# Characterization of the Lil3 protein during deetiolation of *Hordeum vulgare*

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# Characterization of the Lil3 protein during deetiolation of *Hordeum vulgare*

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# Abstract

Angiosperm plants growing in darkness are free of chlorophyll and develop etioplasts instead of chloroplasts the characteristic organelles of green plants. Upon illumination, chlorophyll and the chlorophyll binding proteins of the photosynthetic machinery rapidly accumulate. Recently, chlorophyll has been shown to bind to Lil3 immediately after illumination of etioplasts. Lil3 is a light harvesting like proteins that shares an alpha helix motif with the light harvesting proteins of the photosystem complexes. However, it is unknown how much of the Lil3 protein complex is present during development of the photosystem complexes. Here, we show that Lil3 is a membrane protein and that its amount is constant throughout deetiolation of dark grown barley seedlings. We found that the Lil3 protein immediately assembles into two protein complexes upon the onset of illumination of barley leaves, but is not present as a protein complex in etioplasts. In contrast, equal amounts of the protein were found in etioplasts and in any of the developmental time points during biogenesis of chloroplasts. Our results demonstrate that the Lil3 proteins could provide the missing link for transfer of the chlorophyll free membrane of etioplasts into the chlorophyll rich membrane of chloroplasts. The Lil3 protein could operate as chlorophyll storage and integrate into an enzymatic chlorophyll delivery chain to enable the assembly of the photosynthetic machinery in the membrane of plant plastids. This report is the starting point for a more detailed characterization of the Lil3 function. The next level of further investigations will be directed to understand the composition, folding, and structure of the Lil3 protein complexes.

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# Abbreviations

| | |
|---|---|
| ˚C: | Celsius degrees |
| ACA: | ε -aminocaproic acid |
| APS: | Ammonium peroxodisulfate |
| BN: | Blue native |
| CAO: | Chlorophyllide a oxygenase |
| Chl a/b: | Chlorophyll a/b |
| CN: | Clear native |
| CMC: | Critical micellar concentration |
| Cyt $b_6f$ | Cytochrome $b_6f$ |
| DM: | n-dodecyl-β-D-Maltoside |
| DNA: | Deoxyribonucleic acid |
| DTT: | dithiothreitol |
| ECL: | enhanced chemiluminescence |
| EDTA: | Ethylendiamintetraacetate |
| ELIP | early light-inducible proteins |
| ESI: | Electroprspray ionisation |
| F-ATPase | ATPsynthase from plant leaves |
| Fd: | Ferredoxin |
| FNR: | Ferredoxin-NADPH-reductase |
| HRP: | Horse radish peroxidase |
| IgG: | Immunoglobulin G |
| kDa: | Kilo Dalton |
| LDS: | Lithium dodecyl sulfate |
| Lil3: | Light harvesting like protein 3 |
| LMW: | Low molecular weight |
| m/z: | Mass per charge |
| mA: | Milli Ampere |
| MALDI: | Matrix-assisted laser desorption ionization |
| MS: | Mass spectrometry |
| $NADP^+$/H: | Nicotinamide adenine dinucleotide phosphate/reduced form |
| p.A.: | pro Analysis |
| $P_{680/700}$ | $Pigment_{680/700}$ |
| PAGE: | Polyacrylamide gel electrophoresis |
| PC: | plastocyanin |
| Pchl: | Protochlorophyll |
| POR: | protochlorophyllide oxyreductase |
| Q-TOF: | Quadruple time of flight |
| RF: | Running front (in electrophoresis) |
| RuBP: | ribulose 1,5-biphosphate |
| SDS: | Sodium dodecyl sulfate- |
| TBS: | Tris-buffered saline |
| TEMED: | Tetramethylethylenediamine |
| TMK: | Tris magnesium KCL |

# Contents

# 1 Introduction

## 1.1 Theory

Recently, a protein with sequence similarity to the light harvesting proteins was discovered immediately after the first light rays had hit plants grown in complete darkness. According to its protein sequence the protein was identified as a member of the light-harvesting like proteins with the name Lil3. The protein had shown the remarkable capacity to assemble de novo synthesized chlorophyll; although it did not participate in photosynthesis. In order to characterize the function of this protein, we set out to study the quantitative changes of the Lil3 protein during deetiolation of the plant.

### 1.1.1 An introduction to the photosynthesis

Photosynthesis is the process we today look upon as producing oxygen and organic compounds from water, carbon dioxide and the energy of the sun. This process is carried out in photo autotrophic organisms like plants, algae and bacteria. Photosynthesis is maybe the most important processes for all life on earth. Most organisms are oxygen dependent and almost all the food chains start at the photo autotrophic level. Plants are therefore known as the producers of the land ecosystems. All photo autotrophic cells contain chloroplasts. The chloroplast is the organelle where photosynthesis takes place (Raven et al. 2003).

Photosynthesis has been discovered step by step over the last 350 years. While in the Greek school of thinking the plant got all their nutrition from the soil, the first evidence that soil alone was not sufficient was given by Jan Baptist van Helmont, a Belgian physician (ca 1577-1644). He grew a tree in a pot and added only water to it. In the end of his experiment the weight of the tree had increased by 74.4 kilograms and the soil weight had only decreased by 57 grams. He therefore concluded that the substances of plants were produced from water and not from the soil. In 1771, Joseph Priestley, an English scientist showed by the use of burning candles that used air can be " restored" by vegetation. The Dutch physician Jan Ingenhousz confirmed this theory in 1796. He showed that the air was "restored" by the green parts of the plant in combination with the presence of

sunlight. He also suggested that the oxygen was released from the carbon dioxide and that carbon reacted with water making “carbohydrate”. This theory was widely accepted until 1931. Then Van Niel, a graduent student at the Stanford University, proposed that water and not carbon dioxide was the source of oxygen in photosynthesis. This theory was supported by the experiments of Robert Hill in 1937 (Raven et al. 2003).

Photosynthesis can be divided in two major processes, the light reaction and the dark reaction. The first evidence of both a light and dark dependent step in photosynthesis was found in 1905 by the English physiologist F.F Blackman. The light reaction is light dependent, and the site for the reaction is in the thylakoid membrane. In the light reaction, light energy is used to form ATP from ADP and to reduce $NADP^+$ to NADPH (Nicotinamide adenine dinucleotide phosphate). In the dark reaction (or carbon fixation) the chemical energy from the light reactions ATP and NADPH is then used to drive $CO_2$ fixation. But although the name suggests otherwise, dark reactions occur in the dark and in the light and reactions are mostly localized in the stroma. (Raven et al. 2003).

### 1.1.2 The proteins and membranes of the cell

Proteins consist of amino acids bound together by peptide bonds. The 20 amino acids are characterized by the chemistry of the side groups which are bound to the alpha carbon atom in each amino acid. The sequence of amino acids from the N- to the C- terminus determines the primary structure of the protein. Folding of the primary structure into either alpha helixes or beta folds determines the secondary structure of a protein. The folding may either be spontaneously or is assisted by helper proteins called chaperones. Many steps in the folding of a protein are related to the arrangement of the amino acid side chains relative to the surrounding water molecules with hydrophobic side groups pushed away from the water and arranged more in the interior of the protein. The secondary structures are stabilized by the dynamic interactions of hydrogen bridges between the amino acids, and the hydrophobic interactions called van Der Waals interactions which occur between the aliphatic hydrophobic side chains, and the more long lived covalent disulfide bridges that chemically fix the secondary structures. The

folding of secondary structures into structures of higher complexity is called tertiary structures which are hence an assembly containing different alpha or beta folded units of the protein. The proteins can have an overall more hydrophobic or hydrophilic character and many proteins associate with other highly complex protein assemblies into the multidimensional quarterly structures known from protein complexes. (Campbell and Reece 2002).

All cells contain membranes that separate the inside of a cell towards the surrounding environment and within the cell divides it into different compartments, the organelles. Membranes consist of a lipid bilayer, embedded with membrane proteins of various functions. The membrane proteins are responsible for communication between the cells and the organelles and towards the environment. Molecules are sensed and there uptake and release is regulated utilizing physical forces like e.g. membrane potentials, proton gradients, and the proteins as biogenic factors that are embedded within the membranes. Membranes contain two layers (bilayer) of lipids. One typical type of lipid e.g. the phospholipids that are found throughout living organisms have a hydrophilic (water soluble) head and a lipophilic tail (water repellent). The membrane structures are held together by van der Waals forces between the aliphatic tails of the lipids and the force applied from the water of the cell towards the hydrophilic lipid heads and against the hydrophobic tails. The phospholipids tails are hereby pushed away from the aqueous solution of the surrounding environment. Hydrophilic heads will then turn out on each side of the membrane, “protecting” the hydrophobic tails. (Campbell and Reece 2002). Proteins containing a high number of hydrophobic amino acids are often arranged in hydrophobic alpha helixes that are lipophilic and are therefore often found integral in the lipid bilayer.

### 1.1.3 Chloroplasts

Chloroplasts are the organelles responsible for photosynthesis in a plant cell. Chloroplasts are normally disc shaped and between four and six micrometers in diameter. A single mesophyll cell contains 40 to 50 chloroplasts and a cubic millimetre contains up to 500,000 chloroplasts. In the illuminated cell,

chloroplasts are normally placed randomly alongside the cell walls. However, in the high light intensity of a sunny day, the chloroplasts align along the cell walls oriented parallel to the light rays and provide shadow for each other. They can turn around in the cell due to various strength of light. (Raven et al. 2003).

Plastids are semiautonomous organelles, which mean that some of the proteins are encoded by plastid DNA (Deoxyribonucleic acid) and some by nuclear DNA and that the plastid encoded proteins can be synthesized in the plastids itself (Lopez-Juez and Pyke 2005). The plastid DNA molecule was discovered in the 1960 and the early 1970. Surrounding the plastids are an inner and an outer membrane, called inner and outer envelope (von Wettstein et al. 1995). Plastids reproduce by splitting (fission) similar to bacteria (Lopez-Juez and Pyke 2005). Plastids are classified by the kinds of pigments they contain (Raven et al. 2003). Chloroplast, which carry out photosynthesis are characterized by their specific chlorophyll and carotenoid pigment content (Raven et al. 2003).

Chloroplast contain an inner membrane system called thylakoids (von Wettstein et al. 1995) (see fig. 1.1). The thylakoid membranes are differentiated in two domains; grana and stroma lamella (Albertsson et al. 1990). The grana look like stacks of coins and the stroma lamellae form the connections between the grana (Nelson and Ben-Shem 2004). The chlorophyll and carotenoid pigments are located in the thylakoid membranes (Nelson and Ben-Shem 2004). The pigments are receptors for the light which is used in photosynthesis (Nelson and Ben-Shem 2004). Since plastid pigments absorb light less effectively in the green region of the visible solar energy emission spectrum, light that is passing through the leaves is still green and also the light reflected from the plants is mainly green. (Nelson and Ben-Shem 2004).

Plastids, are also the site within the plant cell for temporary storage of sugar in form of starch. We enjoy the long term storage of starch in the plastids of potato tubers and corn; however, in chloroplasts the starch grains accumulate only when during active photosynthesis more sugar is synthesized then the plant cell needs. When the plants are devoid of light, photosynthesis stops and starch stored in the chloroplast is retransformed into transportable sugar forms to nourish the cell. (Raven et al. 2003).

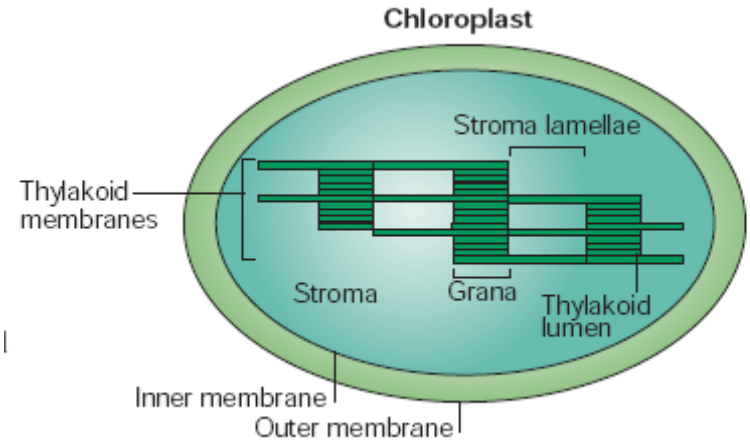


**Figure 1.1 The model of the chloroplast (Nelson and Ben-Shem)**

The plastid proteins are encoded by both nuclear and plastid DNA. The plastid DNA is in circular form, like in bacteria. Proteins encoded in the nucleus are imported in the plastid from the cytosol where they are synthesized (Lopez-Juez and Pyke 2005).

### 1.1.4 Proplastids and etioplasts

Proplastids are small plastids with no pigmentation (Lopez-Juez and Pyke 2005) and the lack of chlorophyll (von Wettstein et al. 1995). They are found in meristematic (dividing) cells of roots and shoots (Lopez-Juez and Pyke 2005). All the other plastids like chloroplasts, chromoplasts or amyloplasts are differentiated proplastids (Lopez-Juez and Pyke 2005). If proplastids are kept in the dark they may turn into etioplasts by the forming of prolamellar bodies, which are tubular membranes (Lopez-Juez and Pyke 2005; Muhlethaler and Frey-Wyssling 1959; von Wettstein et al. 1995). If the etioplast is exposed to light, the prolamellar bodies are dissolved and thylakoid membranes are formed (Kanervo et al. 2008; Muhlethaler and Frey-Wyssling 1959; von Wettstein et al. 1995). In the embryonic cells in seeds, the proplastids first develop into etioplasts and then into chloroplasts after being exposed to light (Muhlethaler and Frey-Wyssling 1959; von Wettstein et al. 1995). The structural difference of the etioplast and the chloroplast can be seen in fig 1.2.

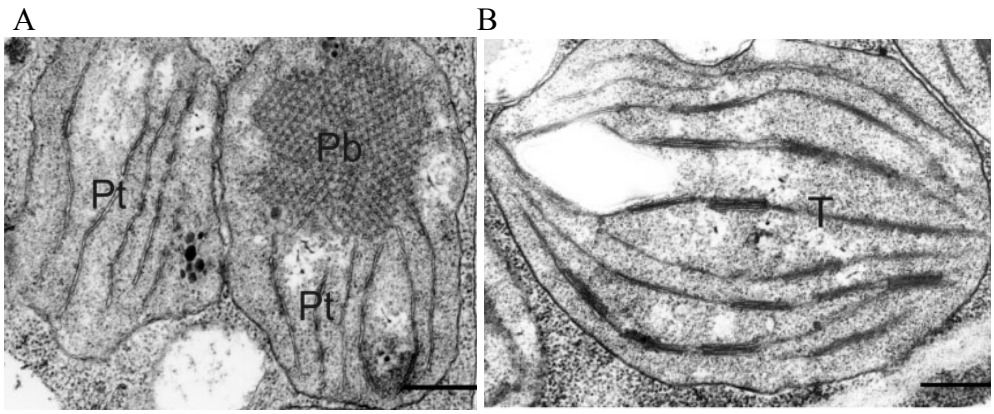


**Figure 1.2A: Electron microscopy of deetiolating etioplasts of pea.**
The two plastids contains prolamellar body (Campbell and Reece) and prothylakoids (Pt) (Kanervo et al. 2008).

**B: Electron microscopy of chloroplast of Pea, after 24 hour light exposure.**
The chloroplasts have developed thylakoid membranes (T) (Kanervo et al. 2008).
In figure A prolamellar bodies and prothylakoids of etiolated plastids can be seen. In figure B is a fully developed chloroplast with thylakoid membranes (T).

### 1.1.5 Pigments

The chlorophyll of the chloroplast absorbs light in the violet and the blue and also in the red region of the visible solar radiation. An absorption spectrum can therefore be measured between the wavelengths of 400 and 725 nanometres. Chlorophyll is embedded in special units called photosystems. There are two photosystems involved in the light reaction, photosystem I and II (PSI and PSII). Each photosystem contains 200-400 pigment molecules and is composed of two linked components; the antenna complex and the reaction centre (RC). (Raven et al. 2003).

There are two kinds of chlorophyll (chlorophyll *a* and chlorophyll *b*) that absorb the photons of the light for photosynthesis. Chlorophyll *a* however is the only one used to extract the chemical energy from the light energy. Chl *a* is located in the reaction centre of both photosystems. Chl *b* which is located in the antenna pigments funnels the photons into the Chl *a* in the reaction centre. Some carotenoid pigments are also located in the antenna complex. They are thought to function as protection against photo-oxidative stress. Two Chl *a* molecules of the

PSI reaction centre are known as $P_{700.}$ P stands for pigment and 700 is the wavelength of the absorbance peak for the pigment. Two Chl *a* of the PSII reaction centre are believed to form the $P_{680.}$ (Nelson and Yocum 2006).

### 1.1.6 The light reaction

Thylakoid membranes are the site of the light reaction. The overview of the main complexes of the light reaction and the distribution in the thylakiods can be seen in figure 1.3.

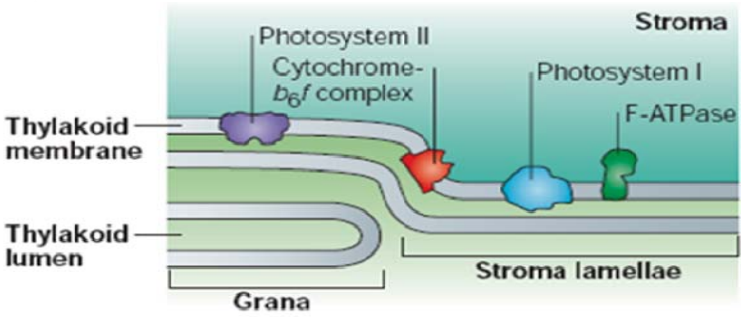


**Figure 1.3: The location of the four membrane-protein complexes in the thylakoid membrane of the chloroplast** (Nelson and Ben-Shem 2004).

