greening process of etiolated seedlings (Schoefs and Franck 2008)

It is of great importance to consider the heterogeneity of the etioplast population with respect to ultrastructure, composition and developmental potential of the material used in a study. A prerequisite for the relevance of chloroplast developmental studies is to choose etioplasts that are readily transformed into chloroplasts upon illumination. PLBs become more prominent while the distribution of PTs decrease with increased age of the tissue. The greening process is hampered in older tissues (Lütz 1981; Wellburn 1983; Rascio et al. 1984, 1986; Bergweiler et al. 1986). Thus, care must be taken in order to distinguish events involved in plastid development from those of plastid ageing. The plant organ, from which the etioplasts are isolated, is another important factor needed to be taken under consideration. Dicotyledons, like Arabidopsis, do not develop true leaves in darkness, simply small cotyledons. Such cotyledon is not a representative tissue for studying of the greening process of leaves. The cotyledons have a limited lifespan and were shown to have an altered protein import compared to true leaves (Kim and Apel, 2004). Grass seedlings develop true leaves in darkness. Tissues of different ages are found along the leaf axis representing different developmental stages, from proplastids to ageing etioplasts (Robertsson and Laetsch 1974). Besides the leaf, etiolated grass seedlings are composed of two other organs, namely the coleoptile, which covers the leaf during the first days of growth, and the mesocotyle, which is well developed in darkness. Like the cotyledon of dicotyledons, neither the coleoptile nor the mesocotyle is a representative tissue for studying chloroplast development (Hinchman 1972; Virgin 1996; Savchenko et al. 2005).

Care was taken to meet with the important matters described above, when designing the experiments (**I, II, III**). Wheat, the model organism of these experiments, is a very important agronomic crop worldwide. Dark-grown seedlings of wheat have been extensively studied with regards of ultrastructure and pigment composition, both before and during greening (for review, see e.g. Ryberg and Sundqvist 1991). Etioplasts were isolated from that part of dark-grown wheat leaves which contains mature etioplasts and which are quickly transformed into chloroplasts upon illumination (Wellburn et al. 1986; Gunning and Steer 1996). Technical difficulties in using wheat as a model organism include that it is has an allohexaploid genome composed of circa 16000 Mb of DNA (Armuganthan and Earle 1991),

about 40 times the size of the rice genome. The complete wheat genome has not yet been sequenced. However, the significant amount of identity/homology generally shared between proteins of different plant species enables very well proteomic studies of wheat.

3.2. Etioplast inner membranes (EPIMs)

The unique ultrastructure of etioplasts was detected by electron microscopy studies more than 50 years ago (Leyon 1954; Heitz 1954). The most notable feature of etioplasts is the highly regular 3-D lattices of PLBs. The flat membranes of PTs radiate from the PLBs. The relative amount of PLBs and PTs varies with age, species and growth conditions of the plant (Lütz 1981; Wellburn et al. 1983; Bergweiler et al. 1984; Rascio et al. 1984; 1986). The separation of EPIMs into PLBs and PTs offers a lateral heterogeneity in structure as well as in lipid and protein composition. However, there is no strict border between PLBs and PTs (Gunning 1965). Nonetheless, recent results based on divergent behaviour during salt stress indicated that the lumen of PLBs and PTs may not be completely continuous (Abdelkader et al. 2007). Upon illumination the PLBs are dispersed and are reorganized together with PTs into thylakoid membranes, thus proteins and lipids from both membrane types contribute to the photosynthetically functioning chloroplast (Ryberg and Dehesh 1986; Selstam 1998; von Wettstein et al. 1995). The lateral heterogeneity in protein composition of PLBs and PTs was examined in paper (**III**) and is presented in section 3.4 and 5.1.

The lipid composition of PLBs and PTs resembles that of thylakoids (Ryberg et al. 1983; Selstam and Sandelius 1984). The two major lipids are the galactolipids monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) constituting approximately 50% and 30% of the total membrane acyl lipids, respectively. The PLBs have a higher MGDG to DGDG ratio (1.6-1.8) than the PTs (1.1-1.4) as well as a higher lipid to protein ratio (Ryberg et al. 1983; Selstam and Sandelius 1984). The slightly higher MGDG/DGDG ratio in PLBs than in PTs favours the formation of the cubic structure of PLBs (Brentel 1985; Selstam 1998) even though PLBs can still be formed in the Arabidopsis *MGD synthase 1* mutant, which has a 75% reduction in *MGD1* mRNA formation (Jarvis et al. 2000). Instead, the existence of POR, a key enzyme in chlorophyll biosynthesis, was shown to be of major importance for the formation of PLBs (Ryberg and Sundqvist 1988; Böddi et al. 1989; Sperling et al. 1997; 1998; Frick et al. 2003; Masuda et al. 2003; 2009). POR accounts for at least 90% of the protein content in PLBs (Ryberg and Sundqvist 1982; Dehesh and Ryberg 1985; Dehesh et al. 1986; Lindsten et al. 1988) and the aggregation of POR-Pchlide-NADPH complexes is believed to be associated with the highly regular structure of PLBs (Ryberg and Sundqvist 1988; Böddi et al. 1989).

3.3. Pigments of EPIMs

In angiosperms, chlorophyll biosynthesis is a light-dependent process and in etioplasts this process is arrested at the stage of Pchlide. Etioplasts hold significant reservoirs of Pchlide. Whatever the plant species, Pchlide occurs in two functionally different states referred to as non-photoactive and photoactive Pchlide. Upon illumination, the photoactive Pchlide is quickly transformed to Chlide, a reaction catalysed by POR, and further esterified by chlorophyll synthase to form chlorophyll (for review, see Rüdiger 1997; Sundqvist and Dahlin 1997). The phototransformable form of Pchlide is present in ternary complexes of POR, Pchlide and NADPH. The aggregation of these complexes generates a typical fluorescence emission peak at 657 nm ($Pchlide_{F657}$) in a low temperature (77 K) fluorescence emission spectrum. The non-phototransformable form of Pchlide is not part of such ternary complexes and is referred to as “free” Pchlide (Oliver and Griffiths 1982). The non-photoactive Pchlide

form generates a fluorescence peak at about 633 nm ($Pchlide_{F633}$). Minor spectral forms of Pchlide can be distinguished (Böddi et al. 1992). 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 the POR-pigment complex (Ryberg and Sundqvist 1991; Schoefs and Franck 2003). Another reason for formation of different spectral forms may be due to interactions between the Pchlide and its membrane environment (Böddi et al. 1989). $Pchlide_{F657}$ is by far the most abundant Pchlide form in PLBs while $Pchlide_{F633}$ is localized mainly to the PTs (e.g. Ryberg and Sundqvist 1982; 1988; 1991).