PSI localizes to the stroma lamellae. PSII is almost exclusively found in the grana (Nelson and Yocum 2006). F-ATPase is mainly located in the stroma lamellae and the Cyt $b_6f$ complex is found in the grana, and the grana margins (the connecting thylakoid between the grana) (Nelson and Yocum 2006). According to the reaction which they catalyse, PSII is defined as a water–plastoquinone oxidoreductase, the Cyt $b_6f$ complex as a plastoquinone–plastocyanin oxidoreductase, PSI as a plastocyanin–ferredoxin oxidoreductase and the F-ATPase as a proton motive force (pmf)-driven ATP synthase (Nelson and Ben-Shem 2004).

Early biochemical studies showed that the thylakoid membrane of the chloroplast is capable of NADP reduction, ATP formation and oxidizing water by a light dependent reaction (Nelson and Ben-Shem 2004; Whatley et al. 1963). In further studies two separate systems were found to catalyse these reactions, hence the photosystem I (PSI) and photosystem II (PSII) (Nelson and Ben-Shem 2004). An F-ATPase (ATPase) was also found to produce the ATP, driven by a proton

motive force (pmf) formed by the light reaction (Jagendorf and Uribe 1966; McCarty et al. 1971; Nelson and Ben-Shem 2004). The cytochrome (Cyt) $b_6f$ complex is involved in the electron transport between PSII and PSI (Nelson and Ben-Shem 2004). Together with the PSII, Cyt $b_6f$ contributes to the pmf that drives the ATPase (Cramer and Butlera 1967; Nelson and Ben-Shem 2004).

In the light reaction, a photon from light is absorbed by the pigments including Chl *a*, Chl *b* and the family of carotenoids in the antenna complex (LHCII) of PSII. One photon is then captured by Chl *a* ($P_{680}$) in the reaction centre (RC) of PSII. The exited Chl *a* enables a directed electron flow. The remaining $P_{680}^{+}$ is then able to extract an electron from water molecules leading to its photolysis after $P_{680}$ has been excited two times. This step creates oxygen and hydrogen and starts the build up of a proton motive force in the thylakoid lumen (pmf). (Raven et al. 2003).

The electron flow initiated at $P_{680}$ now passes an electron transport chain that links PSII with PSI via the mobile carrier plastoquinone (PQ) and the Cyt $b_6f$ complex and a plastocyanin (PC). The Cyt $b_6f$ is the second proton pump in the electron chain and amplifies the level of protons in the thylakoid lumen relative to the stroma. Cyt $b_6f$ contributes to the proton motif force (pmf) in a mechanism known as the Q-cycle. The result of the mechanism is the release of two protons and of two electrons from plastoquinone to the lumen. While the protons increase the pmf and enable the synthesis of ATP from ADP and inorganic phosphate, the electrons reduce plastocyanine (PC) and hence enable electron transfer to photosystem I (PSI). (Nelson and Ben-Shem 2004).

Also, in PSI the light is captured in the antenna proteins (LHCI) and funnelled to the chl *a* in the reaction centre ($P_{700}$) (Raven et al. 2003). An electron of the $P_{700}$ is injected into a redox chain of the cofactors $A_0$ $A_{1,}$ $F_x$ $F_A$ and $F_B$ (Nelson and Yocum 2006). The electron reduces a ferredoxin (Fd) on the stroma side of the PSI (Nelson and Ben-Shem 2004) and electron can be captured by NADP reductase which produces NADPH of the $NADP^{+}$ (Nelson and Ben-Shem 2004). The exited $P_{700}$ replace the lost electron, by translocating one from the plastocyanin (PC) on the inside of the thylakoid membrane (Nelson and Ben-Shem 2004).

The PSI can also work independently of photosystem II in a reaction called the cyclic electron flow. Here the electrons are transferred from $P_{700}$ back to Cyt $b_6f$ via ferredoxin (Fd) and the plastocyanin and the reaction centre of the PSI. This reaction powers a PSII independent proton translocation via the cytb6f and enables ATP synthesis. However, no NADPH is produced in cyclic electron flow. (Raven et al. 2003).

### 1.1.7 The dark reaction

The dark reaction which is also called the carbon fixation reaction fixes carbon diaoxide from the air (in terrestrial plants) and enables sugar synthesis. The ATP and the NADPH molecules made in the light reaction fuel the carbon fixation. The net product of photosynthesis is glucose ($C_6H_{12}O_6$, but the primary product of carbon fixation is either a three carbon or a four carbon molecule in the C3 and C4 plants respectively). (Raven et al. 2003).

Carbon fixation is carried out in the Calvin cycle in the stroma of the plastids. The Calvin circle is similar to other metabolic cycles, because in the end of the cycle the starting molecule is regenerated. This molecule is named ribulose 1,5-biphosphate (RuBP). In the first stage of the cycle the carbon dioxide is covalently bound to RuBP. The resulting intermediate is hydrolyzed to two molecules of 3-phosphoglycerate (PGA). This first reaction is catalyzed by the enzyme RuBP carboxylase/oxygenase, also known as Rubisco. Then 3-phosphoglycerate is reduced to 3-phosphoglyceraldehyde (PGAL) which can be transferred to the cytosol of the cell. There it is either transferred to sucrose which is the transport sugar in the plant, or it is converted to starch within the plastids and stored.(Raven et al. 2003).

### 1.1.8 The complexes of the light reaction

In figure 1.4 an overview of the complexes involved in the light reaction is given.

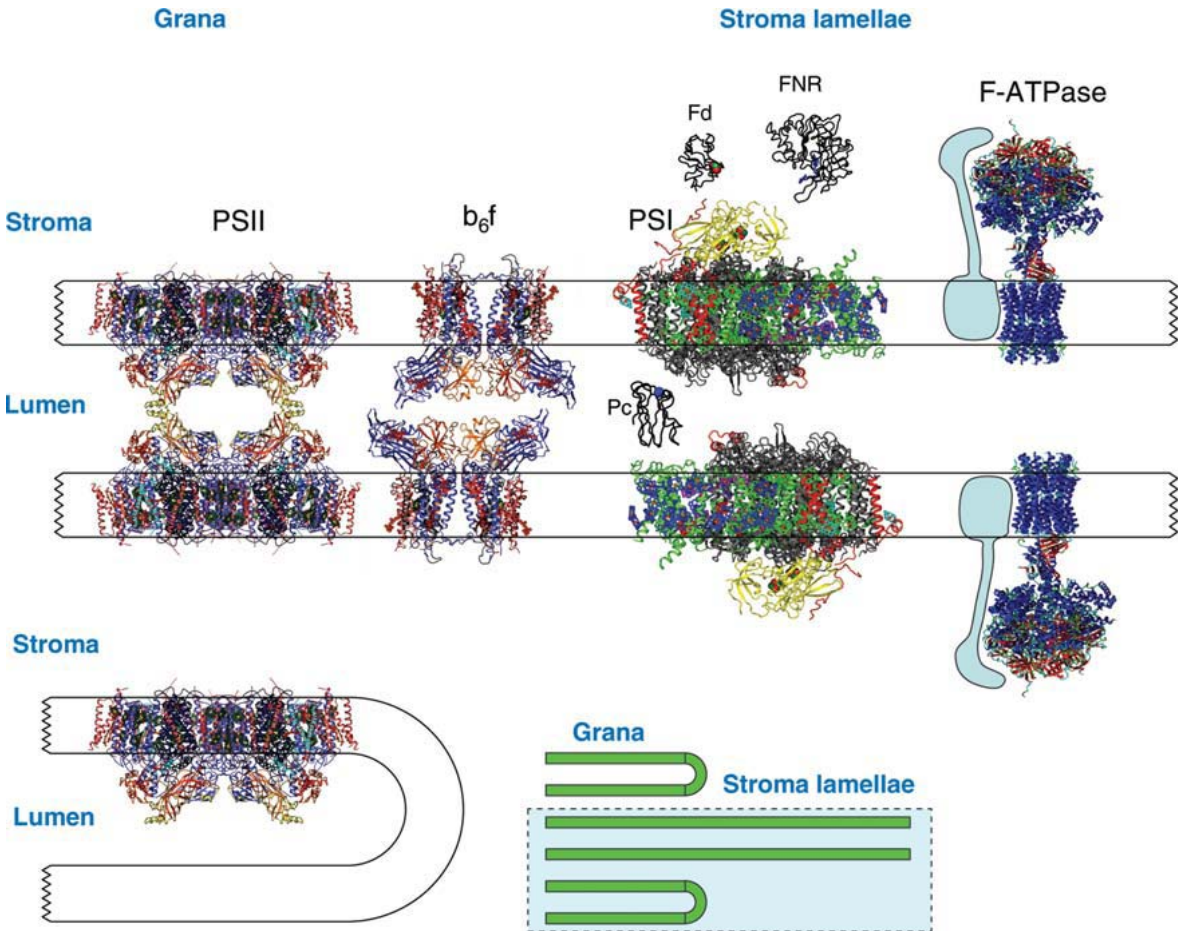


**Figure 1.4** ”**The architecture of thylakoid membrane complexes and soluble proteins based on high-resolution crystal structures”** (Nelson and Yocum 2006).

Photosystem I (PSI), photosystem II (PSII), cytochrome (Cyt) *b6f* and ATPase (F-ATPase in plants) are the four main complexes of the light reaction. Plastocyanin (PC), Fd and FNR are proteins that assemble with the photosystems to perform the red-ox reactions (Nelson and Yocum 2006).

Photosystem I was the first of the photosystems to be discovered (thereby the name). It forms a supercomplex with the light harvesting complex I, LHCI and the reaction centre (RC). PSI also forms complexes with other soluble electron

donors and acceptors (Dekker and Boekema 2005). The 14 subunits of PSI reaction centre in higher plants are PsaA ($P_{700}$) –PsaL (Nelson and Yocum 2006).

The light harvesting chlorophyll binding proteins of PSI are named with Arabic numbers attached to the end of the gene name LHCa for the PSI complex LHCa1, LHCa2, LHCa3 and LHCa4 (Ben-Shem et al. 2003; Jensen et al. 2003; Scheller et al. 2001). The PSI binds a high number of about 100 chlorophyll molecules. All subunits constituting PSI e.g. like the PsaF which is responsible for the binding of PC to the PSI have a specific function (Nelson and Yocum 2006). For assembly of the proteincomlex a high number of additional proteins are needed (Nelson and Yocum 2006).

The composition of the subunits in Photosystem II (PSII) is even more complex (Nelson and Yocum 2006). The PSII forms a supercomplex consisting of the light harvesting proteins, LHCII and the PSII (Dekker and Boekema 2005). The membrane proteins D1, D2 together with PsbE and PsbF form the reaction centre (Nelson and Yocum 2006; Minagawa and Takahashi 2004), where the core antenna proteins CP47 and CP43 are attached (Nelson and Ben-Shem 2004). Also some small polypeptides like, PsbI, PsbT and PsbW have been predicted to be attached (Minagawa and Takahashi 2004). The PSII consists of a RC and a minor and a major antenna (Minagawa and Takahashi 2004). The minor antenna is the CP24, CP 26 and CP29 and the major antenna is the LHCII (Minagawa and Takahashi 2004). PsbZ is believed to connect the RC to the minor antenna and PsbS has been predicted to be located in the peripheral side of the complex (Minagawa and Takahashi 2004).

Plastocyanin (PC) is a soluble copper-containing protein present in the thylakoid lumen (Nelson and Yocum 2006). The PC is a mobile Cyt *b6f*-PSI electron donor that transfers electrons to PSI (Nelson and Yocum 2006). In the model plant *Arabidopsis thaliana* there was no other electron donor to PSI has been found (Weigel et al. 2003). PC is therefore very likely the only mobile PSI electron donor in higher plants (Nelson and Yocum 2006).

Fd functions as electron acceptor on the donor side of PSI. It reduces $NADP^+$ via FNR. Fd also reduces the Cyt *b6f* complex or plastoquinone in the cyclic pathway (Buchanan and Balmer 2005; Nelson and Yocum 2006). Fd is a

soluble protein on the stroma side of PSI (Nelson and Yocum 2006). Fd has one 2Fe-2S cluster that accepts electrons from the PSI (Nelson and Yocum 2006). The Fd-PSI interaction involves PsaC, D and E subunits (Andersen et al. 1992; Lushy et al. 2002; Minai et al. 2001).

Isolation of the chloroplast Cyt $b_6f$ complex showed it's similarity to the mitochondrial cytochrome $bc_1$ complex (Nelson and Ben-Shem 2004). The complex is a dimer with the molecular weight of 217 kDa (Kurisu et al. 2003; Stroebel et al. 2003). It contains four large subunits (of 18-32 kDa) (Nelson and Ben-Shem 2004). The units are cytochrome *f*, cytochrome $b_6$, the Rieske iron-sulphur unit and subunit IV (Nelson and Ben-Shem 2004). It also has four small hydrophobic subunits; PetG, PetL, PetM and PetN (Nelson and Ben-Shem 2004). The Cyt $b_6f$ complex is shown to bind one Chlorophyll molecule per monomeric complex (Reisinger et al. 2008a).

The ATP synthase is found e.g. in membranes exhibiting a proton potential like the chloroplast thylakoid membrane and the inner membrane of the mitochondria. In autotrophic organisms the ATPase, catalyses ATP synthesis by the pmf generated in the electron transport chain (McCarty et al. 2000). ATP synthase complex is an enzyme consisting of many subunits, see fig 1.5, of Nelson and Ben-Shem (Nelson and Ben-Shem 2004).

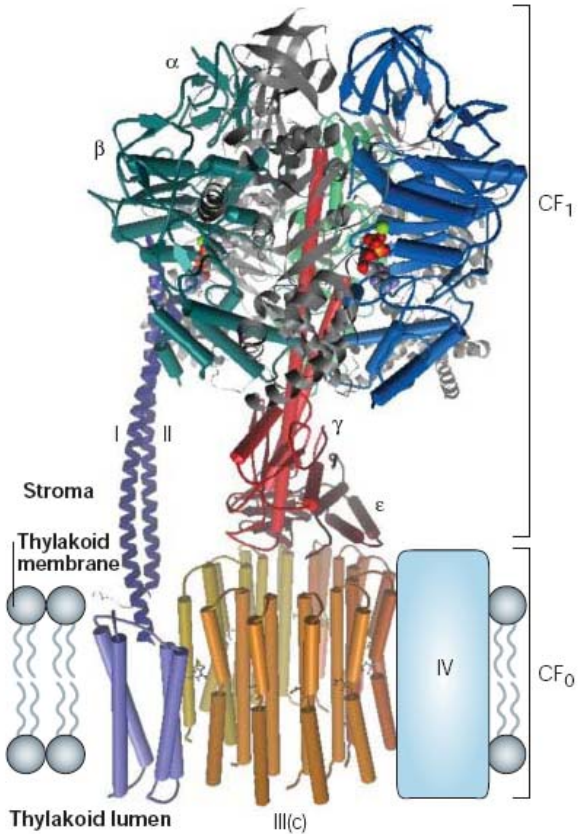


**Figure 1.5: The subunits of ATP synthase.**

"This model was created by W. Frasch (Arizona State University Arizona, USA) using available structural data for mitochondrial F-ATPase subcomplexes, as well as biochemical information" (Nelson and Ben-Shem 2004). The figure shows 12 III-subunits, but it is an ongoing dispute whether the complex contains 14 subunits. Subunit IV is added in a schematic way. The location of the δ subunit is not known so it is missing in the figure.

ATPase has two parts, one transmembrane and one stromal unit, known as $CF_0$ and $CF_1$ respectively. The $CF_0$ is composed of one subunit I, II, and IV and 14 subunits of subunit III which forms a ring-like structure (McCarty et al. 2000). In the $CF_1$ there are subunits called α, β, δ, γ and ε. Helixes connecting the complexes $CF_0$ and $CF_1$ are also found. The whole complex has been shown to function as a rotating, proton driven motor. Proton movement through the $CF_0$ results in ATP synthesis by the β-subunits of the $CF_1$. The subunits I, II, IV, δ, α and β are thought to be the stationary, and the III, γ and ε are the rotating units (Nelson and Ben-Shem 2004).

### 1.1.9 Chlorophyll synthesis

The pigment chlorophyll exists in all photosynthetic organisms. They harvest the light energy and drive the electron transfer in the light reactions. The synthesis of chloropyll is induced by light in angiosperms. If plants are grown in the absence of light, plastids in angiosperms develop into etioplasts (von Wettstein et al. 1995).

In etioplasts, the chlorophyll biogenesis is halted upon synthesis of protochlorophyllide. For reduction of protochlorophyllide *a* into chlorophyllide *a*, light is required as a substrate for the catalysis by NADPH: protochlorophyllide oxyreductase (Weigel et al.) (Apel et al. 1980; Griffiths 1978). NADPH is the hydrid donor of the reaction (Apel et al. 1980; Griffiths 1978). The two types of POR enzymes, POR A and the POR B have been characterized in detail. The mRNA (Messenger ribonucleic acid) level and the protein amount of POR A are declining during deetiolation while the POR B mRNA and the protein level remain constant. It is therefore suggested that POR A is active in the beginning of the deetiolation, while POR B maintains active during all developmental stages (Holtorf and Apel 1995).

The next step of the Chl *a* synthesis is an esterification of chlorophyllide *a*, to chlorophyll *a* via chlorophyll synthase (Rudiger et al. 1980) and thereafter an oxygenation of Chla to by chlorophyll *a* oxygenase. Accumulation of Chl is paralled by accumulation of the Chl *b* binding LHC proteins (Dreyfuss and Thornber 1994; Mathis and Burkey 1987). Chlorophyll *a* oxygenase (CAO) was also shown to convert chlorophyllide *a* to chlorophyllide b, by a two step oxygenation (Oster et al. 2000).