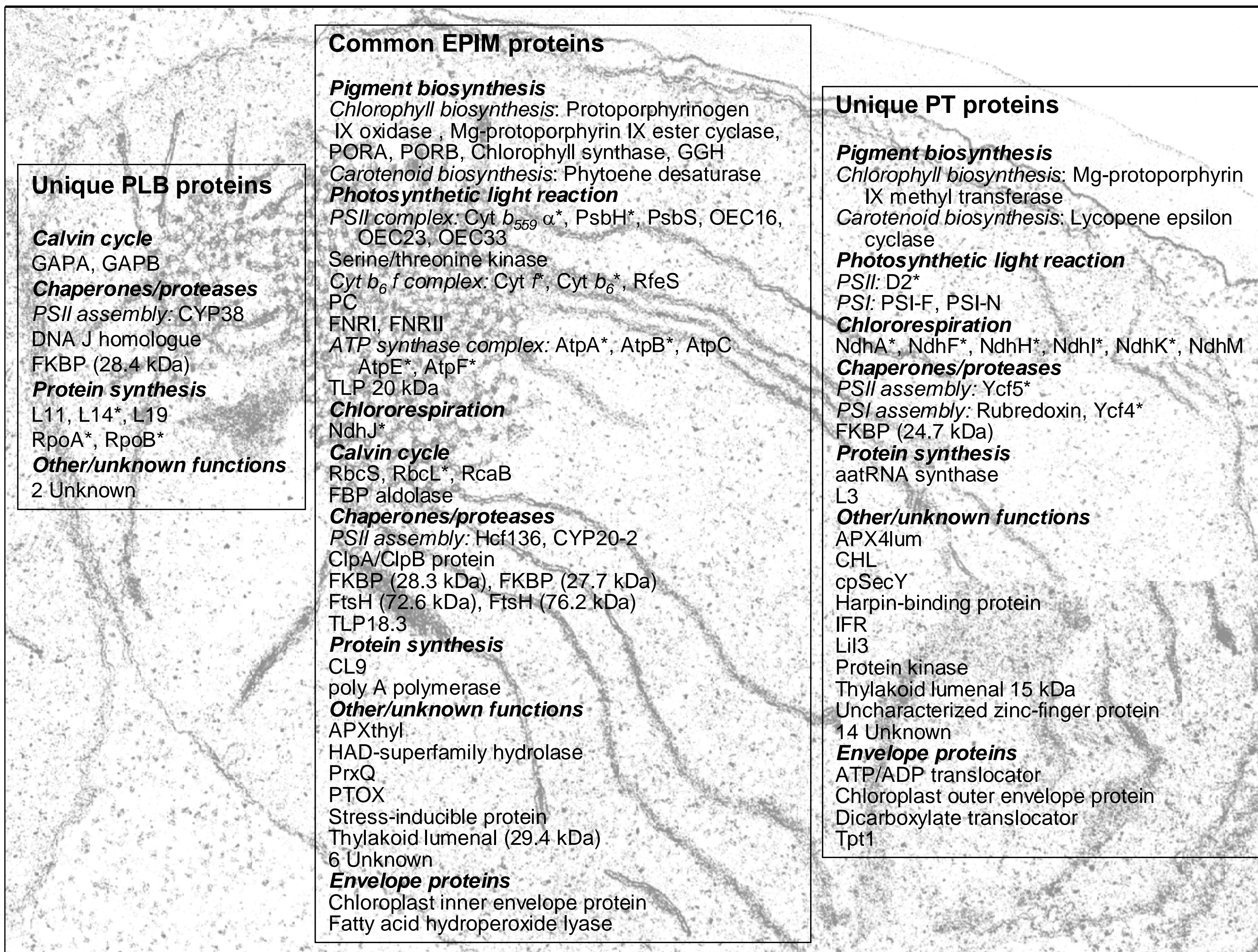
Carotenoids, typically lutein and violaxanthin, are present in dark-grown seedlings. The carotenoid composition of PLBs and PTs are very similar (Böddi et al. 1989). Carotenoids are important for photoprotection and are thus part of the protective system of etioplasts that is ready for light (**III**). It is not known whether carotenoids have a function already in darkness or not. However, a possible influence of carotenoids on the PLB structure is under debate (for review, see Solymosi and Schoefs 2008).

3.4. Proteins of EPIMs

A number of different proteins are known to accumulate in EPIMs. The proteins of EPIMs have been separated by 1-D gel electrophoresis and described in several papers (e.g. Høyer Hansen et al. 1977; Selstam and Sandelius 1984; Lindsten et al. 1988). However, in these early studies most proteins were not identified. The presence and distribution of a few proteins were verified immunologically, e.g. POR and ATP synthase (e.g. Shaw et al. 1985; Ryberg and Dehesh 1986). Besides POR, the α- and β-subunits of ATP synthase constitute the main part of the EPIM proteome (Lütz et al. 1981; Santel and Apel 1981; Ryberg and Sundqvist 1982). The presence of other proteins in etioplasts has been reported, although the localization of these proteins within the EPIMs has not been determined (for references, see paper **I**).

Systematic analyses of the EPIM proteome by modern proteomic techniques have revealed the presence of huge amounts of proteins with various functions (**I, II, III**). A 2-D gel electrophoresis of EPIM proteins (Figure 2) resulted in the separation of more than 200 protein spots of which 46 protein spots were identified (**I**). Several proteins, e.g. POR, subunits of the ATP synthase, and the oxygen evolving complex, were found to be separated into different isoforms (due to distinct pI values). These findings are interesting since this phenomenon, seen as a train of spots, might be caused by post-translational modifications of the proteins. The possibility of POR being post-translationally modified by phosphorylation has been discussed (**I**, see section 4.4). Subfractionation of EPIMs into PLBs and PTs, followed by a highly sensitive proteomic approach, generated the identification of 64 PLB proteins (**II**) and totally 111 PLB and PT proteins (**III**) summarized in Figure 3. The lateral heterogeneity in protein composition of PLBs and PTs is discussed in connection to etioplast to chloroplast transition in section 5.