### 1.1.10 The assembly of chlorophyll binding protein complexes

If angiosperm seedlings grow in the dark etioplasts will form instead of chloroplasts. “The etioplast accumulate large amounts of thylakoid lipids with the complex of protochlorophyllide and a form of the enzyme responsible for its light-driven reduction,
protochlorophyllide reductase A” (Lopez-Juez and Pyke 2005). Etioplasts lack many of the protein complexes found in the chloroplast since many of the proteins

need light to be transcribed in the nucleus of the cell or the plastid and many light induced proteins are required to assemble to the functional PS complexes. As an example, nuclear encoded chloroplylll binding proteins (LHCI/II) of the chloroplast are not transcribed in the absence of light (Adamska et al. 1999).

Plastid DNA codes for at least six Chl *a* binding proteins including $P_{700}$ of PSI and the D1, D2 and CP47/43 of PSII. All plastid encoded chlorophyll binding proteins are expressed (by RNA) in the etioplast. In the etioplast, plastid encoded chlorophyll binding proteins are synthesized despite the absence of Chl but proteins are degraded (Eichacker et al. 1990). Specifically, chlorophyll *a* synthesis is required to stabilize these proteins against degradation (Eichacker et al. 1990). Interestingly, the D2 protein appears as an exception to this rule (Müller and Eichacker 1999). The D2 protein accumulates in etioplasts at least in low amounts without the presence of Chlorophyll. D2 is a reaction centre protein of the PSII, and is crucial for its assembly. (Müller and Eichacker 1999).

Besides NADP: Protochlorophyllide oxyreductase (Weigel et al.), also the ATPsynthase (ATPase) the cytochrome (Cyt) $b_6f$ complex, could be isolated with the same molecular mass and subunit position as in chloroplasts in the etioplast (Griffiths 1978; Reisinger et al. 2008a). Cyt $b_6f$ is shown to bind chlorophyll in the chloroplast however, in the chlorophyll lacking etioplast, the pigment stabilizing the chlorophyll binding protein complex Cyt $b_6f$ is the phytyllated chlorophyll precursor, protochlorophyll *a* (Reisinger et al. 2008a).

Parts of both photosystem I and II are already visible after one hour of illumination of the etiolated plant (Reisinger, personal communication). The LHC I and LHC II part of the PSI/PSII appear after two hours respectively (Dreyfuss and Thornber 1994; Mathis and Burkey 1987). After four hours all the complexes of the PSI/II are assembled (Reisinger, personal communication).

Early studies of the electron transport chain in barley showed photochemical activity of PSI and PSII after one and one and a half hour respectively, after illumination was started. However the activity of PSI combined with PSII, hence NADP production was not seen before after four hours, when PSI was able to reduce ferredoxin. The electron transport chain was found to be complete after four hours (Ohashi et al. 1989).

Figure 1.6 and 1.7 show pictures of silver stained second dimension SDS gels, containing etioplasts and chloroplasts respectively. It illustrates that the protein composition of the plastid membranes change qualitatively and quantitatively during deetiolation of etioplasts to chloroplasts.

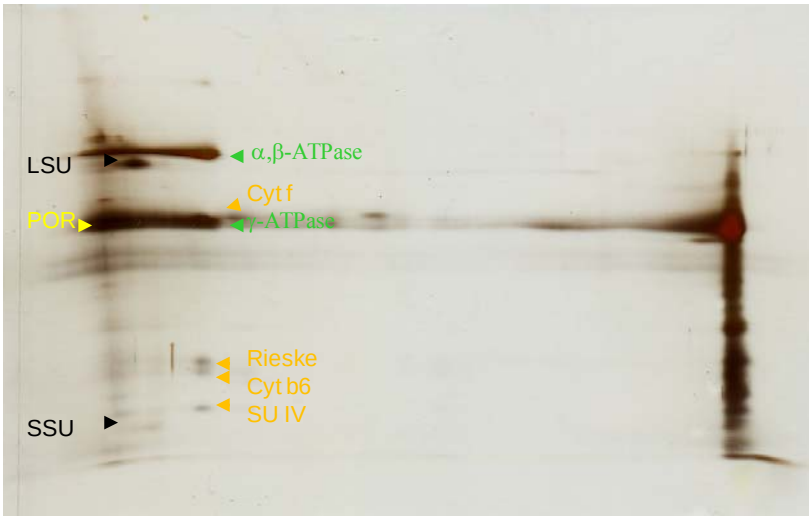


**Figure 1.6: Etioplast inner membranes of barley on a silver stained second dimension SDS gel.**

The colours orange and green indicate the units of the cytochrome $b_6f$, ATPase complexes respectively. POR is the NADPH: protochlorophyllide oxyreductase. The SSU and the LSU are the two subunits of the enzyme ribluose 1,5 bisphosphate carboxylase/oxygenase responsible for $CO_2$fixation in the plastid. The complexes were named after the proteomic mapping of Granvogl et al. (2006).

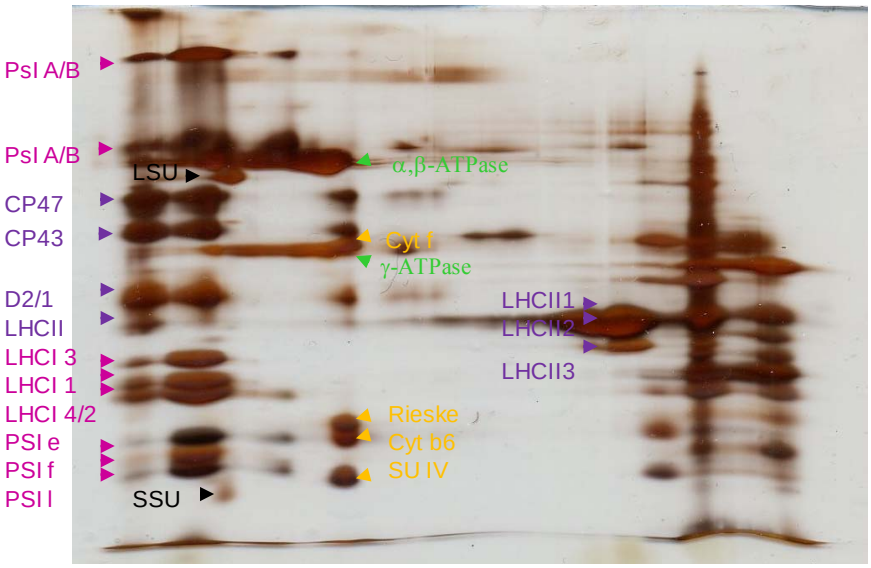


**Figure 1.7: Thylakoid membranes of barley on a silver stained second dimension SDS gel.** The colours orange, green, pink and purple indicate the units of the cytochrome $b_6f$, ATPase, PSI and PSII complexes respectively. The SSU and the LSU indicates the small- and the large subunit of the RubisCo, respectively. The complexes were named according to Granvogl et al. (2006).

### 1.1.11 The model plant barley, *Hordeum vulgare*

The plant used in this work is a cultivar of barley, (*Hordeum vulgare*) called Steffi. Barley is a member of the grass family (Brandstveit et al. 2004). It is a annual plant, which means that its growth cycle is over in one year (Brandstveit et al. 2004). The plant is grown for animal feed, and in central Europe it is used for production of malt in the brewing industry and in healthy food products (Brandstveit et al. 2004).

The reason for using barley in these experiments is its ability to germinate with high efficiency and to grow in complete darkness. In contrast to the graminaceae, dicotyledonous plants like Arabidopsis need light for germination and continued growth. Barley seeds are a rich source of starch and nutrients to enable efficient growth for up to nine days in darkness without any light and the supply of any growth media. After 4.5 days of growth in darkness, plants can be harvested during a phase of constant growth. (Eichacker, personal communication).

### 1.1.12 The Lhc super-gene family

The light harvesting chlorophyll (LHC) binding proteins are represented by the LHC I and II (Adamska et al. 1999). Ten types of LHC proteins have been recognized in higher plants. Accumulation of LHC proteins are known to be controlled by light (Mathis and Burkey 1987). LHC proteins represent the antenna system of the photosystems (Jansson 1999). The corresponding genes have been named Lhc genes. The Lhc super-gene family encodes the light harvesting chlorophyll *a*/b-binding (LHC) proteins. Some of them are part of photosystem I (PSI) and some are part of photosystem II (PSII). (Jansson 1999)

All LHC proteins have three membrane-spanning helixes where helix one and three are homologues. The helices have a *LHC motif* region consisting of 22 amino acids. The majority of the pigments like the chlorophyll *a* and *b* and also some carotenoids bind to this region. The LHC motif is highly lipophilic. (Jansson 1999).

Lhc genes also code for some proteins where the function is more or less unknown (Jansson 1999). Two proteins with homology to the LHC proteins are the early light-inducible proteins (ELIP`s) which have previously been found in higher plants. The ELIP`s belong to the family of light-harvesting-like (Lil) proteins. The function of the family of Lil proteins is only partly known. Some of the proteins are thought to be associated with photosystem II, and they have the LHC motif in their sequence like all LHC proteins (Jansson 1999).

Lil proteins have also been linked to protection against photo-oxidative stress (Jansson 1999; Klimmek et al. 2006). mRNA levels increased for all the proteins in the Lil family under high light conditions, except for one of them where mRNA levels remained constant (Jansson 1999). The mRNA encodes for the Lil3 protein, which was therefore thought to have an another function in the plant plastid (Jansson 1999).

### 1.1.13 Lil3

In *Arabidopsis,* Lil3 is a 262 amino acid protein, homologue to the LHC proteins (Jansson 1999). Recent experiments in barley showed that after the illumination of dark grown seedlings, two new complexes containing Lil3 are de novo

accumulated. Light induced synthesis of chlorophyll therefore appears to trigger the assembly of the Lil3 protein complexes (figure 1.8, labelled as complex 1 and 2) (Reisinger et al. 2008c).

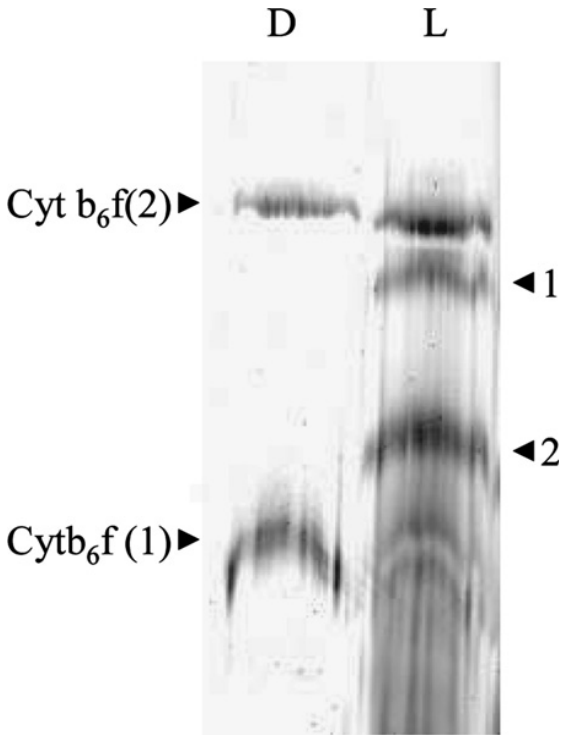


**Figure 1.8**: **The assembly of the Lil3 complexes after illumination of etiolated plants**
The complex 1 and 2, hence the Lil3 complexes assemble in the plastid by the exposure to light (L). The complex was not seen in the etioplast (D), kept in the dark. Cyt $b_6f$ is also seen in the figure. (1) and (2) mark the monomeric and dimeric cytochrome *b6f* complex, respectively **(Reisinger et al. 2008c)**.

As pigment binding complex subunit exclusively Lil3 was identified in both complexes. The complexes were determined with a molecular mass of 210-250 kDa and 160-180 kDa respectively. Lil3 complexes were not assembled in the etiolated state (D). Absorbance spectroscopy and thin layer chromatography showed that both de novo assembled complexes bind the pigments Chl. Also, Pchl (protochlorophyll) and carotenoids were identified. The function of the Lil3 is not clear at the present time. It has been discussed that the complexes may function as an intermediary pigment storage complex, between the chlorophyll synthase and other pigment binding proteins (Reisinger et al. 2008c).

## 1.2 Methods

In the following experiments, several separation and identification methods for proteins were used. For extraction of the proteins, the plastids were first isolated from the leaves. Membrane proteins were then isolated from the thylakoid membranes and separated by native gels, SDS gels or a combination of these methods.

The proteins were then visualized by directly staining of the gels, or identified by antibodies after Western blotting. Lil3 was also identified in the SDS-gel by mass spectrometry. The assembly of Lil3 complex was studied in colorless native gels by antibody and auto-fluorescence scan.

In the quantification of the Lil3 the proteins were isolated from the leaves by grinding. Antibody was used for the detection of the protein in SDS-gels. The signal strength was analyzed by a program, able to convert the signal to a numeric report. The signals were then compared between different stages of deetiolation by graphic illustration in Excel.

### 1.2.1 Isolation of plastids and the thylakoid membrane

The protocol for plastid isolation was described by Klein *et al.* (1986) and modified by Eichacker *et al.* (1990; 1996b). It is important to carry out all the isolation steps on ice (Klein and Mullet 1986; Eichacker et al. 1990) to prevent loss of enzymatic plastid activity and degradation of proteins.

The harvested plants are first homogenized in isolation medium to release the plastids from the leaf cells. The homogenate is then filtered through 22 µm pores, to separate the plastids from plant cells and cell wall fragments. Plastids are then centrifuged three times. First they are centrifuged to separate them from cell debris and organelles of low density of the plant cell. Plastids are then centrifuged through a Percoll gradient to separate the broken and intact plastids (Klein and Mullet 1986) fig 1.9.

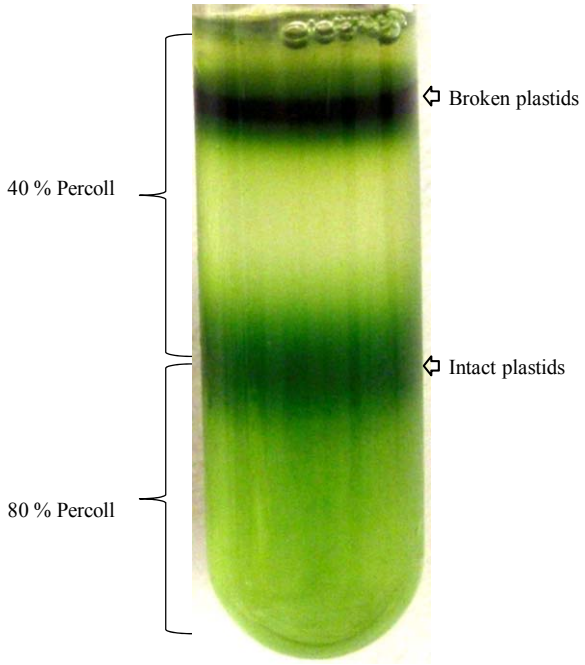


**Figure 1.9: Isolation of intact chloroplast by Percoll density centrifugation**
The intact plastids were found between the 40 % and the 80 % Percoll layer after centrifugation. The broken plastids were on top of the Percoll solutions.

In the Percoll gradient, intact plastids will be concentrated on the top of the 80 percent Percoll layer, because of the high density in the 80 percent Percoll. The broken plastids however, are not able to get through the 40 percent Percoll layer, since they have lost the plastid stroma and are of lower weight. In the end plastids are centrifuged in a washing medium to separate them from the Percoll. After final centrifugation the plastids are found in a pellet since the Percoll has been diluted strongly.

All solutions used for isolation of plastids consist of D-sorbitol, Hepes and EDTA (Klein and Mullet 1986). The D-sorbitol is used to keep the same osmotic pressure in the solutions relative to the interior of the chloroplast. The Hepes buffer is used to maintain the pH stable at 8.0 which correspond to the internal pH of the plastidic stroma (Jagendorf and Uribe 1966).

After isolation plastids are resuspended in a low volume and counted in a haemocytometer. Counting of the isolated plastids is necessary for loading a known amount of plastid proteins on a gel which is then used for the separation of proteins. Instead of counting, the amount of proteins could also be determined

colourmetrically by Bradford test or by chlorophyll determination. But in etioplasts there is no chlorophyll present and the amount of chlorophyll is increasing during the deetiolation in the plastids (see sec 1.1.9), therefore it is not suitable for a comparison of different developmental stages like in the following experiments. In contrast to plastid counting, the Bradford test (1976) for determination of plastid protein content is much more time consuming and is not working very precisely with membrane proteins. For these reasons, plastids are counted in a Thoma counting chamber designed for counting blood cells.

The volume of plastid solution needed from the isolation will vary due to difference in the concentration of the isolated plastids. If the isolation is successful a density of about 1 x $10^7$ plastids per micro liter will be obtained. Plastids are first diluted 500 times to make the counting more easily. The number of plastids counted in four squares (P) (typically around 100), is put in to following formula to calculate the amount of plastids in one micro liter; Px4x500x10. P is multiplied by four, because the counting chamber has 16 squares in total. It is then multiplied by 500 due to dilution before counting. Last it is multiplied by ten since the volume of the 16 squares corresponds to 0.1 µl, to get the amount of plastids in one micro liter. 1x$10^8$ plastids are used for one sample in the electrophoresis. Hence to find the volume of isolated plastids needed for one sample, 1x$10^8$ is divided by the amount of plastids in one micro liter.

After the counting, 1x$10^8$ plastids are dissolved in 1xTMK-buffer for lysis of the outer membranes as the TMK-buffer is hypo-osmotic compared to the plastid stroma (Müller and Eichacker 1999). The plastids are then incubated for at least ten minutes on ice to enable the lysis. After the incubation, the sample is spun down to remove the envelope and soluble stromal proteins of the plastid. The membranes are then washed two times in 1xTMK. Two consecutive washing steps in TMK buffer are important to ensure that remaining soluble and peripheral proteins are removed. (Müller and Eichacker 1999).

After isolation of the thylakoid membranes, the proteins must be extracted from the membranes. This is the most critical step of the isolation. Here, different detergents are used for the various kinds of separations (Reisinger and Eichacker 2007).

### 1.2.2 Harvesting leaves for quantification of Lil3

For the quantification of Lil3, ground leaves are used instead of isolated plastids to simplify the experiments. The protocol is based on the plastid isolation explained in section 1.2.1 with a few modifications. Barley leaves are illuminated for a different length of time: E, 10s, 1h, 2h, 4h and C. The leaves are then harvested. Instead of isolating plastids, the leaves are directly ground with a pestle in 1xTMK. The samples are further prepared by the protocol for SDS electrophoresis with a few modifications. The main difference is that centrifugation is carried out at a higher speed. This is to ensure that all the inner plastid membranes are pelleted and only the soluble plant cell material is removed during the single sample preparation steps, since the pellet tends to resuspend very easily from in ground leaf samples.

### 1.2.3 Protein solubilisation

For the extraction of native membrane complexes from membranes, non ionic detergents are normally used. All detergents are amphiphilic, meaning that they have both hydrophilic and hydrophobic abilities (a hydrophilic head group and a hydrophobic tail) which enables the formation of micelles in an aqueous solution (Reisinger and Eichacker 2008). The solubility of the detergents depends on the chemical properties of these parts separately. If the hydrophobic tail is long the detergent is generally believed to be less harmful for dissociation of protein complexes. The head group of the detergent largely determines the solubility of the whole detergent molecule in the buffer system, whereas the hydrophobic tail is the driving force for the interaction of the single detergent molecules. It is the balance between the strength of the interaction of these groups with water molecules that makes detergent form micelles at different concentration and sizes in an aqueous solution (Reisinger and Eichacker 2008).

Micelles are small “molecules” where the hydrophobic tails of the detergent are arranged together protected by the hydrophilic head. When the membrane proteins are correctly solubilised they will form a micelle containing one protein complex per micelle. Important for the solubilisation is the critical micelle concentration (CMC) of the detergent (Garavito and Ferguson-Miller 2001; Reisinger and Eichacker 2007).

If the concentration of the detergent is higher than the CMC, micelles start to form. The lower the CMC value, the more stable micelles can be retained during complex isolation which is important to keep membrane proteins dissolved (Reisinger and Eichacker 2007). The CMC and thereby the solubilisation, can be affected by the type of detergent and its concentrations, the sample buffer, the salts, the temperature and the forces applied. These factors need to be adjusted experimentally in different biological systems to get the best conditions (Reisinger and Eichacker 2007).

For the blue native gel, ACA-buffer and n-dodecyl-β-D-maltoside *(DM)* detergent is used. DM is a nonionic detergent, which means that it will not be charged at different pH values. Detergents are thought to most likely break the lipid-protein and the lipid-lipid interactions. However, also the interactions between the proteins can be affected dependent on the protein structure and chemical composition of the detergent. In the BN samples, Coomassie, a negative molecule used to charge the protein micelles is added. This gives a negative charge to the proteins (Reisinger and Eichacker 2008, 2007).

Clear native (CN) electrophoresis has been described as useful for further analysis of fluorescence in the protein complexes after electrophoresis (Wittig and Schägger 2005), since Coomassie blue has been shown to interfere with some detection methods (Wittig and Schägger 2005). For the clear native gels of this experiment, the LDS-system set up by Reisinger et al. (2008b) was used. “In comparison to BN-PAGE it is compatible with spectroscopic methods enabling analysis of fluorescent complexes after electrophoresis” (Reisinger et al. 2008c). In the LDS-system 1xTMK buffer and detergent mix are used. The detergent mix consists of lithiumdodecylsulfate (LDS), DM and digitonin (DIG). The running buffer of the LDS system is clear, since no Coomassie is added. Without the

Coomassie blue in the buffer, the charge must be added in another way. The anionic detergent LDS is therefore used. This detergent is also used in the samples for directly adding charge to the proteins. A very low amount is used because LDS it is very efficient detergent, which may solubilize the proteins completely by destroying the complexes. The LDS is therefore combined with DM and DIG. A combination of digitonin and DM is used because it turned out by practical approaches that this combination solubilizes the sample almost completely and keeps the complexes intact at the same time. Digitonin alone is not able to solubilize the sample completely, (it is one of the mildest detergents known) and therefore it is not so efficient. If only the DM was used, some complexes were destroyed. (Reisinger et al. 2008a).

For SDS-PAGE analysis, thylakoid membrane samples are resuspended in 1xTMK-buffer and 3xSB buffer. The buffer contains sodium dodecyl sulfate (SDS) and dithiothreitol (DTT). SDS is a anionic detergent which opposed to the nonionic detergents (used for the native electrophoresis) breaks the protein-protein interactions of the complex (Reisinger and Eichacker 2008). The SDS in the 3xSB disrupts the non-covalent bonds in the proteins. It binds to the backbone of the aminoacids in the protein. DTT is added to break disulfide bonds in the protein structure. Finally, the sample is heated to completely denature the proteins .(Westermeier et al. 1997).

In the CN-gels the complexes have been found to move less far indicating a higher molecular mass (Wittig and Schägger 2005). This is also true for the LDS-detergent system, indicating that the low content of LDS does not charge the micelles as effectively as the Coomassie in the blue native, and therefore the complexes carry less charge. However, the charge density in the system cannot be increased since LDS is a very strong detergent which otherwise would destroy the protein interactions of the complexes. This was explained by Reisinger, who setup the LDS-system. (Reisinger et al. 2008a)

### 1.2.4 Electrophoretic separation

Electrophoresis means the transport of charged particles, like proteins along an electric field gradient (Eichacker and Reisinger 2007). In native and SDS-PAGE

(sodium dodecyl sulfate-polyacrylamide gel electrophoresis) the proteins/complexes are separated according to their mass to charge ratio. An excess amount of negative charges is added to the protein or protein complexes, the internal charges are masked and the protein are separated relative to each other according to their different molecular masses while they move towards the anode in the electric field. Smallest fragments with a low molecular mass travel faster than the bigger molecules with high molecular mass. The protein separation is proportional with the charge of the protein and the strength of the electric field. It is inversely proportional to the size/mass of the protein and the viscosity of the separation gel (Reisinger and Eichacker 2007). Small proteins with less size and resistance in the gel therefore travel faster than the larger proteins. This results in a gradual separation due to the size/mass of the proteins (Reisinger and Eichacker 2007). Electrophoresis is carried out at a constant temperature of 4 °C for the Native and 15°C for the SDS. This ensure that heat generated during the movement of the ions is constantly removed (Reisinger and Eichacker 2007, 2008).

### 1.2.5 SDS-PAGE

The SDS-PAGE was invented by U.K. Laemmli (1970). The method was then used for cleavage of proteins in the bacteriophage T4.

In SDS-PAGE, a negative charge is applied to the proteins by the loading of the SDS. 1.4 g SDS bind to about one gram of protein and the charge/mass ratio is therefore equal in all proteins. The proteins can now travel towards the positively charged anode guided by the field gradient. Hence, separation in SDS-PAGE is brought about by the higher resistance of larger molecules to move through the pores of the PAA gel and therefore is highly dependent on the pore size of the gel. In SDS PAGE polyacrylamide gels are used for separation of proteins (Westermeier et al. 1997).

Polyacrylamide gels consist of acrylamide monomers, normally cross linked by bisacrylamide. The total amount of acrylamide and cross linking bisacrylamide is normally given in percentage. The pore size is dependent on the concentration of the cross linker and is smallest when the percentage of the

bisacrylamide is four percent. Higher and lower percentages of bisacrylamide give larger pores. The heavy proteins will travel slower than the small proteins because they are retained by small pores. The smaller proteins are, the faster they can travel trough the gel, and there is a separation due to protein size. When the gel is stopped after some time, the smaller proteins have travelled further than the larger ones (Westermeier et al. 1997).

The SDS-gels have two phases, the stacking and the separating gel. The stacking gel focuses all the proteins to one starting point. The pores are big and separation therefore is due to charge and largely independent of size. The effect of charge for the separation of the proteins in the stacking gel is strongly influenced by the pH of the stacking gel which is 6.8 in SDS-PAGE. This pH is close to the isoelectric point of glycine and glycine is therefore uncharged or even slightly cationic (tracking ion). Proteins travel in the stacking gel according to their charge obtained from binding SDS or by endogenous charge at this pH in native PAGE. In any case, the ionic proteins have a higher mobility than the glycine; although the proteins are much larger in size. However, a second factor is important during this separation phase. While the proteins start to move slowly into the stacking gel, chloride ions in the separation gel travel very fast to the anode and generate and additional field between the chloride free (leading ion) (lack of negative charges) separation gel and the neutral glycine molecules in the stacking gel. This field directs the movement of the proteins between the tracking and leading ion and proteins move with a constant velocity (iso-tachophorese). This will lead to a separation of the proteins according to their molecular mass, into thin and sharp layers in the stacking gel. However, the concentration of glycine at the border of the separating gel, will be slightly more anionic then within the stacking gel and now the negatively charged glycine molecules will take the lead overtake the proteins and slow down the protein movement. At the same time proteins hit the higher polyacryamide concentration of the separation gel and their movement is strongly retarded. Now the sieving effect of the polyacrylamide net is the dominating force for the separation of the proteins in the overall electric field of the electrophoresis system (Eichacker and Reisinger 2007)

The separating gel separates the proteins according to size/molecular weight. In the separation gel the pH is 8.8. At this pH a much higher percentage of the glycine will be ionized. This increases the mobility of glycine and decreases the mobility of the proteins. The glycine will accelerate through the concentrated layers of proteins, which then start to separate (Eichacker and Reisinger 2007).

SDS-loading buffer contain sucrose, Tris and SDS. SDS is added, to solubilize the proteins and to charge them negatively. The temperature at centrifugation and the electrophoresis is always kept at minimum 15ºC. In a lower temperature the SDS starts to precipitate. Sucrose or glycerol is added to simplify the application of the sample in the stacking gel wells and Tris is added to keep the pH constant. SDS electrophoresis can be used as both two-dimensional and one-dimensional separation technique (Schägger and Jagow 1991).

### 1.2.6 Native PAGE, polyacrylamide gel electrophoresis

The protein complexes of the chloroplast can be separated by native electrophoresis. The native electrophoresis separates protein complexes in the native state (thereby the name). The blue native electrophoresis as one method of native gel electrophoresis was first described by Schägger and Jagow (1991).

The native electrophoresis gel consists of two phases; a stacking gel and a separating gel. The stacking gel focuses the proteins by size and the separation gel separates the proteins by the same principle as the SDS electrophoresis. Due to the negative charge mediated by the blue Coomassie dye molecule, a charge shift is imposed which leads to migration of the charged proteins to the anode during electrophoresis (Reisinger and Eichacker 2008). In CN gels this charge is induced by LDS (Reisinger et al. 2008a).

“The cathode buffer is supplemented with Coomassie to ensure constant binding of Coomassie to the proteins and mobility of the proteins” (Reisinger and Eichacker 2008). The cathodic dye buffer is replaced by a non coloured cathode buffer when the running front is half way through the electrophoresis. This minimizes the background staining and is important for immunodetection of protein complex (Reisinger and Eichacker 2008).

### 1.2.7 Combination of native and SDS electrophoresis

The native electrophoresis was combined with a second dimension SDS electrophoresis according to the method of Schägger et al. (1994). This combination gives the opportunity to get a very high separation capacity for membrane proteins and to get some information about the interactions of the proteins. The protein complexes are first separated in a native electrophoresis. The SDS gel then separates the protein subunits of the single complexes. The proteins in the second dimension SDS-gel can easily be related back to the complexes in the native state (Reisinger and Eichacker 2007). The native gel-lane is put in solubilisation buffer prior to the SDS- electrophoresis, to break the non covalent bonds between proteins in the complexes (Reisinger and Eichacker 2007).

### 1.2.8 Coomassie staining

For Coomassie staining the protocol of Neuhoff et al (1988) was used. The purpose of the fixing is to denature and precipitate the proteins in large insoluble aggregates within the gel. Coomassie dye bind to proteins by Van der Waals attractions, as well as ionic interactions between the dye sulfonic acid groups and the amine group of the protein. The gel is destained to get a clear background (Eichacker and Reisinger 2007).

### 1.2.9 Silver staining

The method of silver staining used in the experiments was developed by Blum et al. (1987). Silver staining results in immobilization of a small number of silver ions by the proteins in the gel. The silver is reduced by the amino acid side chains of the proteins, creating an amine silver complex (Eichacker and Reisinger 2007).

### 1.2.10 Immunodetection

The electrophoretic blotting procedure (the transfer of protein from polyacrylamide gels to a nitrocellulose membrane) was first described by Towbin et al. (1979). The transferred proteins were detected by immunological procedures (thereby the name immunodetection). The excess binding sites in the membrane was first blocked by a protein. The transferred proteins were then detected by a first and a second antibody. The second antibody was either radioactively marked

or conjugated to a peroxidase or a fluorescein (Towbin et al. 1979). The immunodetection of protein is also known as Western Blot. The name western blot was given by Neal Burnette and is related to Southern blot developed earlier by Edwin Southern. Towbin improved the solubilisation of membrane proteins for transfer by the addition of 20 % methanol.

The proteins migrate out of the gel and onto a membrane by electric current. The nitrocellulose membrane should never dry out. The solutions must not be poured directly on the blot and the blot should only by handled by a pinsetter. This is important in order to avoid contamination by other proteins or removing of the attached proteins. After the blotting the blot is stained in Ponceau to fix the protein to the membrane and to check the success of the blotting process.

After the transfer the blot is blocked by protein (five percent milk in this experiment) to reduce non-specific protein interactions between the membrane and the antibody. After the blocking, the membrane is incubated in the first antibody. Antibodies are proteins with receptors or binding sites for specific antigens. Primary antibodies bind to the target protein (Towbin et al. 1979). In the following experiments the detection of the Lil3 proteins was carried out by using a Lil3 primary antibody. The antibody was made prior to this experiment. The Lil3 antibody was constructed to bind an amino acid sequence previously found in Lil3 by Reisinger et al. (2008c).

Secondary antibodies are constructed to bind to the primary antibodies (Towbin et al. 1979). Secondary antibodies can be used to detect different primary antibodies, and can therefore be used in several different experiments. In these experiments HRP- (horseradish peroxidase) or Cy3- linked secondary antibody is used. The HRP is detected by a film, the Hyperfilm ECL (High performance chemiluminescence film). HRP catalyze the oxidation of luminol, which results in the emission of light. Hyperfilm ELC (enhanced chemiluminescence) is a blue-light sensitive film that is used to detect the emission from this reaction (wavelength of 428 nm). The Cy3-linked antibody is detected in a Typhoon laser scanner. (Eichacker and Reisinger 2007).

### 1.2.11 Scanning gels and blots for auto-fluorescence

Auto-fluorescence scan is used to detect the chlorophyll binding complexes by the fluorescence of bound chlorophyll after excitation by the same principle as for the Cy3-dye, explained in the section 1.2.10. The gels/blots were scanned by the wavelength where the chlorophyll absorbs light (400-700 nm) (see section 1.1.4).

### 1.2.12 Mass spectrometry

Mass spectrometry has a wide range of application opportunities. It is used for analyzing structures of inorganic and organic molecules, elemental compositions, rates of atomic isotopes in samples and composition of mixtures. The method was first developed in the petroleum industry to analyze mixtures of hydrocarbons in the oil. It is now also widely used among chemists and biologists for the studying of for instance, the composition of proteins and molecules. In the following experiment MS is used to identify Lil3 by looking for special peptides expected to be found in this protein. (Skoog et al. 2007).

In MS, the analyte (in this case the peptide solution from the protein) is vaporized. Based on an electric field collision and a coloumbic explosion of the analyte droplets shrinking by solvent evaporation, charged analyte species are generated. The positively charged molecular ions that enter the mass spectrometer can then be fragmented by interactions with the molecules of the collision gas. (Skoog et al. 2007).

The fragmentation can be influenced by the energy applied to accelerate the analyte ions. If the applied energy is too high, the peptides in the sample may be completely broken down. Within the MS the positive ions produced can be sorted according to their mass/charge ratio. The numbers of molecules accumulated with a specific m/Z value are then presented in a mass chromatogram. In the mass spectrum, the largest peak, the base peak, is given the value of 100. The heights of the other peaks are presented in a percentage of this peak. (Skoog et al. 2007).

The formation of gaseous ions can be done by different ion sources. The ion source highly influences the appearance of the mass spectra. There are two different categories of ion sources; gas-phase and desorption sources. In the gas-

phase ion source, the sample is first vaporized then ionized. The formation of gaseous ions can be done by different ion sources. The ion source influences the appearance of the mass spectra. Electro spray ionization/mass spectrometry (SEE/MS) is the ion source used in this experiment. SEE is a desorption ion source, meaning that the sample is directly converted from liquid to gaseous ions. It is one of the most important techniques for analyzing biologic molecules like peptides and proteins in a size of 100 Da or more. ESI takes place under atmospheric pressure and temperatures. The solution is pumped through a stainless steel capillary needle, by only a few micro liters per minute. The spray is charged because the needle is surrounded by an electrode which maintains the needle at a charge of several kilovolts. Charged spray is evaporated in a capillary where the attachment of the charge to the analyte molecules also happens. The charge density increase as the droplets of the spray becomes smaller. The ions formed in ESI can be detected within a mass/charge (m/z) range of 1500 or less. (Skoog et al. 2007).

The type of mass spectrometer used in this experiment was a Q-TOF premier. This is a combination of two types of MS methods thereby the name MS/MS. By combining two types of MS, the resolution in the MS spectra is improved. The Q stands for Quadruple mass analyzer. A quadruple MS has four rods of electrodes. The number may vary, as for eight rods or less. The ions are accelerated trough the space between the rods. Only the ions with a certain m/z get through the "tunnel" of electrical fields. The others are trapped by crashing on the electrodes. In the TOF (time of flight) the positive charged ions are accelerated by an electric pulse in a tube and separated by the time they need "to fly" to the detector. The ions with a high m/z ratio will be accelerated less before traveling down the TOF than the ions with a lower m/z rate. (Skoog et al. 2007).