Figure 3. Proteins identified in prolamellar bodies (PLBs) and prothylakoids (PTs). The etioplast inner membranes (EPIMs) of dark-grown wheat leaves were fractionated into PLBs and PTs. Proteins were separated and identified as described in (**II**, **III**). * Encoded by the plastid genome. Abbreviations used: APX4lum, lumenal ascorbate peroxidase 4; APXthyl, thylakoid-bound ascorbate peroxidase; Atp, ATP synthase; CHL, chloroplast lipocalin; CL9, plastid ribosomal protein CL9; cpSecY, chloroplastic SecY; CYP, cyclophilin-type peptityl-prolyl cis-trans isomerise; Cyt b_{559} α, b_6, *f*, cytochrome b_{559} alpha chain, b_6, *f*, respectively; FBP aldolase; fructose 1,6-bisphosphate aldolase; FKBP, FK506 binding protein-type peptidyl-prolyl cis-trans isomerise; FNR, ferredoxin-NADPH oxidoreductase; GAP, glyceraldehyde-3-phosphatase; GGH, geranylgeranyl hydrogenase; IFR, isoflavone reductase; L3, L11, L14, L19, ribosomal protein L3, L11, L14, L19, respectively; Ndh, NAD(P)H:plastoquinone oxidoreductase; OEC, oxygen evolving complex; PC,



plastocyanin; POR, NADPH:protochlorophyllide oxidoreductase; PrxQ, peroxiredoxin Q; PTOX, plastid terminal oxidase; RbcL, S, ribulose-1,5-bisphosphate carboxylase large and small subunit, respectively; Rca, ribulose 1,5-bisphosphate carboxylase activase; RfeS, Rieske Fe-S protein; Rpo, RNA polymerase; TLP, thylakoid lumen protein; Tpt1, triose phosphate translocator

Proteins of EPIMs (Figure 3) are to a huge extent directly or indirectly involved in the generation of a photosynthetically active plant upon illumination. The identified proteins represent diverse functions such as pigment and protein biosynthesis, photosynthesis, chaperone and protease activity. Many proteins with unknown functions were identified as well. It is possible that such proteins are candidates for etioplast-specific functions, not yet characterized (von Zychlinski et al. 2005). Some of the identified proteins were of special interest. PORB was identified for the first time in dark-grown wheat and chlorophyll synthase was concluded to be a true constituent of PLBs (**II**) as indicated before (Lindsten et al. 1990; 1993). Both these proteins are plausible constituents also of PTs (**III**). D2 of PSII, subunits of PSI and six out of seven identified subunits of the NAD(P)H:plastoquinone oxidoreductase (Ndh) complex were concluded to be localized exclusively to the PTs (**III**).

The overall protein composition of EPIMs revealed in my studies points to a high readiness for a rapid formation of the photosynthetic apparatus (see section 5). However, some of the proteins which accumulated in darkness were previously shown to decrease during etioplast to chloroplast transition, e.g. Ndh subunits, ATP synthase, FtsH (Guéra et al. 2000; Kleffman et al. 2007; Kanervo et al. 2008a). This may indicate that these proteins are needed at the very beginning of the greening process or that they have specific roles in darkness. ATP synthase, Clp and FtsH protease complexes were suggested to have a crucial role for etioplast and chloroplast biogenesis (Kanervo et al. 2008a). The Ndh complex has a possible role in energizing of the etioplast (Fischer et al. 1997; Guéra et al. 2000)

4. NADPH:PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR)

4.1. POR isozymes

Gymnosperms, mosses, algae and cyanobacteria possess a light-independent pathway for the reduction of Pchlide to Chlide (Fujita et al. 2000). In angiosperms this process is strictly light-dependent and catalysed by the POR enzyme. POR is a nucleus-encoded protein, translated in the cytosol and imported to the plastid (for review, see Aronsson et al 2003a; b). The first sequenced POR polypeptide was isolated from barley (*Hordeum vulgare*, Schulz et al. 1989). Since then, the POR polypeptide from a number of different plants has been sequenced e.g. pea (Spano et al. 1992a), wheat (Teakle and Griffiths 1993) and cucumber (*Cucumis sativus*, Kuroda et al. 1995). POR isozymes have been reported in a few angiosperms. PORA and PORB, two structurally similar but differently regulated isozymes, were identified in e.g. barley (Holtorf et al. 1995) and Arabidopsis (Armstrong et al 1995). In Arabidopsis, also a third isozyme PORC was identified (Oosawa et al. 2000). In wheat, only one POR isozyme, PORA, has been sequenced, but the presence of PORB in wheat was shown recently (**II, III**).

The different POR sequences generally share a very high sequence similarity (see Figure 1, **IV**). The main sequence diversities are found within the transit peptide regions of the protein. The mature parts of the POR sequences contain highly conserved regions. As an example, the precursor form of PORA from Arabidopsis and wheat share 70% amino acid (a.a.) sequence similarity, whereas the mature form of the proteins share 80% sequence similarity.

4.2. Structure of POR

The precursor form of POR consists of approximately 400 a.a. and has an apparent mass of 41 to 43 kDa. The mature form of POR is 34 to 37 kDa. Attempts to crystallize POR have failed, thus the precise 3-D structure of POR has not been determined. Based on sequence similarity,

POR has been assigned as a member of the short-chain dehydrogenase (SDR) family (Wilks and Timko 1995). SDRs are NAD(P)(H)-dependent enzymes that typically comprise two different domains, one for nucleotide binding and one for substrate binding, arranged in a Rossmann-fold. The domains are found in highly conserved regions outlined in a multiple alignment of POR sequences, Figure 1 in paper **IV**. The Rossmann-fold is composed of parallel beta-strands linked by alpha-helices. The nucleotide-binding domain corresponds to a glycine-rich motif, being GxxxGxG in POR. The significance of the GxxxGxG motif for NADPH-binding in POR has not been verified experimentally. The substrate-binding domain in SDR proteins is comprised of the highly conserved motif YxxxK. Mutagenesis experiments of the two conserved residues showed that they are essential for POR activity, not only for substrate binding but also for binding of NADPH (Wilks and Timko 1995; Lebedev et al. 2001; Heyes and Hunter 2002). Several other domains important for enzymatic activity of POR have been identified by mutagenesis experiments (Dahlin et al. 1999; Aronsson et al. 2001a; Lebedev and Timko 2002). As an example, a pair of residues, cysteine and arginine, 16 to18 a.a. downstream of the GxxxGxG motif, is involved in nucleotide/substrate binding (Dahlin et al. 1999; Townley et al. 2001).

Secondary structure predictions of POR differ somewhat, but all correspond to a typical Rossman-fold (Birve et al. 1996; Dahlin et al. 1999; Townley et al. 2001; Buhr et al. 2008). The predicted structure agrees with relative amounts of secondary structure elements as revealed by circular dichroism (Birve et al. 1996). POR was estimated to consist of 33% alpha-helices, 19% beta-sheets, 20% turns and 28% random coils. Proteins of the SDR family with known structure have subsequently been used as templates for deriving models of the tertiary structure of POR (Dahlin et al. 1999; Townley et al. 2001; Buhr et al. 2008). The structure is modeled as parallel beta sheets surrounded by helices. There is a 33 to 35 a.a. residue insertion between the fifth and the sixth beta-sheets that separates POR from other proteins of the SDR family. Thus, the lack of a structural template in this region means that the structure of this loop is highly unclear. The loop is generally more hydrophobic than the POR sequence as a whole (see section 4.4.3.). It has been suggested to be involved in membrane-anchoring (Birve et al. 1996) or protein complex formation (Reinbothe et al. 2003).