In the following experiment MS was used to identify Lil3 by looking for specific peptides found in this protein known from former experiments. To prepare the protein for mass spectrometry, it must be released from the SDS-gel, and digested into peptides to be able to perform de novo sequencing. The digestion of proteins by enzymes was described in by Wilm et al. (1996). It is a very time consuming process that takes around 20 hours. However the method

was recently shortened by around 18 hours by the help of a small reactor; the *OMX-S*® described by Granvogl et al. (2007). The digestion of the Lil3 in this experiment was performed by this protocol.

After destaining the gel was centrifuged with the reactor side of the OMX-S® down in the centrifuge, to fragment the gel into small pieces and to get the dispersed gel into the reactor chamber. After adding the trypsin to the reactor the OMX-S® was shortly centrifuged with the reactor faced down in the centrifuge to spin the trypsin solution in the reactor chamber. For digestion of the proteins, the trypsin incubated proteins were put on a shaker at 50 degrees. This temperature was found to accelerate protein cleavage, despite of reduced incubation time compared with the earlier method (Granvogl et al. 2007). In the end the peptide solution was separated from the gel pieces by centrifuging the reactor with the sampler faced down.

A ZipTip® micro column was used for cleaning up the peptide solution before transferring the peptides to the mass spectrometer according to the protocol provided by the Millipore Company. The column was activated by pipetting of acetonitrile and equilibrated by formic acid respectively. Peptide solution was then pulled trough the column to attach the peptides in the column. Formic acid was used for removing salts and other contaminants from the peptide solution bound to the column. Elution solution is used to elute the peptides from the column.

### 1.2.13 Analyzing protein amounts by Tina program and Excel

A standard curve was first made from known amounts of plastids. This was carried out by loading known amounts of plastids on a SDS gel. The films from the respective blots were analyzed in the TINA 2.0 software to relate the amount of plastids to the signal strength of the proteins on the film. A standard curve was then made in Excel.

The films where the proteins were detected by Lil3 antibody were scanned and converted to TINA 2.0 files. The program made a numeric report of the optical density in all regions. The higher the number, the higher was the optical density. The optical density in each region was related to the intensity of the

signal in the band. This number could therefore be calculated back to the signal strength of the antibody, hence to the protein amount in each band. The region report was exported to Microsoft Excel 2007. In Excel, the numbers from each band at every greening stage were put together in a graph. The middle value and standard deviation were calculated. The background and the signal strength could vary between the different films due to the development of the film. This difference was reversed in the calculations in Excel.

In the quantification experiments three parallels from three different greening stages were loaded on each gel. The films from the SDS-gels with three different deetiolation stages were analyzed by TINA 2.0. The parallels of the quantification should be compared to the standard curve. It should then be possible to relate the strength of the signal in the different developmental stages back to the amount of plastids in each leaf. The standard curve should also be used to show if there was a correlation between signal strength and amount of proteins. In Excel the middle value of the etiolated leaves therefore was calculated and used as 100 percent. Middle values of the other stages, hence the ten second-, one hour-, two hour- and four hour illuminated, as well as the green leaves were compared to that of the etiolated leaves. The percentage difference in signal between the stages of deetiolation was then calculated. The data was analyzed considering standard deviations.

## 1.3 Objectives of the thesis: quantification of Lil3 proteins

Little is known about how the plants are able to turn from heterotrophic to photoautotrophic metabolism. In order to understand this dramatic change completely, more information about the protein complexes involved is needed. Lil3 is one of the few complexes assembled right after light exposure (Reisinger et al. 2008c). Since Lil3 is assembled so early in the deetiolation, it may have a crucial role in the metabolic shift of the plant(Reisinger et al. 2008c). It has been suggested that Lil3 has a role as a temporary pigment storage complex, between the chlorophyll synthase and other pigment binding proteins (Reisinger et al. 2008c). The amount of protein may therefore increase as pigments accumulate during the deetiolation.

In the following experiments, the Lil3 protein was studied by looking at the assembly of the protein during deetiolation in barley leaves. The protein amount was quantified in several stages of the greening period. The proteins were identified by Lil3 antibody and the ECL system. Signal strength of the antibody was then related back to the amount of Lil3 protein present in each stage.
The following questions were asked: Is the amount of Lil3 protein changing during deetiolation? Is an increase observed? Is there a change in the assembly stages of the Lil3 complexes during greening?

# 2 Material and methods

## 2.1 Chemicals

The chemicals where of p.A.-quality and applied from Applichem, Arcus, Fluka, GE Healthcare, J.T. Baker, Merck, Roth, Serva, Sigma and VWR.

## 2.2 Plant material

Barley, *Hordeum vulgare L*, cultivar Steffi Saatzucht Ackermann & Co, Irlbach

## 2.3 Antibodies

Lil3 ab (from rabbit) Innovagen, Lund

Anti rabbit IgG, HRP-linked F(ab')$_2$ Fragment (from donkey) GE healthcare, Uppsala

Anti rabbit IgG, Cy3-Linked (from goat) GE healthcare, Uppsala

## 2.4 Molecular mass standard

Low molecular weight (LMW) marker for SDS page

| Protein | kDa |
|---|---|
| Phosphorylase b | 97 |
| Albumin | 66 |
| Ovalbumin | 45 |
| Carbonic Anhydrase | 30 |
| Trypsin inhibitor | 20.1 |
| α-Lactalbumin | 14.4 |

## 2.5 Instruments

| | | |
|---|---|---|
| Block thermostate | HBT 130 | Biotech, Bovenden |
| Blot apparatus | Nova Blot Kit (Multiphor) | GE Healthcare,Uppsala |

| | | |
|---|---|---|
| Centrifuge | 5415R | Eppendorf, Hamburg |
| | Sorvall RC-5B | S. Dupont, Wilmington |
| Electroph. power supply | EPS3500/EPS3501XL | Biotech, Freiburg |
| Electrophoreis unit | 2059 Midget | LKB, Bromma |
| Gel dryer | Gelairdryer | Bio Rad |
| | Slab gel dryer GD2000 | Hoefer, San Fransisco |
| Homogenisator | Ultra thurax PT 10-35 | Kinematica |
| Incubators | Rumed thermostate cabinet | Rubarth Apperate |
| Magnetic stirrer/hot plate | TypMRII | Heidolph, Schwabach |
| Mass spectrometer | Maldi Q-TOF Premier | Waters, Eschborn |
| Microscope | Leitz Diavert | Leitz Wetzlar, Wetzlar |
| PH-meter | inoLAB pH level1 | WTW, Weilheim |
| Refrigerated Cirulator bath | K20 | Haake, Karlsruhe |
| Rocking platform | WT15 | Biometra, Gottingen |
| Scanner | Epson perfection 1640 SU | Epson, Meerbusch |
| | Typhoon Trio | GE Healthcare,Uppsala |
| Shaker | Kl 2 | Bachhofer, Reutlingen |
| Software | Image Quant 5.0 | GE Healthcare,Uppsala |
| | Microsoft Excel | Microsoft corporation |
| | TINA 2.0 | Softpedia, Bucharest |
| | MassLynx 4.0/4.1 | Waters, Eschborn |
| Thermomixer | HTM 130L | Biotech, Bovenden |

## 2.6 Growing conditions

The plants used in this experiment were barley, *Hordeum vulgare L*. The barley seeds were planted on a three cm layer of vermiculite. A layer of 0.5 cm vermiculite was also covering the seeds. The seeds were incubated at 25˚C for 4.5 days in a light tight incubator. The protocol was based on the method by Klein et al (1986), modified by Eichacker et al (1990).

For the deetiolation, the different groups were exposed to light for varying length of time, hence ten seconds (10s), one hour (1h), two hours (2h) and four hours (4h). Green chloroplast containing leaves were grown in a light (50μE/$m^2$s)

incubator. The isolation of the etiolated plastids and membranes were carried out in complete darkness. For the other groups the isolation process was carried out in light.

## 2.7 Harvesting leaves for quantification of Lil3

The protocol was based on the method for the isolation of plastids. Barley leaves were illuminated for different length of time (sec. 2.1). Leaves were harvested for the following illumination points: E, 10s, 1h, 2h, 4h and C. The leaf was cut two centimeter from the top. The upper first centimeter was discarded, only the second centimeter was used. Three different leaves were used in each sample.

The leaves were transferred to a micro centrifuge tube containing 50 micro liters 1xTMK-buffer. Leaves were ground completely with a plastic pestle. After grinding, 50 micro liters 1xTMK-buffer were used for rinsing the pestle to collect all the plant material. The samples were further processed by the protocol described in section 2.10.

### 2.7.1 Solutions

| | |
|---|---|
| 10 xTMK buffer | (20 ml) |
| 100 1M Tris/HCl, pH 8.5 | 2 ml (1M Tris/HCl pH 8.5) |
| 100 1M $MgCl_2$ | 2 ml (1M $MgCl_{2)}$ |
| 200 1M KCl | 2 ml (2M KCL) |

## 2.8 Isolation of plastids

The protocol for the plastid isolation was described by Klein et al (1986) and modified by Eichacker et al (1996a) and Muller et al (1999). All the steps during the isolation were performed on ice. Seedlings were cut one cm above the seed. After cutting, the leaves were directly put in isolation medium where they were cut into pieces of one cm. The plants were then homogenized with an ultra thorax. The homogenate was filtered through four layers of gauze bandage and one layer of nylon gauze with a pore size of 22 µm. After filtering to a 500 ml centrifuge beaker, the liquid was centrifuged for two minutes (4100xg, 4˚C) in the RC-5B

centrifuge. The supernatant was discarded and the pellet was resuspended on ice in the remaining isolation medium.

The sample was then filtered through the nylon gauze (pore size 22 µm) to a Corex glass tube with a two-step Percoll gradient. The upper layer in the glass-tube contained a 40 % Percoll solution and the lower layer contained an 80 percent layer. After centrifuging the samples for eight minutes (4100xg, 4˚C) the intact plastids were found between the two concentration layers. Intact plastids were transferred by a pipette to a clean Corex glass-tube. The tube was filled up by washing medium and centrifuged for three minutes (4100xg, 4˚C). The supernatant was discarded and the pellet was resuspended on ice. Intact plastids were transferred to a micro centrifugation tube.

### 2.8.1 Solutions

| | |
|---|---|
| 1M Hepes/KOH pH8.0 | (500 ml) |
| Hepes | 119.2 g |
| KOH | 21 g |
| 0.5 M EDTA-disodium | 18.615 g |
| 1M KOH | to pH 8.0 |

| | |
|---|---|
| Isolation medium | (1000 ml) |
| 400 mM D-sorbitol | 72.88 g |
| 50mM Hepes/KOH pH 8.0 | 50 ml (1M Hepes/KOH pH 8.0) |
| 2mM EDTA | 4 ml (0.5 M EDTA) |

| | |
|---|---|
| 40% (v/v) Percoll | (120 ml) |
| Percoll | 48 ml |
| 400 mM D-sorbitol | 8.74 |
| 50mM Hepes/KOH pH 8.0 | 6 ml (1M Hepes/KOH pH 8.0) |
| 1 mM EDTA pH7.5 0.5 M | 240 µl (0.5 M EDTA) |

| | |
|---|---|
| 80 % (v/v) Percoll | (60 ml) |
| Percoll | 48 ml |
| 400 mM D-sorbitol | 4.37 g |
| 50 mM Hepes/KOH pH 8.0 | 3 ml (1M Hepes/KOH pH 8.0) |
| 1mM EDTA pH7.5 0.5 M | 120 µl (0.5 M EDTA) |

| | |
|---|---|
| Washing medium | (1000 ml) |
| 400 mM D-sorbitol | 36.4 g |
| 50mM Hepes/KOH pH 8.0 | 25 ml (1M Hepes/KOH pH 8.0) |

### 2.8.2 Counting of plastids

Two micro liters of the isolated plastids were diluted with 998 micro liter of washing medium. Ten micro liters of the diluted plastids was transferred to a Thoma counting chamber. The plastids were counted in four squares.

The number of plastids in four squares P (typically around 100), was put in to following formula to calculate the amount of plastids in one micro liter; f.ex100 plastids x4x500x10. $1x10^8$ plastids were used for one sample. To find the volume of isolated plastids needed for one sample, $1x10^8$ was divided on the amount of plastids in one micro liter.

## 2.9 Isolation of the inner membrane proteins

All steps of the sample preparation were performed on ice. $1x10^8$ plastids were transferred to a microcentrifugation tube. The plastids were first centrifuged for three minutes (7500xg, 4ºC). The supernatant was discarded and the plastid pellet was resuspended in 100 micro liters 1xTMK-buffer (pH 8.5 for clear native gels). Plastids were then incubated for ten minutes on ice. After the incubation, the sample was spun down for three minutes (7500xg, 4 ºC). The supernatant was discarded. In the next step (the washing step) the plastids were diluted in 1xTMK and centrifuged for three minutes (7500xg, 4 ºC). After centrifugation the supernatant was discarded and the pellet was resuspended in 1xTMK-buffer. The washing step was repeated twice.

The step of the isolation of the membrane proteins was different with respect to the kind of gel electrophoresis that the samples were to be used in. The protocols are therefore described separately in the following sections.

### 2.9.1 Isolating membrane complexes for blue native electrophoresis

For the blue native (BN) samples the pellet of $1*10^8$ plastids was resuspended in 70 micro liters ACA-buffer. Ten micro liters of n-dodecyl-β-D-maltoside (DM) was added for solubilisation of the membrane protein complexes. The sample was incubated for ten minutes on ice to solubilize the membrane proteins. At the end the sample was centrifuged for ten minutes at (16000xg, 4 ºC), to pellet the unsolubilised material. After the centrifugation, five micro liters of charging buffer was added to the sample. $2.117647*10^7$ plastids were loaded in each well.

#### *2.9.1.1 Solutions*

| | |
|---|---|
| ACA-buffer | (20 ml) |
| 750 mM ε-aminocaproic acid | 1.968 g |
| 50 mM Bis-Tris pH 7.0 | 2 ml (50mM Bis-Tris) |
| 0.5 M EDTA-$Na_2$ | 0.2 ml (0.5 M EDTA) |
| | |
| DM | (5 ml) |
| 10 % (w/v) n-dodecyl-β-D-maltoside | 0.5 g |
| | |
| Charging buffer | (5 ml) |
| 750 mM ε-amino caproic acid | 0.492 g |
| 5% (w/v) Coomassie-G 250 | 0.250 g |

### 2.9.2 Isolating membrane complexes for clear native electrophoresis

For CN gels, the pellet of $1*10^8$ plastids was resuspended in 70 micro liters 1xTMK (Tris Magnesium KCL) and ten micro liters detergentmix were added. The sample was then incubated for ten minutes on ice. It was then centrifuged for ten minutes (16000xg, 4ºC) to pellet the insoluble material. Then the supernatant was then ready for applying to the gel. $2.25*10^7$ plastids were loaded in each well.

*2.9.2.1 Solutions*

| | |
|---|---|
| Detergentmix | (92 µl) |
| Digitonin | 60 µl |
| DM | 30 µl |
| LDS | 2 µl |

| | |
|---|---|
| Digitonin | (5 ml) |
| 10 % (w/v) Digitonin | 0.5 g |

| | |
|---|---|
| DM | (5 ml) |
| 10 % (w/v) n-dodecyl-β-D-maltoside | 0.5 g |

| | |
|---|---|
| LDS charging buffer | (5 ml) |
| 750 mM ε-aminocaproic acid | 0.492 g |
| % (w/v) Lithium-dodecylsulfate | 0.5 g |

### 2.9.3 Isolating proteins for SDS electrophoresis

The pellet of $1*10^8$ plastids was resuspended in 60 micro liters 1xTMK-buffer and 30 micro liters 3xSB. The sample was then heated for two minutes at 72ºC. After the heating step, the sample was centrifuged for five minutes at (16000xg, 15ºC) to pellet unsolubilised material. $1.11*10^7$ plastids were loaded in each well.

## 2.10 Isolating proteins from ground leaves

The protocol was based on the method for preparation of samples for SDS electrophoresis (sec 2.9 and 2.9.5), with a few modifications. The centrifugation was performed at a higher speed, to be able to pellet all the plant material. Leaves were ground (see preparation in sec. 2.7), and spun down for three minutes (16000xg, 4ºC). In the washing step, 200 micro liters 1xTMK was added to the pellet and centrifuged for three minutes (16000xg, 4ºC). The washing step was repeated twice. The pellet was resuspended in 50 micro liters 1xTMK-buffer and 30 micro liters 3xSB. The sample was then heated for two minutes at 72ºC in the block thermostat. After the heating step the samples were centrifuged for five

minutes at (16000xg, 15ºC) to remove the excessive unsolubilised material. Ten micro liter of the supernatant was loaded in each well for the SDS electrophoresis.

### 2.10.1 Solutions

| | |
|---|---|
| 1xSB | (10 ml) |
| 2 % (w/v) SDS | 0.2 g |
| 10 % (w/v) sucrose | 1 g |
| 0.03 % (w/v) bromphenol blue | 0.0003 g |
| 66 mM $NaCO_3$ | 0.071g |
| 66 mM dithiothreitol | 0.103g |

## 2.11 Isolation of the stroma

Isolation of the stroma part of the plastid was easily done during the isolation of the thylakoid membrane (section 2.9). This protocol was followed to the centrifugation step, after the sample was incubated on ice for ten minutes. The supernatant is the stroma part of the plastid. Ten micro liters of the supernatant was added 20 micro liters 3xSB. The sample was heated for two minutes at 72˚C. It was then centrifuged for five minutes (16000xg, 15ºC) to pellet the external membranes and other insoluble material. The sample was then ready for loading to the SDS-gel.

### 2.11.1 Solutions

| | |
|---|---|
| 10 xTMK buffer | (20 ml) |
| 1M Tris/HCl pH 8.5 | 2 ml (1M Tris/HCL pH 8.5) |
| 1M $MgCl_2$ | 2 ml |
| 2M KCl | 2 ml |

| | |
|---|---|
| 1xSB | (10 ml) |
| 2 % (w/v) SDS | 0.2 g |
| 10 % (w/v) sucrose | 1 g |
| 0.03 % (w/v) bromphenol blue | 0.0003 g |
| 66 mM $NaCO_3$ | 0.071 g |
| 66 mM dithiothreitol | 0.103 g |

## 2.12 Native-PAGE

The protein complexes of the chloroplast were separated by native-PAGE (polyacrylamide gel electrophoresis). Two methods of native separation was used, hence the blue native PAGE described in by Schagger et al (1991) and the clear native PAGE or LDS-page described by Reisinger et al (2008a).