4.3. Import and localization of POR

POR is imported into the plastid by passing through the translocons at the outer and inner chloroplast envelope membranes (Toc and Tic; Aronsson et al. 2000). The question whether the import of POR is dependent on the presence of Pchlide has been a matter of controversy. In a series of papers Reinbothe et al. (1995b; c; 1997; 2000; 2005) claimed that POR import into plastids is strictly Pchlide-dependent. On the other hand, several other reports indicate that Pchlide is not required for import (Teakle and Griffiths 1993; Dahlin et al. 1995; 2000; Aronsson et al. 2000; 2001b). The import of PORA was reported to be substrate-dependent in cotyledons but not in true leaves (Kim and Apel 2004). Recently, Reinbothe et al. (2008) described a pentapeptide motif in the transit peptide of pPORA in barley responsible for substrate-dependent import of PORA. However, Plöscher et al. (2009) showed that the transit peptide of e.g. barley PORA is considerably shorter than previously reported. Thus, Pchlide cannot bind to PORA transit peptide as proposed (Reinbothe et al. 2008), since the pentapeptide is located in the mature part of PORA.

In etiolated seedlings of angiosperms, most of POR and its substrate Pchlide are localized to the PLBs (Ryberg and Sundqvist 1982; Dehesh and Ryberg 1985; Shaw et al. 1985; Ryberg and Dehesh 1986; Arthus et al. 1992) although some amounts of POR are also detected in PTs. In wheat, PORA and PORB were present in PLBs (**II, III**) and plausibly also

in PTs (**III**). In light-grown seedlings, the amount of POR is substantially lower. In chloroplasts, POR was concluded to be localized to the stromal side of the thylakoid membranes (Teakle and Griffiths 1993; Dahlin et al. 1995).

4.4. Association of POR to the membrane

Several different studies have concluded the POR protein to be of major importance for the formation of the highly regular structure of PLBs (Ryberg and Sundqvist 1988; Böddi et al. 1989; Sperling et al. 1997; 1998; Frick et al. 2003; Masuda et al. 2003; 2009). Thus, POR must exert some influence on the PLB lipids to induce the formation of the 3-D lattice of tubular membranes. Since the crystal structure of POR has yet to be determined the membrane-association of POR is left for suggestions derived from various washing and mutagenesis experiments as well as from structural predictions. Proteins may be attached to the membrane by permanent interaction, i.e. integral membrane proteins, or temporarily membrane interaction, i.e. peripheral membrane proteins. Integral membrane proteins are defined as those membrane proteins which require a detergent or some other apolar solvent to be displaced. Integral TM membrane proteins possess one or more regions spanning the membrane while integral monotopic membrane proteins are permanently attached to the membrane on one side (Lomize et al. 2007).

4.4.1. Experimental behaviour of POR

Several attempts have been performed to try to elucidate the strength of the membrane association of POR. The behaviour of POR upon various washing experiments has been studied both *in vivo* and *in vitro*. Various salts, detergents, and proteases have been used to study the solubilisation of POR from PLB, PT and thylakoid membranes. These experiments are unanimous in the conclusion that POR is firmly attached to PLBs and PTs (Lütz and Tönissen 1984; Grevby et al. 1989; Selstam and Widell Wigge 1989; Widell Wigge and Selstam 1990; Engdahl et al. 2001). However, most of the POR attached to thylakoid membranes was removed by salt or detergent treatment (Dahlin et al. 1995). It was speculated that membrane binding properties of POR might change, i.e. that affinity for the membrane declines, during greening (Aronsson et al. 2003b).

In addition, mutagenesis experiments of POR were used to elucidate the ability of POR to bind to thylakoid membranes (Dahlin et al. 1999; Aronsson et al. 2001a; b) and etioplast membranes (Engdahl et al. 2001). These experiments demonstrated the requirement of NADPH and ATP for membrane association. Also, charged a.a., cysteine residues and the C-terminal were also shown to be important for membrane association (see section 4.4.3.).

4.4.2. POR - an integral transmembrane (TM) protein?

Early hydropathy plots reported that no hydrophobic segment, long enough to span the membrane, could be found in any of the POR sequences tested (Benli et al. 1991; Spano et al. 1992b; Timko 1993). These papers have been persistently referred to ever since, in connection to the rejection of POR being an integral TM protein, notwithstanding the obvious firm membrane attachment of POR. However, during the last decades the methods for prediction of TM segments (most commonly helices) have been immensely refined. Hydropathy scales simply search a sequence for a sufficiently hydrophobic stretch of residues traversing a membrane. Refinements of such methods have included statistical and structural information of known TM helices, such as the presence of specific sequence patterns and motifs, the packing of helix bundles and the properties of inter-helical residue interaction (for review, see Chen et al. 2002; Cuthbertsson et al. 2005). One such method, HMMTOP (Túsnady et al. 2001), was used to search for possible TM helices in PORA from wheat and PORB from

barley (**II**). The analysis was performed in order to try to understand why PORB was not detected by 2-D gel electrophoresis (**I**). The method predicted one TM helix in PORA from wheat and three in PORB from barley (**II**). These surprising findings induced us to extend this study to incorporate more POR sequences and to apply more available TM prediction tools. Twenty different methods for TM helix prediction, including statistical or structural information, were used to elucidate the possibility of POR being an integral TM protein (**IV**). Eleven of the methods predicted one or several of the 14 POR sequences to contain one to three TM segments. Three predicted TM segments were common for all POR sequences. Phylogenetic studies of TM prediction pattern revealed some evolutionary trends (see Figure 3, **IV**). We found that POR is most likely traversing the membrane by either the first or the third predicted TM segment with the C-terminal located to the stromal side of the membrane. A possible scenario is that POR is reversibly traversing the membrane, i.e. the TM segment is released due to conformational changes of the POR-pigment complex during light-dependent reduction of Pchlide. POR being a plausible integral TM protein is logic concerning its experimental behaviour and intricate role for PLB formation (**IV**). Our results open for a renewed discussion concerning the role of POR for PLB formation and dispersal.

4.4.3. POR – an integral monotopic or peripheral membrane protein?

It is possible that POR is anchored to the membrane rather than spanning the membrane. POR has been described as a peripheral or globular protein in many studies (e.g. Dahlin et al. 1995; Townley et al. 2001; Buhr et al. 2008). However, the experimental behaviour of POR clearly shows that PLB- or PT-anchored POR cannot be assigned as a peripheral membrane protein, which according to definition is readily solubilised by water (Lomize et al. 2007). Thus, if POR is not an integral TM protein, POR must be assigned as an integral monotopic membrane protein. One or several of the predicted TM helices may be monotopically inserted in the membrane. Several other possibilities of membrane anchoring domains have been proposed over the years. However, the orientation and likelihood for these suggested anchores to be monotopically inserted in the membrane are not known. Very few structures of monotopic membrane proteins are known, since the membrane-anchoring elements are often lost during protein extraction (Lomize et al. 2007). It should also be mentioned that the possibility of POR being an integral TM protein does not exclude that it is also anchored to the membrane by one or several of the following suggested domains.

The lack of thylakoid membrane association of POR after pretreatment of the membranes with the non-specific protease thermolysin indicated an interaction with a membrane protein (Dahlin et al. 1995). However, this putative protein has not been identified.

The long extra insertion-loop of 33 to 35 a.a. (see Figure 1, **IV**), that separates POR sequences from the rest of the short-chain dehydrogenases, was discussed as contributing to membrane anchoring (Birve et al. 1996). Secondary structure of this hydrophobic region is predicted as a loop followed by a short (Birve et al. 1996) or longer (Townley et al. 2001) alpha-helix. In the POR model by Townley et al. (2001) this helix-turn-strand is surface exposed. A multiple alignment of POR sequences in the insertion segment (Figure 1, **IV**) shows that 11 conserved hydrophobic a.a. (4 identical for all sequences and 7 with conserved hydrophobicity) are found in this segment. In barley PORA and PORB, this segment contains 14 and 16 hydrophobic a.a., respectively.

A.a. 362-395 in pea were shown to be important for proper membrane association, though the thermolysin-sensitive part, approximately the last 19 a.a., of pea POR (382-400) was stated not to be in close association to the membrane (Aronsson et al. 2001b). Thermolysin treatment of membrane associated POR protein in etioplasts resulted in a 2 to 4 kDa POR degradation product (Lütz and Tönissen 1984; Engdahl et al. 2001), suggesting that this is also true for POR associated to etioplast inner membranes. Thus a.a. in the C-terminal

region corresponding to approximately 362-381 in pea POR is involved in membrane association. The secondary structure in this segment is predicted as part of a small beta-sheet followed by loop section or loop and an alpha-helix (Birve et al. 1996). The predictions made by Townley et al. (2001) and Buhr et al. (2008) do not include the last 25 to 30 a.a. in the C-terminal. The membrane anchor has been suggested to be mediated by tryptophan (Trp) residues (Aronsson et al. 2001b). The possibility of Trp mediating membrane anchoring was first suggested by Birve et al. (1996). POR has four conserved Trp residues (see Figure 1, **IV).** Trp residues were shown to play a particularly important role in mediating interactions between the lipid glycerol and the protein in the membrane/solution interface (Woolf et al. 1994). Furthermore, Trp residues was shown to be frequently involved in membrane binding in a comprehensive study of peripheral/monotopic membranes with known 3D structure (Lomize et al. 2007). Studies of the accessibilities of POR Trp to fluorescence probes indicate that the Trp residues are located at the polar heads of the lipids (Denev et al. 1997). Trp1 (the first Trp residue) is found in the Rossman-fold region and modelled by Townley et al. (2001) as buried in the hydrophobic core of POR. The other three Trp residues were modelled as being surface exposed. Trp4 is located in the C-terminal, within the thermolysin-sensitive part of POR (appr. the last 19 a.a. in pea POR) and thus not tightly associated to the membrane (Lütz and Tönissen 1984; Aronsson et al. 2001b; Engdahl et al. 2001). Trp2 and Trp3 lies within the C-terminal area, showed by C-terminal mutants to be important for membrane association.

4.5. Regulation of POR

4.5.1. Isozyme regulation

The isozymes, PORA, PORB and PORC, are differently regulated with respect to light. PORA appears to be present and functional only during the first few hours of greening, whereas PORB is present and active throughout the life of the plant (Armstrong et al. 1995; Holtorf et al. 1995; Oosawa et al. 2000). In contrast, PORC was not detected in etioplasts. High light irradiation was shown to induce the accumulation of PORC (Masuda et al. 2003). In some plant species, POR has not been possible to categorize into any of the isozyme groups neither by homology nor from regulation pattern (for review, see Masuda et al. 2004).

4.5.2. Is POR regulated by phosphorylation?

Several investigations indicated that POR is a phosphorylated protein. Covello et al. (1987) found that the main phosphorylated protein of etioplasts was a protein of 35 kDa, most likely being POR, since this protein disappeared upon illumination. Furthermore, several isoforms of POR, with different pI, were separated by 2-D gel electrophoresis (Ikeushi and Murakami 1982; Dehesh et al. 1986; **I**) possibly reflecting various modes of phosphorylation of POR. In my studies, five different isoforms of PORA were found in wheat EPIMs (**I**). In the mature part of wheat PORA, fifteen possible phosphorylation sites are predicted by the web-tool NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos/). However, the separation of different POR isoforms may be an effect of other types of post-translational modification as well such as glycosylation, deamination or acetylation. Those post-translational modifications shift the pI and/or mass of a protein (Rodriguez-Pineiro et al. 2005).

Reversible protein phosphorylation is suggested to play a regulatory role in PLB formation and dispersal by affecting the aggregation of the POR-pigment complexes (Wiktorsson 1995; Wiktorsson et al. 1996; Kovacheva et al. 2000). This suggestion is based on observations of pigment and protein behaviour in the presence of e.g. inhibitors of protein phosphatase or kinase activity. However, it is not known from these studies whether the effects of different treatments are due to a direct or an indirect influence on POR, i.e. it is

possible that another protein is phosphorylated/dephosphorylated mediating a regulatory effect on POR.

The possibility of POR being a phosphorylated protein was further studied using a MS-based strategy (unpublished results). Wheat EPIM proteins were separated by 1-D gel electrophoresis and a band corresponding to the size of POR (~34 kDa) was analysed. Various methods for enrichment of phosphorylated peptides were used prior to MS; IMAC (Phos-Select, Sigma and ZipTipMC, Millipore) and MOAC (in-house made colons according to Larsen et al. 2005). However, no phosphorylated peptide was detected by MS analyses in any of the enriched samples. I can only speculate on whether these results are due to technical problems of the methods (see section 2.1.2.), low stoichiometry of phosphorylated peptides in the sample, or if the results show that POR is indeed not a phosphorylated protein, at least not before illumination.