The glass plates and alumina-plates (10.1 cm x 8.2 cm), with spacers between (0.75 mm) were cleaned with ethanol and assembled in a casting chamber. Plastic clamps were attached to the chamber to seal the lid. The solution for the separating gel (section 2.12.1) was made by adding Temed and APS to the acrylamide to start the polymerization (under the hood). The gel solution was filled in the chamber between the glass/alumina sandwiches. Water saturated butanol (0.7 ml) was filled in on top of every gel (between the glass plate and alumina-plate) to make a straight surface. The gels were polymerized after one hour.

After polymerization the water saturated butanol was removed by water and the gels were carefully dried with filter paper. Clamps were then placed on each side of the gel. Agarose was filled in each of the corners of the gel to make sure that there was no opening between the gel end the spacer. A comb was put on top of the spacers to form the wells of the stacking gel. Each well was marked with a permanent marker for easily loading the samples after removing the comb. The stacking solution was then filled in with a pipette on top of the gel. The comb was carefully removed after for 15 minutes when the gel was polymerized.

The gel was attached to the electrophoresis unit. LDS-cathode buffer or 1xcathode buffer (blue) was filled in the upper chamber (cathode) for the clear native (CN) and the blue native gels, respectively. The wells were rinsed with a syringe and the samples were added (18 µl in each well). 1x anode buffer was then added to the lower chamber (anode). The lid attached to the electrophoresis power supply was put on top of the electrophoresis unit. A refrigerated circulator bath was attached to the electrophoresis unit to maintain a temperature of 4˚C during the electrophoresis. The electrophoresis was carried out at 1200 V, six mA, 24 W until the running front reached the bottom of the gel (around one hour). For

the blue native (BN) gels the 1xcathode buffer (blue) was replaced with clear 1x cathode buffer after half time of the run.

### 2.12.1 Solutions

| | |
|---|---|
| Agarose agarose (low EEO) | 1 spatula |
| Final volume | 20 ml |
| | |
| 6x gel buffer | (100 ml) |
| 3 M ε-aminocaproic acid | 39.36 g |
| 0.3 M Bis-Tris pH 7.0 | 30 ml |
| | |
| APS | (5 ml) |
| 10 % (w/v) APS | 0.5 g |
| | |
| Separating gel 7.0 % | (30 ml) |
| 12 % PAA (37.5:1) 30 % | 7.35 ml |
| 6xGel buffer | 5.25 ml |
| APS | 15 µl |
| Temed | 60 µl |
| | |
| Stacking gel 4 % | (5 ml) |
| 4 % PAA (37.5:1) 30 % | 0.665 ml |
| 6xGel buffer | 0.833 ml |
| APS | 5 µl |
| Temed | 50 µl |
| | |
| 5x LDS-cathode buffer | (500 ml) |
| 10xcathode (clear) | 50 ml |
| dodecyl lithium sulfate | 0.05 g |

| | |
|---|---|
| 10x (clear) cathode buffer | (500 ml) |
| 500mM Tricine | 44.8 g |
| 150 mM Bis Tris pH 7.0 | 15.69 g |
| HCl | to pH7.0 |

| | |
|---|---|
| 10x (blue) cathode buffer | (500 ml) |
| 0.2 % Coomassie 250 G | 1 g |
| 10x cathode buffer for BN | 500 ml |

| | |
|---|---|
| 10x anode buffer | (500 ml) |
| 0.5 M Bis-Tris | 52.3 g |
| HCl | to pH 7.0 |

## 2.13 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by the method of Laemmli 1970 was used to separate the proteins of the chloroplast. SDS-running buffer was loaded in the upper chamber of the electrophoresis unit (cathode). The wells were rinsed with the syringe before the sample was loaded. In the quantification experiments, three parallels from three different greening stages were loaded on each gel.

SDS anode buffer were then poured in the lower chamber (anode). The power supply was attached to the electrophoresis unit. The refrigerated circulator bath was then attached to keep a temperature of at 15˚C during the electrophoresis. The electrophoresis was run at 1200 V, 15 mA, 24 W until the running front reached the bottom of the gel.

### 2.13.1 Solutions:

| | |
|---|---|
| Agarose gel with 1xSDS | (100 ml) |
| Agarose (low EEO) | 0.5 g |
| SDS running buffer | 100 ml |
| Solve by heating | |

| | |
|---|---|
| 8xTris pH 8.8 | (500 ml) |
| 3 M Tris | 181.65 g |
| $dH_2O$ | 300 ml |
| HCL | 20 ml |
| | |
| 0.25M Tris pH 6.8 | (500 ml) |
| Tris | 15.125 g |
| $dH_2O$ | 300 ml |
| HCl | to pH 6.8 |
| | |
| APS | (5 ml) |
| 10 % (w/v) APS | 0.5 g |
| | |
| SDS separating gel | (25 ml) |
| 4 M Urea | 7.21 g |
| 12.5 % PAA (37.5:1) 30 % | 12.50 ml |
| 375mM (8x)Tris, pH 8.8 | 3.75 ml (375mM (8x)Tris) |
| 0.016 % APS | 50 µl |
| 0.05% Temed | 15 µl |
| | |
| SDS Stacking-gel | (5 ml) |
| 5 % PAA (37.5:1) 30 % | 0.80 ml |
| 0.25 M Tris pH 6.8 | 2.48 ml |
| 0.05% APS | 50 µl |
| 0.015% Temed | 5 µl |
| | |
| 10x SDS running buffer | (1000 ml) |
| 1.92 M glycine | 144.13 g |
| 0.25 M Tris | 30.3 g |
| 1 % (w/v) SDS | 10 g |

## 2.14 Combination of native- and SDS-PAGE

The native PAGE was combined with SDS-PAGE by the method of Schagger et al (1991). The protocol for SDS-PAGE was followed, with some exceptions. Instead of a comb, 0.5 ml water saturated butanol was put on top of the stacking gel-solution. The water saturated butanol was rinsed of by tap water after polymerization.

The line cut from the native gel was first put in solubilisation buffer and incubated on a moving platform for five minutes. The lane was then put on top of the stacking-gel. Air bubbles were removed between the SDS-gel and the native gel-lane by using a plastic spacer. Finally the gel-lane was covered by SDS-agarose to seal the gel.

### 2.14.1 Solutions

| | |
|---|---|
| SDS-Solubilisation buffer | (300 ml) |
| 2% (w/v) SDS | 6 g SDS |
| 66 mM $Na_2CO_3$ | 2.12 g |
| 0.66 % (v/v) β-mercapto-ethanol | 2 ml |

## 2.15 Protein detection

For detecting the proteins or protein complexes a few methods were used. The proteins in the gels were either stained by Coomassie colloidal or by silver staining. Auto fluorescence (chlorophyll-fluorescence) scan was used on gels and immunoblots to detect the pigment associated complexes/proteins.

### 2.15.1 Coomassie staining

For the Coomassie staining the protocol of Neuhoff et al (1988) was followed. The gel was kept on a shaker during all steps of the staining process. The gel was first put in fixing solution for one hour. The gel was then incubated in staining solution for 24 hours and destained in $dH_2O$ until the background was blank. After destaining, the gel was put in a plastic envelope and scanned by the Epson perfection 1640 scanner. For drying the gel was placed on a piece of blotting

paper and covered with plastic wrap. The gel was then kept in the gel dryer for 30 minutes.

*2.15.1.1 Solutions*

| | |
|---|---|
| Fixing solution | (1000 ml) |
| 40 % ethanol | 400 ml |
| 10 % acetic acid | 100 ml |
| | |
| Solution A | (900 ml) |
| 2% (w/v) ortho-phosphoric acid | 20 g |
| 10 % (w/v) ammonium sulfate | 100 g |
| | |
| Solution B | (20 ml) |
| 5 % (w/v) Serva-Blue G250 Brilliant | 1 g |
| | |
| Staining solution | (125 ml) |
| 98 % (w/v) Solution A | 98 ml |
| 2% (w/v) Solution B | 2 ml |
| 25% (v/v) ethanol | 25 ml |

### 2.15.2 Silver staining

The method of silver staining was developed by Blum et al. (1987). The first step was to put the gel in the fixing solution for two hours. After the fixing step, the gel was washed three times for 20 minutes. The gel was then put in the thiosulfate solution for one minute. It was then washed for three times 20 seconds in 330 ml $dH_2O$. The gel was then incubated for 20 minutes in the silver nitrate, after which it was washed for two times 20 seconds in 330 ml $dH_2O$. The gel was put in the develop solution for five to ten minutes until the protein bands were clearly visible (formaldehyde was added to the develop solution until right before use). It was then washed in 330 ml $dH_2O$ for two times one minute. In the last steps the gel was put in stop solution for five minutes and then washed for 30 minutes in 330 ml $dH_2O$. The gel was then scanned in a plastic envelope by the Epson

scanner. After being scanned, the gel was fixed in a frame between two cellophane sheets for drying. The cellophane was pre wetted. All air bubbles between the layers of cellophane where removed. The frame containing the gel was then put in the Gel Airdryer for three hours.

*2.15.2.1 Solutions*

| Fixingsolution | (1000 ml) |
|---|---|
| 40 % ethanol | 400 ml |
| 10% acetic acid | 100 ml |

| Wash solution | (1000 ml) |
|---|---|
| 30 % EtOH | 300 ml |

| Thiosulfate solution | (1000 ml) |
|---|---|
| 0.02 % Na-thiosulfate | 200 mg |

| Silver nitrate | (1000 ml) |
|---|---|
| 0.2 % silver nitrate | 2.0 g |

| Develop solution | (1000 ml) |
|---|---|
| 3 % Na-carbonate | 30 g |
| 0.05 % formaldehyde | 0.5 ml |
| 0.0004 % Na-thiosulfate | |

| Stop solution | (1000 ml) |
|---|---|
| 0.5 % glycine | 5.0 g |

### 2.15.3 Scanning for autofluorescence

A typhoon Trio scanner was used for detection of autofluorescence (chlorophyll fluorescence) of native complexes in gels and on blots. Gels were scanned after the native PAGE and the blots were scanned right after the blotting. Both the gels

and the blots were put directly on the scanner. For scanning of the emitted autofluorescence (chlorophyll fluorescence), signal amplification at the detector of 400 and 500 Volt was used. A resolution setting of 100 micrometer was used.

## 2.16 Protein identification

The Lil3 antibody was raised against the peptide sequence: (NH2-) CQSTWQDDSTSGPKK (-COOH), as given in the one letter amino acid standard code language. Lil3 was detected by Cy3 linked and/or HRP-linked second antibody. The HRP- linked antibody was detected on a film after enhanced chemiluminescence reaction. The fluorescence from the Cy3 antibody was detected by scanning with the Typhoon Trio scanner.

### 2.16.1 Immunodetection

The procedure of the transfer of protein from polyacrylamide gels to a nitrocellulose membrane was described by Towbin et al (1979). After the electrophoresis, the gel was put in a paper sandwich on top of a Hybond-ECL nitrocellulose membrane (GE-Healthcare) for semi-dry blotting. The membrane was carefully handled by using tweezers. The paper sandwich consisted of three layers of paper, the nitrocellulose membrane in the fourth layer and the gel on top of the membrane. Three pieces of paper was then put on top of the sandwich. All parts of the where soaked in Towbin buffer before placed on the blot apparatus (between two carbon plates).

Glass plates were placed on top of the upper carbon plate to connect it to the plastic lid. In the end, something heavy with a weight of about one kilogram was put on the plastic lid to improve the blotting efficiency. The electrophoresis apparatus was started at 400 mA and with a Volt maximum of twenty. After the electrophoresis the proteins has migrated out of the gel and onto the membrane. The blots were stained in Ponceau for one minute. The background of the membrane was destained by $dH_2O$. After visualization of the proteins on the membrane, the blot was completely destained by washing in 1xTBS. The staining was not used on blots of native gels, or on membranes scanned for autofluorescence.

In the immunodetection, the blot was always carefully handled. It never dried out and the solutions were not poured directly on the blot. The blot was first incubated in five percent milk for one hour. The Lil3 first antibody was then added to the 5% milk (1:7500 dilution). After incubating for one hour, the blot was washed in 1xTBS for three times five minutes. A secondary (HRP- or Cy3–linked) antibody was then added to the blot. The HRP-linked antibody was diluted 1:10000 and the Cy3-linked antibody was diluted 1:3750. After one hour of incubation, the blot was washed for three times five minutes in 1xTBS.

The HRP-linked antibody was detected by the Hyperfilm ECL (High performance chemiluminescence film. The film was exposed to the blot for 3 minutes. The developing of the film was performed in a light tight room.

The Cy3 linked antibody was detected by drying the blots and scanning for fluorescence by a Typhoon Trio scanner. If both second antibodies were used, the HRP- linked antibody could be used right after the incubation of Cy3-linked antibody. The blot was dried and directly incubated in the HRP-linked secondary antibody.

*2.16.1.1 Solutions*

| 1xTBS | (1000 ml) |
|---|---|
| 10mM Tris/HCL 1 M pH 7.5 | 30 ml |
| 150 mM NaCl 5M | 10 ml |

| Towbin | (1000 ml) |
|---|---|
| 96 mM Glycin | 7.2 g |
| 10 mM Tris | 1.21 g |
| 10 % (v/v) methanol | 100 ml |

| Ponceau | (100 ml) |
|---|---|
| 0.2 % (w/v) Ponceau | 0.2 g |
| 1.0 % (w/v) acetic acid | 1.0 g |

### 2.16.2 Developing of high performance chemiluminescence film

The developing of the film Hyperfilm ECL (High performance chemiluminescence film, GE-Healthcare) was performed in darkroom and by use of red light. The ECL1 and ECL2 were mixed in a plastic box. The blot was shortly kept in the ECL1/ECL2-mixture and placed in a clear plastic pocket within a light tight cassette. The film was cut in a minimum of red light and put on the outside of the plastic pocket covering the blot. The cassette was carefully closed and the film was exposed on the blot for three minutes. The film was then removed from the blot and put it in the Kodak D-19 Developer until the bands started showing (about one minute). After the developing, the film was rinsed in tap water and put in Kodak Rapid fixer (with hardener) for five minutes. The film was again rinsed with tap water and air dried. Finally, the film was scanned to the computer for further processing the results.

*2.16.2.1 Solutions:*

| | |
|---|---|
| Luminol | |
| 0.25 M Luminol | 443 mg |
| DMSO | 10 ml |
| | |
| P-coumaric acid | |
| 90 mM p-coumaric acid | 148 mg |
| DMSO | 10 ml |
| | |
| ECL1 | (15 ml) |
| 0.1 M TRIS/HCL pH 8.3 | 750 µl (TRIS/HCL pH 8.3) |
| 2.5 mM luminol | 250 µl |
| 0.4 mM p-coumaric acid | 250 µl |
| | |
| ECL2 | (15 ml) |
| 0.1 M Tris/HCl pH 8.3 | 750 µl (Tris/HCl pH 8.3) |
| 0.0183 % hydrogen peroxide | 9.15 µl |

### 2.16.3 Scanning blots for Cy3

The blots incubated in the Cy3-linked second antibody was dried and scanned by a Typhoon Trio for autofluorescence of the Cy3. The blots were then scanned for both Cy3 and autofluorescence (chlorophyll-fluorescence) to give an overlay picture of the two signals. The same parameters explained in section 2.15.5 were used in the scan.

### 2.16.4 Mass spectrometry

The type of mass spectrometer used in this experiment was a MALDI Q-TOF premier. The MS was used to identify Lil3 by looking for specific peptides found in this protein. For the preparation of the samples for the MS the protocol of Granvogl et al. (2007) was followed.

The gel for the mass spectrometry (MS) was cut under the carbonic anhydrase marker band (30 kDa) were Lil3 was located (fig 3.2, sec. 3.1). If dried, the gel was rehydrated and rinsed by adding $dH_2O$. Coomassie staining was removed from the proteins by washing in a 50 % (v/v) acetonitrile/ammonium carbonate solution. This was repeated for around three times (five minutes each), until the gel was completely destained. The gel was washed in $dH_2O$ between the steps and at the end of the washing process. Subsequently three gel pieces were picked from the gel by an OMX-S®.

The protocol for OMX-S® was used for the further digestion of the proteins. The gel was centrifuged (two minutes, 1300xg) with the reactor side of the OMX-S® down in the centrifuge. The trypsin solution (20 µl) was then added to the reactor. The OMX-S® was shortly centrifuged at 3800 g with the reactor faced down in the centrifuge. The OMX-S® was then put into a thermo mixer with agitation (500 rpm, 50˚C, 45 minutes). The reactor was still pointed down. After the digestion the OMX-S® was centrifuged with the peptide sampler faced down (three minutes, 1000xg). In the end the peptide solution was transferred to a micro centrifugation tube for further processing.

A ZipTip® micro column was used for cleaning up the peptide solution before transferring the peptides to the mass spectrometer. The column was activated by pipetting of acetonitrile formic acid respectively (see section 2.16.7).

The solutions were pulled through the column for five to eight times by a 20 micro liter pipette. Peptide solution was then pulled trough the column (six times). A rest of solution was always kept on top of the column to avoid drying out. Formic acid (20 micro liters) was then pipette eight times through the column. In the end the column was dried out. Four micro litre of elution solution were then pulled trough the column. The peptide solution was transferred into a nanospray-capillary by a gel loader tip and the solution was shortly centrifuged down into the tip. The capillary was fixed in the needle holder of the ESI-Q-TOF-mass spectrometer for analysis. The fragmentation mode used was ESI-MS/MS. The obtained MS/MS spectra were analyzed by MassLynx 4.0.

*2.16.4.1 Solutions*

| | |
|---|---|
| Acetonitril $NH_4CH_3$ | (10 ml) |
| 50 % (w/w) acetonitril | 5 ml |
| 50 % (w/w) $NH_4CH_3$ | 5 ml |
| | |
| Trypsin | (20 µl) |
| 50mM $NH_4CH_3$ | 18 µl |
| trypsin | 2 µl |
| | |
| Elution solution | (1 ml) |
| 65 % acetonitrile | 650 µl |
| 2 % 2-propanol | 20 µl |
| 0.1 % formic acid | 1 µl |
| | |
| Formic acid | (1 ml) |
| 5 % formic acid | 50 µl |
| 95 % $dH_2O$ | 950 µl |

## 2.17 Quantification of Lil3

A standard curve was first made from known amounts of plastids. This was carried out by loading known amounts of plastids to an SDS gel. Amounts from $3.47*10^5$ to $1.67*10^7$ plastids were used. The films from the respective blots were analyzed in the TINA 2.0 software to relate the amount of plastids to the signal strength on the film. A standard curve was then made in Excel.
In the quantification, the films from the SDS-gels with three different deetiolation stages were analyzed by TINA 2.0. The outcome from the calculations performed with Excel was then compared to the standard curve. The protein amount in the different stages of greening could then be related back to the amount of plastids present in each leaf. The standard curve would also show if there was a correlation between signal strength and amount of proteins.

### 2.17.1 Analyzing protein amounts by the Tina 2.0 software

Scanned films were converted and stored as a .TIF-file by inversing the colours and adjusting to grey tones. Then the file was converted to the TINA 2.0 software. Each of the bands were marked manually and thereby referred to as a certain region (see fig. 2.1).

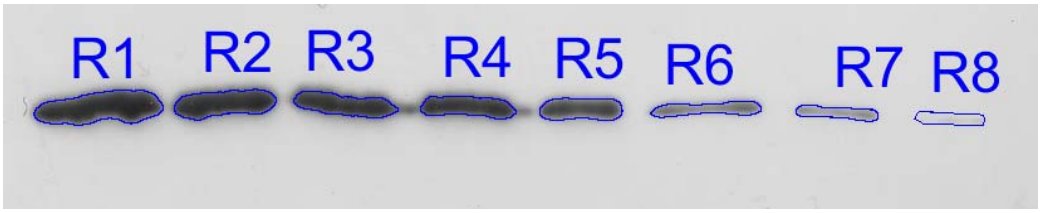


**Figure 2.1: Analyzing the protein amounts by using the TINA 2.0 program.**
Each band was marked and thereby referred to as a certain region. The program then made numeric reports of the optical density in each region. The higher the measured signal value, the higher the optical density.

The program made a numeric report of the optical density in all regions. The region report was exported to Microsoft Excel 2007. In Excel, the numbers from each band at every greening stage were put together in a graph. The middle value and standard deviation were calculated.

The background and the signal strength could vary between the different films due to the development of the film. This difference was reversed in the calculations in Excel. First the middle value of the parallels of etiolated leaves was set to 100 %. The percent difference between each deetiolation stage and the etiolated leaves were then calculated by using the middle values of all the parallels in each stage. The difference of each gel was eliminated by using parallels of one deetiolation stage on to gels. For example the one hour illuminated leaves (1h) were run on the same gel as the etiolated (E) and the ten second illuminated leaves. They were also run on the gel with the two hour (2h) and the four hour illuminated leaves. The difference between these gels was eliminated by the calculating of the difference between the E and the 2h. First the percent difference between the 2h and the 1h on the second gel was calculated. The ratio of this number and the percentage difference between the E and the 1h on the first gel was then calculated as the difference between the E and the 2h. The Excel data was then analyzed considering standard deviations.

# 3 Results

In the first approach, the specificity of the Lil3 antibody for binding to the native state Lil3 protein was tested in a dilution series experiment. Thereafter, the kinetic changes among Lil3 protein complexes were compared. Changes were followed during deetiolation of barley seedlings in order to enable a correlation between the amount of Lil3 protein and the amount of chlorophyll bound to Lil3. In this work, the Lil3 protein was quantified using the characterized Lil3 antibody. In a successive experiment the amount of chlorophyll can then be determined from the auto fluorescence signal strength of the chlorophyll bound in the Lil3 protein complexes. In the following the optimization of the experimental conditions for quantification of the Lil3 protein are described. A standard curve was set up to enable a correlation between the amount of protein and the signal strength of the Lil3 antibody. Thereafter, the Lil3 amount in plastid membranes was determined by the Lil3 antibody. The experimental setup finally enabled a quantification of the kinetic changes in the amount of Lil3 protein during different stages of deetiolation.

## 3.1 Identification of the Lil3 protein

In the first experiments, the specificity of the Lil3 antibody for detection of the Lil3 protein was tested on whole protein extracts from the membranes of barely plastids. Experiments were based on earlier work about Lil3, were the protein had been identified in inner membranes of barley plastids by mass spectrometry(Reisinger et al. 2008c). Proteins from plastids membranes were isolated from etiolated barley seedlings that were either illuminated for ten seconds with white light (10s) and from green plants (C) and the proteins were separated by SDS-PAGE. Finally, mass spectrometry was used to link the antibody based signals to the mass spectra obtained earlier from an etioplast membrane protein band.

The identification of the Lil3 protein by the Lil3 antibody was conducted on nitrocellulose membranes (NCM) by a method called enhanced chemiluminescence (ECL) (see methods). In brief proteins separated by SDS-

PAGE were transferred to NCM by blotting. This process leads to immobilization of the proteins, since the proteins are transferred from the porous network structure of the polyacrylamide network to a rigid polymer structure of the NCM gel-blot. This gel-blot can then be incubated with a primary antibody. If the primary antibody binds to a protein, its presence can be visualized by incubation of the gel-blot with a chemiluminescent enzymatic reaction linked to a secondary antibody. The secondary antibody is directed against the Fab-antibody subunit of the primary antibody and contains the enzyme linked to its own Fab-subunit. In this work, the enzyme horseradish peroxidase was used. The specific antibody based reactions of the plastid membrane and soluble proteins can then be visualized by exposition of the immobilized enzymatic luminescent reaction against a light sensitive film negative and development of silver based chemical depositions in the film negative (fig. 3.1 A and B).

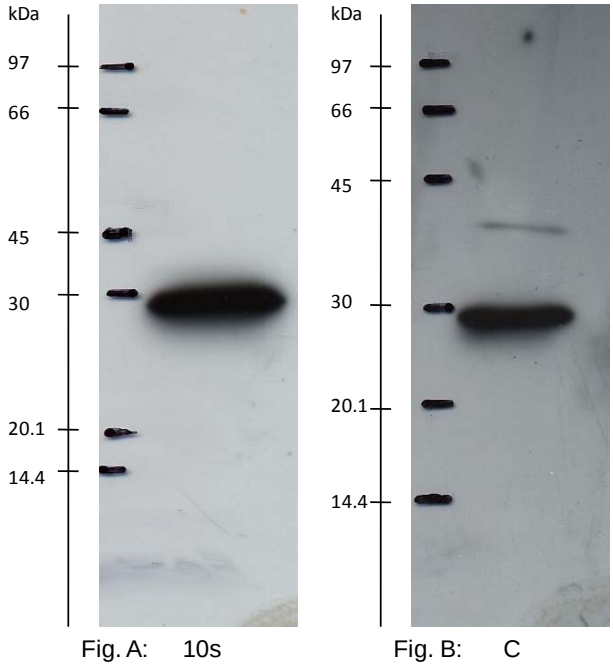


**Figure 3.1: Gel-blot analysis of Lil3**

Gel-blot analysis of Lil3 protein detected by the Lil3 antibody in membrane protein extracts from plastids isolated from barely seedlings after ten second illumination of etiolated seedlings with white light (10s, fig. A) and from green leaves (C, fig. B) by the ECL system.

The film negatives revealed only one main deposition for the proteins isolated from the plastid protein extracts after 10s illumination of the etiolated barley seedlings (10s plastids) and from the plastids isolated from green barley leaves (C). Both antibody based signals were detected close to the *carbonic anhydrase* (CA) marker band of the marker protein set at a molecular mass of 30 kDa. The detected protein revealed a slightly higher mobility relative to the CA marker band indicating a molecular mass of slightly lower then 30 kDa. In the protein extracts C (fig.3.1B) an additional deposition of low signal strength and at a lower mobility relative to the main Lil3 antibody signal could be detected on the film negative. The nature of this antibody based signal could not be determined. However, based on the strong signal strength difference between the main antibody based signal at around 30 kD and the band of lower mobility we considered that the antibody is specific for identification of the Lil3 protein.

In order to corroborate our immunological identification of Lil3 an additional identification of the protein was performed by mass spectrometry. Proteins were extracted from the molecular mass region at which the Lil3 protein had been identified to be localized in the SDS-gel. First, a SDS gel from membrane proteins of 10s plastids (10s) (fig 3.2 A) and chloroplasts (C) was prepared (fig.3.2.B) and the proteins in the gel were stained with Coomassie (see methods). In the molecular mass region of interest directly below the 30 kDa band of the marker protein CA and at which the Lil3 had been detected by the antibody, round gel pieces with a diameter of 1.8 mm were cut utilizing OMX-S$^{TM}$ (see arrows in fig. 3.2). The gel pieces were processed by tryptic in gel-digestion and peptides were extracted from the gel by the OMX-S$^{TM}$ device (see methods). Peptides were then used to determine a Lil3 peptide by mass spectrometry.

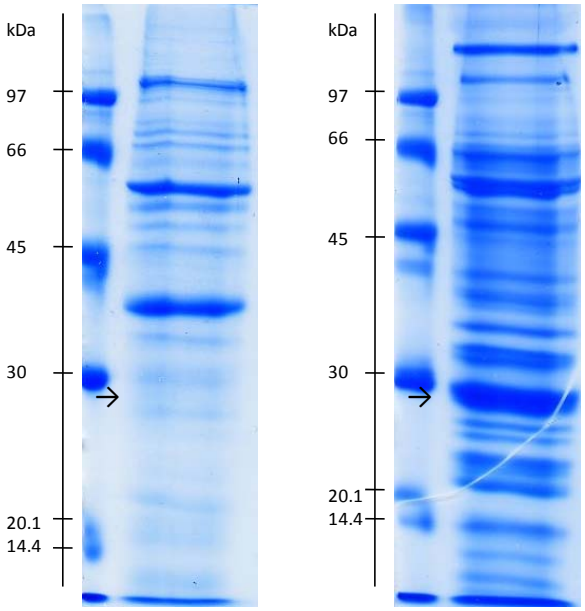


**Figure 3.2: SDS gels of membrane proteins from 10s plastids (10s, fig. A) and from chloroplast (C, Fig.B).**

Proteins in the gel were stained by Coomassie. The gel area processed for analysis by MS has been marked by an arrow.

Proteins were extracted from the gel region that was identified by gel-blot analysis. In this local gel region no peptides could be identified in the proteins from the ten second illuminated plastids (10s). However, among the membrane proteins extracted from chloroplast (C) one of the Lil3 peptides could be identified. The peptide of interest could be identified after fragmentation of a peptide mass signal in the range of 979-993 m/z (fig. 3.3).

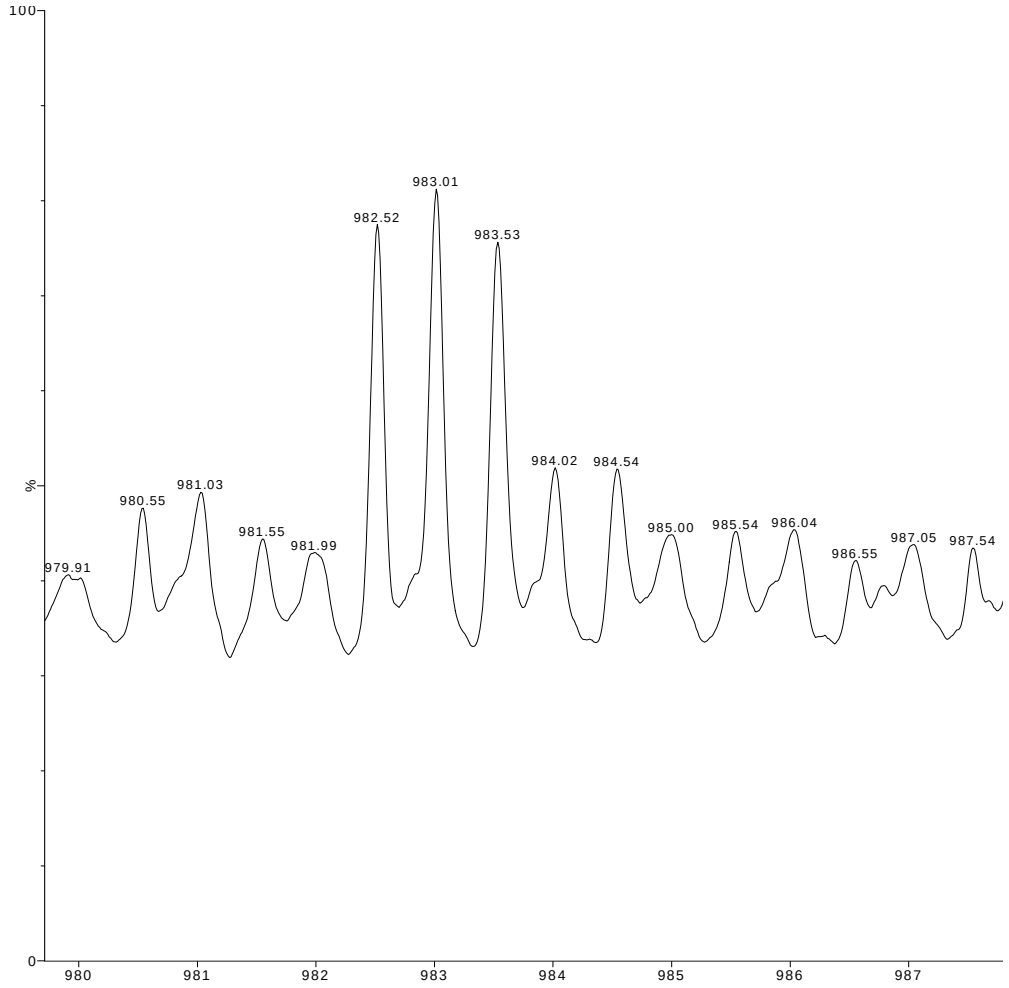


**Figure 3.3: MS spectrum of peptides.**

Three peaks in the mass range of 982.52 to 983.53 represent a double charged peptide molecule. The peptide was selected for fragmentation because of its similarity to the m/z value of already identified Lil3 peptides.

In order to identify a peptide, a peptide mass signal has to be identified. Then the amino acid composition can be determined by fragmentation of the peptide in subfragments containing different numbers of aminoacids. After electrospray ionization of protein in-gel digests, peptide signals often appear as doubly charged mass signals. For specific identification of a Lil3 peptide a list of potential mass signals was compared to the mass signals found in the in-gel extracts. One of the mass signals with a double charged peak and an m/z value of 982.52 were selected for fragmentation. The resulting fragment spectrum was then analyzed by a de novo sequence algorithm embedded in the program MaxEnt3 (fig. 3.4).

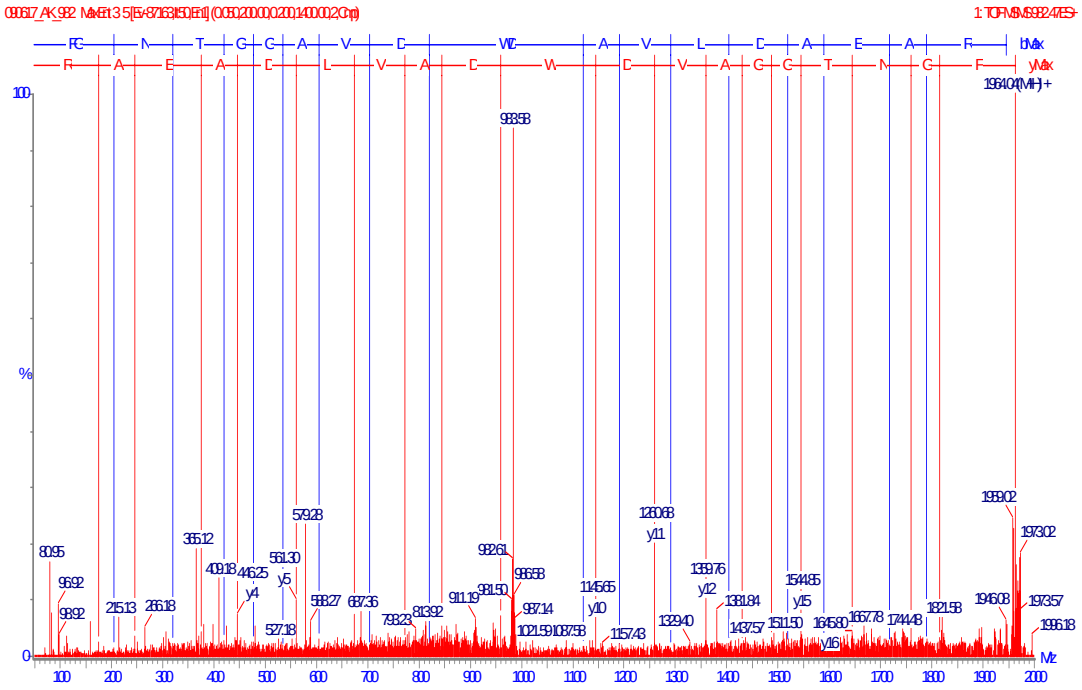

**Figure 3.4: Fragment spectrum of the 982 m/z**

Aminoacid sequence (letters top lines, b-ion fragment series in blue; y-ion fragments series in red) and peptide fragmentation spectrum of peptide 982 m/z (corresponding peptide spectrum fig. 3.3).