4.5.3. POR degradation

The greater part of POR is rapidly degraded upon illumination and disappears within a few hours after onset of illumination (Häuser et al. 1984; Reinbothe et al. 1995a; b; Tziveleka and Argyroudi-Akoyunoglou 1998). However, POR was shown to be protected against proteolytic activity when bound to Pchlide and NADPH. POR in barley was suggested to be degraded upon illumination by a membrane bound protease present in etioplasts (Häuser et al. 1984). In red kidney bean, a thylakoid-bound protease was reported to specifically degrade POR upon illumination (Tziveleka and Argyroudi-Akoyunoglou 1998). In contrast, Reinbothe et al. (1995a; b) showed that pPORA proteins in barley plastids were degraded by a light-induced, nucleus-encoded, ATP- and metal-dependent plastid stromal protease. However, Dahlin et al. (2000) could not confirm any stromal protease activity on POR proteins, neither in etiolated nor in light-grown barley or wheat.

In wheat EPIMs, we found subunits of two different ATP-dependent proteases, FtsH (**I, II, III**) and Clp (**III**). In chloroplasts, the thylakoid-bound Zn-dependent FtsH protease is involved in degradation of unassembled proteins and damaged D1 protein (for review, see Adam et al. 2006; Sakamoto 2006) while the stromal Clp protease is involved in the degradation of numerous stromal proteins (Sjögren et al. 2006; Stanne et al. 2009). FtsH proteases and Clp proteases are isolated as complete multi-protein complexes from pea etioplasts (Kanervo et al. 2008a). Thus, these proteases were suggested to be functionally important already in etioplasts or at the very beginning of greening. Clp proteases were suggested to participate in the degradation of the PLBs and POR upon illumination. Interestingly, FtsH was shown to be down-regulated in pea during greening (Kanervo et al. 2008a), supporting the idea that FtsH might be involved in the degradation of POR upon illumination as previously suggested (**I**). Experiments using various protease inhibitors were performed in order to elucidate whether FtsH is involved in the proteolytic degradation of POR or not (unpublished results). POR degradation and pigment fluorescence were studied in the presence of an ATP source and the metal chelators EDTA or 1,10-phenanthroline. The metal chelators inhibit proteolytic activity of FtsH by chelating the essential Zn-ion. However, these studies generated inconsistent results, probably caused by unspecific chelating action on other divalent ions such as the Mg bound to Pchlide. Such an effect was indicated by the destructible effect of 1,10-phenanthroline on Pchlide as shown by fluorescense emission spectra.

5. ETIOPLAST TO CHLOROPLAST TRANSITION

The transformation of etioplasts to photosynthetically active chloroplasts involves an immense ultrastructural reorganization of the inner membranes of the plastids. This process starts with the reduction of Pchlide to Chlide within milliseconds after onset of light (for review, see Ryberg and Sundqvist 1991) with Pchlide thus acting as the photoreceptor in the etioplast to chloroplast transformation.

5.1. Biogenesis of the photosynthetic apparatus

The synthesis and assembly of the photosynthetic complexes demand concerted interactions between the plastid and the nuclear genetic systems. The assembly process generally involves an assembly-dependent auto-regulation of translation of the plastid-encoded core proteins of the thylakoid protein complexes (for review, see Kanervo et al. 2008b). Chlorophyll is a prerequisite for assembling of many of the photosynthesis proteins (e.g. Kim et al. 1994)

During etioplast to chloroplast transition the PLBs are dispersed and reorganized with PTs into thylakoid membranes. The lateral asymmetries in lipid, pigment and protein (Figure 3) composition of PLBs and PTs give us clues about the possible role of their compositional heterogeneity. A possible model for activation and construction of protein complexes are outlined in Figure 4.

5.1.1. Significance of prolamellar bodies (PLBs) during transition

PLBs are important during etioplast to chloroplast transition for a number of reasons and have been proposed to enhance photomorphogenesis (Sundqvist and Dahlin 1997). PLBs are frequently occurring in plants grown under natural conditions (for review, see Solymosi and Schoefs 2008). Thus, the impact of PLBs during greening of dark-grown plants may also be applicable to understand the role of PLB formation in plants grown under natural light-dark cycles.

PLBs are described as safe storage sites (**II**), functioning like a reservoir of lipids, pigments and proteins, ready to be used for construction of photosynthetically active thylakoid membranes. The pool of Pchlide accumulated in PLBs is of major importance during the transition process. Pchlide is rapidly reduced and esterified to chlorophyll upon illumination (Rüdiger et al. 1980; Ryberg and Sundqvist 1988; 1991). Chlorophyll stabilizes the chlorophyll-binding proteins and enables the assembly of the photosynthetic multiprotein complexes (Klein et al. 1988; Kim et al. 1994; Eichacker et al. 1996). The lipids of PLBs are reused for construction of the thylakoid membranes (Selldén and Selstam 1976; Selstam 1998).

The proteome of dark-grown wheat PLB membranes (**II, III**) has revealed a far more complex protein composition than previously suggested (see section 3.4 and Figure 3). POR is the most abundant protein in PLBs and the protein of most significant importance during transition and for the formation of the highly regular structure of PLBs. The novel detection of several potential TM helices in POR (**IV**) is intriguing in this sence (see section 4.4.2.). Other proteins important during transition are subunits of PSII, Cyt b_6f-complex and ATP synthase, as well as chaperones involved in assembly of the photosynthesis complexes, e.g. CYP38.

PLBs have a well developed system ready for protection against the detrimental effects of light. Pchlide, POR and carotenoids are known to be involved in photoprotection (Franck et al. 1995; Runge et al. 1996; Sperling et al. 1997; Erdei et al. 2005; Buhr et al. 2008). My studies have revealed the presence of several other proteins, such as plastid

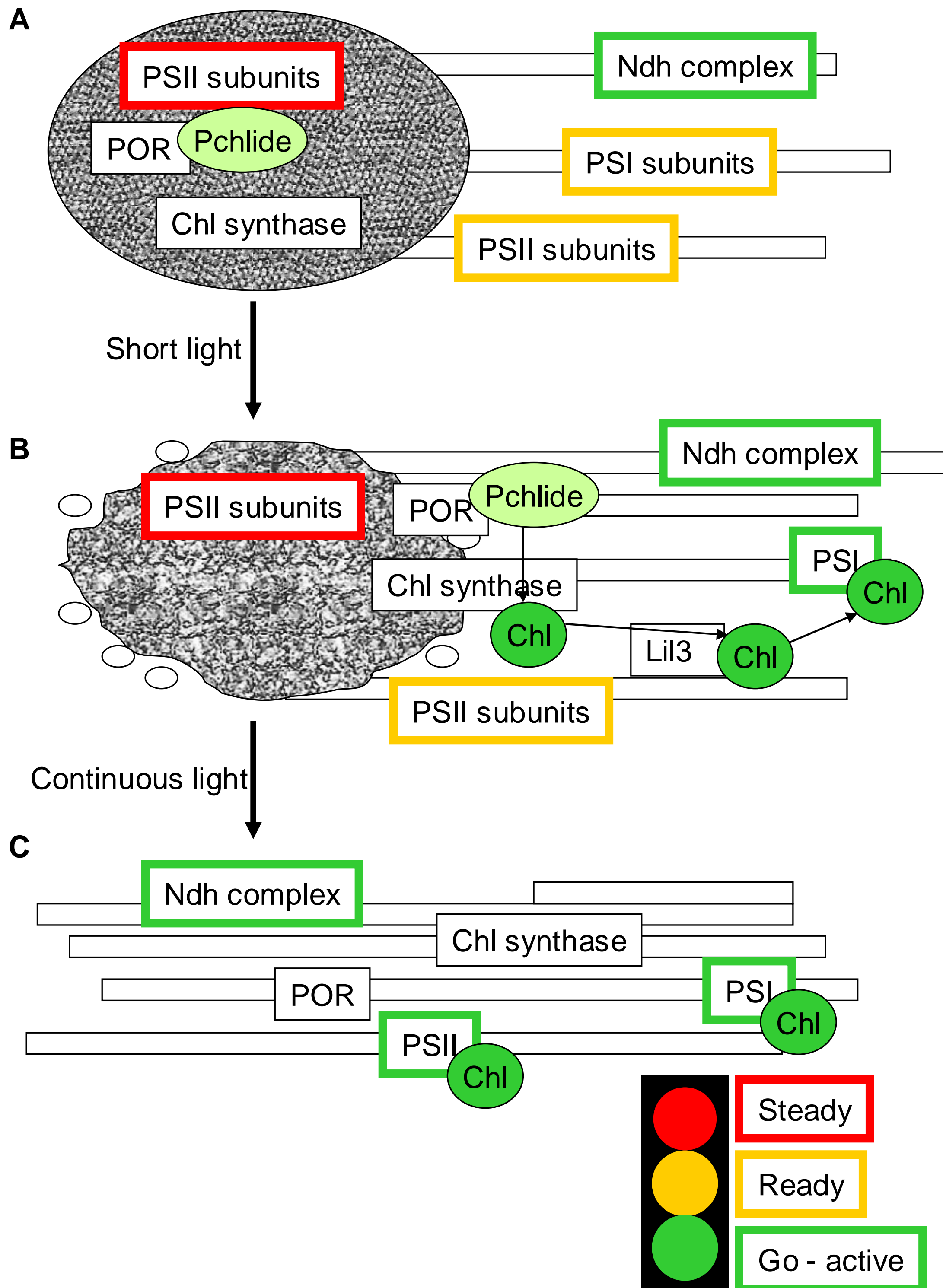


Figure 4. Simplified schematic picture of the activation of protein complexes during etioplast to chloroplast transition. A. PLB and PT membranes in dark-grown etioplasts. **B.** PLBs start to disperse, PTs are increasing in number and length upon short illumination. **C.** Fully functional thylakoid membranes in continuous light. Abbreviations used: Chl, chlorophyll; Ndh, NAD(P)H:plastoquinone oxidoreductase; Pchlide, protochlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase

terminal oxidase, ascorbate peroxidases and PsbS, which may be parts of a sophisticated photoprotective system (**II**, **III**).

Besides the protective role of PLBs, the specific localization of some proteins in PLBs may be of regulatory importance (**III**). Chlorophyll synthase activity is latent while the protein is located in PLBs (Lindsten et al. 1990) but is activated upon illumination and translocated to the PTs at a similar time scale as is POR (Linsten et al. 1993). Whether other proteins are inactive in PLBs remains to be elucidated. Protein subunits and chaperones needed for assembly of PSII are laterally separated in PLBs and PTs. This could mean that the construction of PSII depend on PLB dispersal for the release of arrested proteins (**III**).

5.1.2. Significance of prothylakoids (PTs) during transition

Numerous proteins were concluded to be exclusively located in the PTs in dark-grown wheat (**III**). Three major findings in my studies suggest that photosystem formation is initiated in PTs (Figure 4). Firstly, Lil3, a protein putatively mediating chlorophyll transfer (Reisinger et al. 2008) was shown to be located exclusively in PTs. POR and chlorophyll synthase are translocated from PLBs to PTs upon illumination (Ryberg and Dehesh 1986; Lindsten et al. 1993). Lil3 may well be the protein that mediates the transfer of newly synthesized chlorophyll to the chlorophyll-binding proteins of the photosystems. Secondly, the D2 protein of PSII was found to be a unique PT protein. Accumulation of D2 is the starting point for the assembly process of PSII (Müller and Eichacker 1999; Komenda et al. 2004; Minai et al. 2006). Thirdly, subunits (PSI-F and PSI-N) and proteins involved in assembly (ycf4 and rubredoxin) of PSI were exclusively localized to the PTs. Thus, several subunits and assembly factors are readily available for construction of PSI. Photosynthetic activity of PSI has been measured already within 5 minutes after the onset of illumination (Egnéus et al. 1972). The activation of PSII is much slower (Egnéus et al. 1972; Baker and Leech 1977; Wellburn and Hampp 1979; Ohashi et al. 1989, Krishna et al. 1999). The arrested state of certain subunits and chaperones needed for PSII assembly may explain the slower activation of PSII.

Thus, PTs accommodate a more active protein pool than PLBs during early greening. Moreover, subunits of the Ndh complex are potentially active already in darkness by their involvement in chlororespiration. The Ndh subunits may also be involved in photoprotection during transition of etioplasts to chloroplasts (**III**).

5.2. EPIMs are ready for a life in light – concluding remarks

The revelation of the proteomes of EPIMs (**I, II, III**) supports the idea that etioplasts represent a sophisticated strategy for increased survival and adaption (Wiktorsson 1995). The etioplast stage implicates several advantages compared to the proplastid stage in terms of transition into chloroplasts. Fully developed etioplasts requires a relatively short period of illumination to be transformed into mature, functional chloroplasts. Proplastids requires much longer period of illumination for transition into mature chloroplasts (Robertsson and Laetsch 1974).

To finally summarize my thesis in a few sentences I will come back to the title. PLBs are **steady** in darkness and constitute a role of protective and regulatory importance for the proteins they comprise. PLBs and PTs are **ready** for utilization of as well as protection against light. EPIM proteins are reorganized upon illumination – **go** for a fast onset of photosynthesis!

6. Acknowledgements - Tack!

Financial support was provided by Lars Hiertas Memory foundation, Royal Society of Arts and Sciences in Göteborg and the Swedish Research Council and PA Larsson fond.

Först av allt vill jag tacka **Christer Sundqvist** som varit min huvudhandledare under största delen av min doktorandtid. Tack för att du introducerade mig i etioplasternas fascinerande värld. Din dörr har alltid stått öppen för frågor och funderingar. Jag önskar att du hade kunnat vara med i slutfasen av mitt arbete. Jag vet att du önskar detsamma.

Jag känner en enorm tacksamhet till **Henrik Aronsson** som hoppade in som min huvudhandledare sista halvåret. Du har varit engagerad, peppat och pushat på ett sätt som jag inte vågade drömma om. Utan din hjälp hade det inte blivit någon avhandling (än).

Jag är också mycket tacksam till **Margareta Ryberg** som varit min biträdande handledare under alla mina år som doktorand. Tack för alla diskussioner, idéer och korrekturläsning. Din klarsynthet och noggranhet har varit ovärderlig.

Jag vill tacka **Mats Töpel** för samarbetet under arbetet med artikel IV. Det var roligt att få vidga vyerna lite. Jag vill även tacka andra vänner och kollegor på Botan för trevliga stunder vid lunch och fika. Tack alla som svarat på en miljon frågor under de senaste månaderna. Ingen nämnd, ingen glömd.

Den person som har gjort mest för att denna avhandling ska bli till, utan att skriva ett enda ord, är min make, **Daniel**. Du har näst intill fått leva som ensamstående förälder de senaste månaderna. Du har stöttat och uppmuntrat samtidigt som du har jobbat, skött hushållet och barnen. Mina små godingar **Klara** och **Hilda**, ni har gett mig små stunder av lycka! Från och med nu ska jag vara lite mer maka och mamma än jag varit de senaste månaderna. Ni är mitt allt! Jag älskar er!

Jag vill även rikta ett stort tack till resten av min underbara familj som har uppmuntrat mig, bjudit på middag, passat barn och bett en och annan bön; mamma **Karin**, pappa **Lasse**, mina systrar med respektive, **Hanna** och **Jerker**, **Matilda** och **Sebastian** samt **Jossan** och **Jonathan**, mormor **Rakel**, svärfar **Ruben** och alla ni andra i tjocka släkten. Tack också till alla vänner för välbehövda pauser från forskandet och skrivandet.

Till sist ett tack till den store prolamellärkropps-designern.

Bakom allt finns du Gud, finns din tanke och din mening….
(Roland Utbult)

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8. Populärvetenskaplig sammanfattning på svenska

Steady, ready, go…? Det heter ju ready, steady, go! Ordföljden är vald för att väcka uppmärksamhet och samtidigt peka på de viktigaste slutsatserna från min forskning. Under årens lopp har jag ofta blivit ombedd att förklara vad min forskning går ut på. Svaren har jag varierat beroende på vem som frågat. Ibland har det räckt att jag nämnt ordet växtfysiologi så har folk slagit dövörat till. Jag tänkte i alla fall försöka mig på en förklaring, som jag hoppas att mina vänner, min familj och andra intresserade förstår.

Proteiner är livsviktiga för alla celler, i växter såväl som i djur. De består av långa sekvenser av aminosyror och är en direkt översättning av det budskap som finns i generna i en cell. Proteiner förekommer i cellens samtliga processer, där de utför en rad viktiga funktioner. Då man vill förstå hur en viss typ av celler eller organeller (motsvarande cellens organ) fungerar är det alltså viktigt att fastställa vilka proteiner som finns på plats. Forskning som syftar till att ta reda på vilken proteinsammansättning som finns i t.ex. en cell kallas modernt för proteomik. Denna forskningsgren inom biologin har ökat explosionsartat under de senaste tio-femton åren. Ökningen beror framförallt på två saker, dels på att den teknik som används för proteinidentifiering har utvecklats och blivit känsligare, dels på att man känner till aminosyra-sekvensen för fler och fler proteiner. Eftersom likvärdiga proteiner från olika organismer liknar varandra, finns det idag stora chanser att identifiera de proteiner som finns i en cell eller organell.

Fotosyntesen är en biologisk process som är av grundläggande betydelse för allt liv på jorden. Genom denna process används energin från solljuset till att omvandla vatten och koldioxid till socker och syre. I växter sker fotosyntesen i organeller som kallas kloroplaster. Dessa kloroplaster innehåller stora mängder av det gröna pigmentet klorofyll, som tar upp solljuset. Klorofyll är alltså en av de viktigaste molekylerna på jorden. Bildningen av klorofyll kräver ljus. Finns det inte ljus så bildas ett förstadium av klorofyll som kallas protoklorofyllid. En gräsplätt som fått växa under t.ex. en badpool eller en brädhög på sommaren är som bekant inte grön utan gul. Det är dock inte bara förekomsten av klorofyll som skiljer det gula gräset från det gröna gräset, som fått växa i solljus. Det gula gräset innehåller inga kloroplaster utan en annan typ av organeller som kallas etioplaster. Etioplaster omvandlas snabbt till kloroplaster vid belysning. Etioplastens inre struktur är väldigt olik kloroplastens. Den största skillnaden utgörs av ett märkligt ”klot” (se bild på framsidan av denna avhandling) som kallas prolamellärkropp, en membranstruktur som förkortas PLB på engelska. Den uppmärksamme har märkt att det gula gräset blir grönt ganska snabbt när brädhögen flyttas. Vad som händer är att ljuset aktiverar proteinet POR som sköter omvandlingen av protoklorofyllid som i slutändan bildar klorofyll. Denna process startar på mindre än en sekund från det att man lyfter på brädhögen och är själva startpunkten för omvandling av etioplaster till kloroplaster. PLB-strukturen löses upp, omstruktureras och proteinerna som fanns i PLB omlokaliseras. Efter några timmar är kloroplastomvandlingen fullbordad. Etioplaster finns naturligt även i t.ex. knoppar och groddar och dessa genomgår omvandling till kloroplaster vid knoppsprickning respektive då grodden tränger upp genom jordytan. Omvandlingsprocessen har studerats mycket i syfte att öka förståelsen för både klorofyllbildning och kloroplastutveckling.

Trots intensiv forskning finns det många obesvarade frågor som berör t.ex. funktionen hos PLB-”kloten” och POR-proteinet. Syftet med min forskning har varit att försöka komma steget närmare svaren på några av dessa frågor. Jag har utfört flera olika proteomikstudier på etioplastens inre fraktioner. Vid dessa studier, som är de första i sitt slag, har jag identifierat drygt ett hundratal olika proteiner. Proteinerna som identifierats representerar en mängd olika funktioner, allt från pigmentbildning till nedbrytning av

proteiner. Den funktion som är mest representerad är dock fotosyntesen. Det finns även en mängd proteiner som är inblandade i skydd mot solljusets ofta alltför starka strålning. Sammanfattningsvis kan man säga att PLB är en stadig (**steady**) struktur som skyddar proteiner och pigment. Proteinsammansättningen visar att etioplastens inre struktur är redo (**ready**), dels på att utnyttja, dels på att skydda sig mot solljusets energirika men farliga strålning, detta för att snabbt kunna omvandlas och fotosyntetisera så fort det finns tillgång till ljus (**go!**).