Fragmentation of the peptide mass signal at m/z 982.52 revealed a well resolved spectrum of fragments with different aminoacid length. From the mass differences between these fragments an aminoacid sequence could be determined from the N- to the C- terminus of the peptide by analyzing the y-ion fragments (fig 3.4, dashed lines, y-ion series, N-terminus high molecular mass fragments (right side), C-terminus low molecular mass (left side)). Also, the program MaxEnt3 proposed a series of corresponding b-ion fragments that corroborated the aminoacid sequence read from the y-ion fragment series. According to the two fragment series of corresponding ions an aminoacid sequence was determined to read from the N- to the C-terminus as FGNTGGAVDWDAVIDAEAR (fig. 3.5). This peptide sequence had already been identified as Lil3 by BLAST similarity search in a previous work (Reisinger et al. 2008c). When the sequence was aligned against the sequence of the Lil3 protein from rice, *Oryza Sativa*, the first four aminoacids were found not to be conserved between rice and barley (fig. 3.5).

**FGNT**GGAVDWDAVIDAEAR

**Figure 3.5: Sequence of the Lil3 peptide**

Sequence of Lil3 peptide with mass signal at m/z 982.52 identified from the Lil3 protein of barley chloroplast membranes. The barley sequence differed from the sequence of Lil3 in rice (*Oryza Sativa) by the first four* amino acids of the peptide (bold letters) which hence were not conserved in the two organisms (Reisinger et al. 2008c).

## 3.2 Optimum specificity for the Lil3 antibody/protein interaction

In order to prepare for a determination of different amounts of Lil3 protein in the different developmental phases of the plastid membranes, the optimum reaction specificity for the interaction between the Lil3 antibody and the Lil3 protein was tested. A dilution series of the Lil3 antibody from 1:1000, 1:2000, 1:5000, 1:7500, 1:10000, 1:15000 and 1:20000 was set up and the antibody dilutions were incubated with a defined amount of Lil3 protein. Membrane proteins from $1.11*10^7$ 10s plastids were separated by SDS-PAGE. The gel was blotted onto NCM and incubated with Lil3-antibody concentrations increasing 20-fold. Clearly, the increasing concentrations of Lil3 antibody resulted in an increase of the luminescence signal (fig. 3.6). This indicated that a higher amount of antibody could bind to the immobilized Lil3 protein if more Lil3-antibody was offered for the primary antibody/protein interaction. It was completely unclear in which directions the protein concentration of Lil3 per plastid would change during the deetiolation of the barley plastids. We therefore choose to work with a dilution of the antibody that would enable us to follow a change toward higher and lower protein concentrations. Hence, an antibody dilution of 7500-fold was selected for the further experiments. An additional factor influencing this decision was a saturation of the signal stored in the film negative. Saturation was evident from the “bleeding” of signal to the adjacent areas around the Lil3 protein band, giving the signal a “fuzzy” appearance.

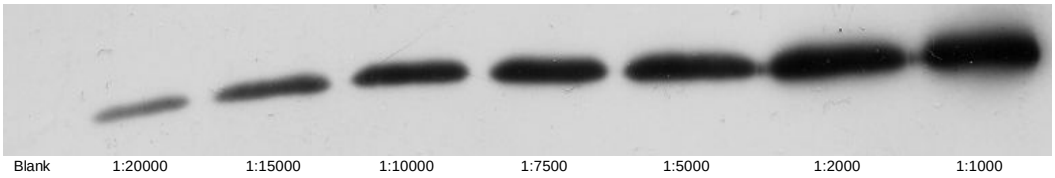


**Figure 3.6**: **Dilution series of the Lil3 antibody/Lil3 protein interaction.**
$1.11*10^7$ 10s plastids were separated per SDS-PAGE lane and the gel-blot incubated with the specified Lil3 antibody dilutions (ratio numbers). As a control, one lane of the SDS-PAGE gel was not loaded with protein extracts from plastids (blank).

## 3.3 Binding of the Lil3 antibody to Lil3 protein in its native state.

The next step to set up the experimental system for determination of changes among the Lil3 protein was the test of the ability of the Lil3-antibody for binding of the Lil3 protein in its native state. This was tested by native gel electrophoresis, gel-blot transfer of the native protein to the NCM and subsequent antibody incubation (fig. 3.7).

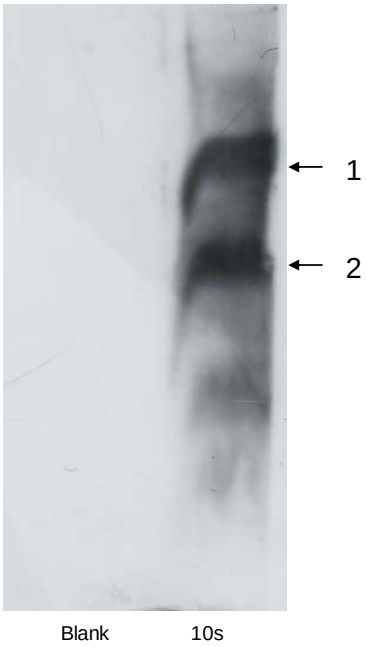


**Figure 3.7: Gel-blot analysis of Lil3 protein after native PAGE separation of membrane protein extracts from 10s plastids (10s).**

Two major protein bands containing the Lil3 protein were determined (arrows labelled 1 and 2). As a control, the reactivity of the secondary antibody against the 10s plastid extract was tested (blank).

After native PAGE separation of the membrane protein extracts from 10s plastids, two major protein bands were detected by the Lil3 antibody (fig. 3.7). In addition, three minor bands became visible at a higher and lower molecular mass relative to the two major bands (fig. 3.7, not labeled). This indicated that the antibody reacted well also against the native Lil3 protein. As a negative control, the membrane protein extract from 10s plastids was only incubated with the secondary antibody to test for unspecific binding of the secondary antibody against the proteins and protein complexes of the plastid membrane. The missing of any signals in the film negative clearly indicated that no reactivity of the secondary antibody against plastid proteins was present. This experiment was a strong basis for the study of a qualitative presence of native Lil3 in protein complexes and for a quantification of the Lil3 protein amount during deetiolation

## 3.4 Difference of antibody signal in blue native and clear native gels

For the determination of native Lil3 protein, an additional experiment was performed in order to enable a detection of the protein by immunological methods and in addition a fluorescence read-out of the pigment binding of the native Lil3 protein. To this end, the separation of native proteins by the more simple and stable but fluorescence quenching blue native PAGE (BN) was compared with the more complicate but fluorescence sensitive clear native (CN) gels. Both native PAGE systems were compared by utilizing membrane protein extracts from 10s plastids and chloroplasts. Interestingly, the separation of the membrane proteins by BN- and CN-PAGE systems revealed striking differences. For both the 10s plastids and chloroplasts, the Lil3 protein was detected in two major and in the 10s plastids also a less intense protein band of lower molecular mass was found after CN-PAGE (fig 3.8 A, arrows 1 and 2). However, after BN-PAGE separation only one protein complex was detected at lower molecular mass. The ion mobility

front line of the BN electrophoresis gel also revealed an antibody signal (fig 3.8 B, arrows 1 and 2).

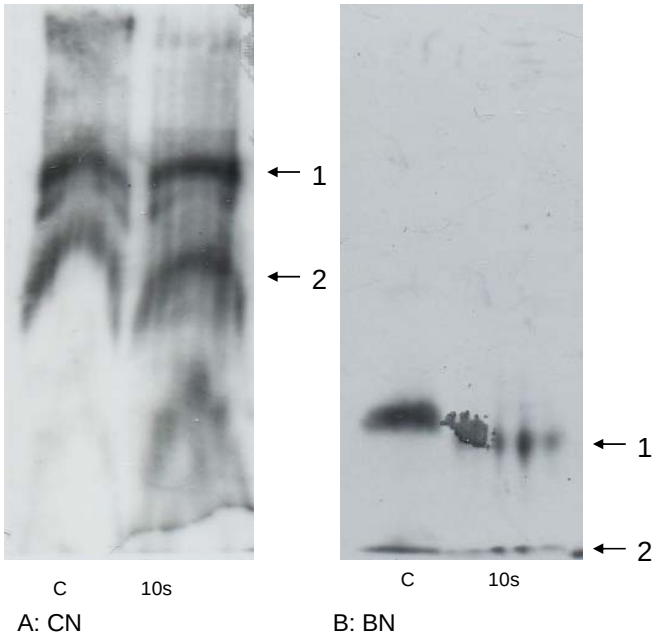


**Figure 3.8**: **Lil3 gel-blot analysis after separation of membrane proteins from chloroplasts (C) and 10s plastids (10s) by clear native PAGE (CN) (A) and blue native PAGE (BN) (B).**

Results indicated that the Lil3 protein is well detected by the Lil3 antibody after separation of the protein by both electrophoretic systems. Hence, both systems would be useful to investigate the development of the protein during deetionlation; however, the mobility of the Lil3 protein was clearly lower in CN. This in turn could indicate that the Lil3 protein was preserved in higher molecular mass structures in the CN electrophoretic system. It was therefore concluded that more information about the Lil3 protein was obtained from separation of the membrane proteins by CN.

In order to investigate the differences between the CN- and BN-PAGE gels further, the protein complexes of the 10s plastids from both native gel types were further separated into protein subunits by SDS-PAGE (fig 3.9).

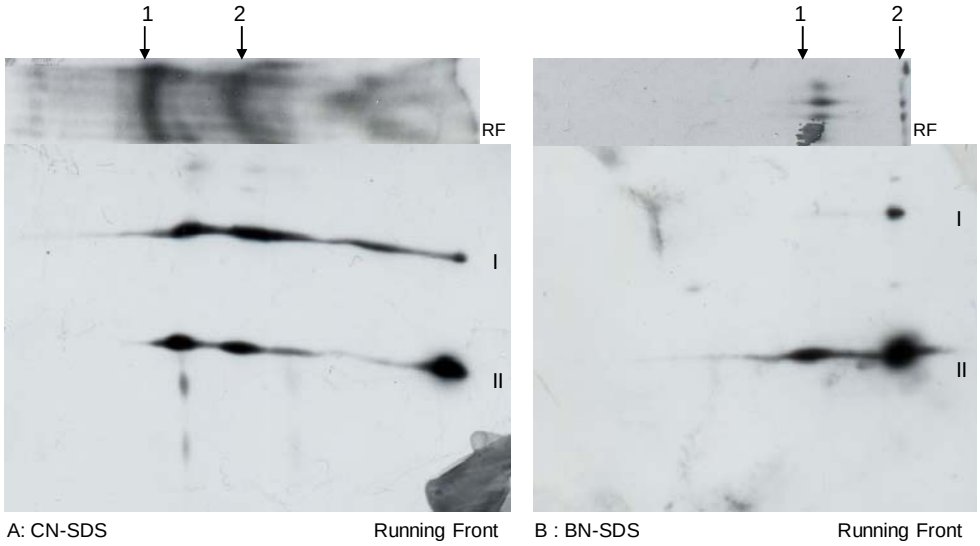


**Figure 3.9 : Gel-blot analysis of membrane proteins from 10s plastids after separation of proteins by two dimensional (2D)-native/SDS PAGE either starting with CN-PAGE**
(A, top of figure with CN-gel, placed perpendicular to the SDS-PAGE, lower part of figure) or with BN-PAGE (B, top of figure with BN-gel, placed perpendicular to the SDS-PAGE, lower part of figure) as first dimension. Proteins from both 2D-gels were transferred to NCM and incubated with Lil3 antibody.

In the gel-blot of the second dimension SDS-gel, the differences between both native gel types for separation of the Lil3 protein complexes were pronounced. After CN-SDS, two Lil3 protein spots could be aligned to complexes 1 and 2 of the native CN-gel (fig. 3.9, A1, A2). This molecular mass line of antibody reactive signals was labeled with a roman two (fig. 3.9, A II). An additional protein spot containing the highest amount of Lil3 protein was localized at the same molecular mass and could be well aligned to the buffer front of the CN-PAGE gel (fig. 3.9, A II, RF). However, the signal strength of the Lil3 reactive antibody in buffer front of the native gel (RF) was low, whereas it was high after SDS-PAGE separation of the protein (fig. 3.9, A, RF and II). Interestingly and to our surprise, the SDS-gel revealed a second molecular mass level of proteins reactive against the Lil3 antibody (fig. 3.9, A I). Lil3 proteins at this molecular mass level were located close to corresponding Lil3 protein at the lower molecular mass level indicating that both Lil3 proteins should be aligned to the two major Lil3 protein complexes detected in the first dimension. However, at the higher

molecular mass level two additional Lil3 reactive proteins were identified. There either aligned to a Lil3 protein complex of lower molecular mass and lower overall concentration in the native CN-gel and to the buffer front (fig. 3.9, A, RF and I). This finding was remarkable since the lowest Lil3 protein complex gave rise only to one Lil3 protein of high molecular mass after SDS-PAGE. In the case of the 2D BN/SDS-PAGE separation and gel-blot analysis, also two levels of molecular mass were found for the Lil3 protein. Here the proteins were well aligned to the single native protein band and the Lil3 proteins in the buffer front of the native gel (fig. 3.9, B, I and II and RF). Interestingly, the lower molecular mass Lil3 proteins revealed the highest antibody signal strength in the second dimension; whereas the Lil3 protein hardly reacted in the buffer front of the BN-PAGE system. The most likely cause for the separation of the Lil3 proteins into two different molecular mass levels in the second dimension SDS-gel could be an incomplete solubilisation of the native protein complexes by SDS. However, further experiments will be necessary to determine whether the unexpected information from this experiment will be of use to better interpret the native protein structure of the Lil3 protein. In summary, the results indicated that the CN-PAGE system was superior to the BN-PAGE for separation of the Lil3 protein complexes.

## 3.5 Lil3, a membrane protein

The high specificity of the Lil3 antibody for the Lil3 protein enabled it to investigate its localization in the sub compartments of the plastids. To find out, intact plastids were isolated and then sub fractionated into a soluble and membrane phase. First, plastids were incubated in a hypotonic buffer. In this buffer system, the plastids rapidly take up water via the inner envelope membrane system and the envelope around the plastid is torn leading to lysis of the plastid. If this lysis extract of plastids is centrifuged at high speed, the presence of a water soluble and water insoluble fraction becomes visible. All water insoluble proteins of the plastids concentrate on the bottom of the centrifuge tube while the water soluble remains in the water phase. The water soluble proteins are associated with the plastid stroma while the water insoluble proteins are associated with the

plastid membrane systems consisting of the envelope membranes and the thylakoid membrane system. In order to test in which of the two sub fractions the Lil3 protein is localized and whether the localization is maintained throughout the plastid development, membrane and soluble proteins from an equal number of 10s plastids and chloroplasts were separated by SDS-PAGE. Proteins were then transferred to a NCM for gel-blot analysis (fig 3.10). On the gel-blot, Lil3 proteins were selectively detected by the Lil3 antibody only in gel lanes loaded with membrane proteins. The signal strength of the antibody appeared independent of the developmental phase of the plastid and could be well detected in both the chloroplasts and 10s plastids. The molecular mass of the Lil3 protein was again determined with a slightly increased mobility relative to the 30 kD molecular mass marker protein CA (fig. 3.1, marker). In gel lanes loaded with the soluble proteins from the plastid stroma fraction of both plastid types, no antibody reaction could be detected. This indicated that the Lil3 protein is localized in the membrane fraction of 10s plastids and chloroplasts and maintains its localization throughout development of the plastid.

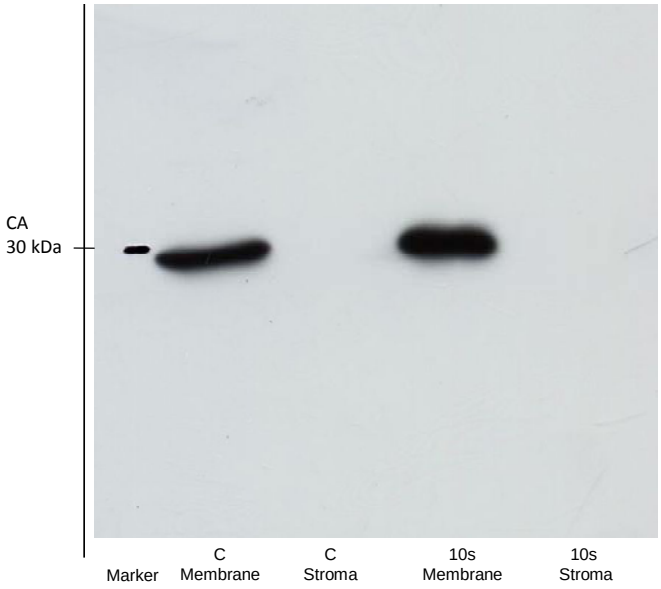


**Figure 3.10**: **Gel-blot analysis of Lil3 proteins from chloroplast (C) and 10s plastids (10s) from the membrane and stroma plastid sub fractions.**

The mobility of a molecular marker protein CA is indicated on the left side of the gel-blot (pencil dash).

## 3.6 Assembly of chlorophyll binding complexes during deetiolation

Based on the preceding experiments, the Lil3 antibody was an excellent tool with very high specificity for the identification of the Lil3 protein in the denatured and the native state. It was therefore concluded that the kinetic changes among the Lil3 proteins could be investigated utilizing this antibody. Especially of interest were the different membrane fractions of plastids during the development of chloroplasts from etioplasts. In addition, earlier experiments had shown that the Lil3 protein complex was well detectable via its fluorescence emission upon excitation with a wavelength of 633 nm (autofluorescence). The combination of both analysis techniques therefore offered the chance to investigate the amount of Lil3 protein and of pigment bound per Lil3 protein. Furthermore, it was anticipated that fluorescence could be a straightforward tool to characterize all changes among protein complexes binding chlorophyll during plastid development. In a first attempt, the kinetic changes among autofluorescent protein complexes were followed between etioplasts, 10s, 1h, 2h, and 4h plastids, and chloroplasts (fig. 3.11). For the analysis, plastids were isolated from barley seedling grown in darkness for 4.5 days or grown in darkness and thereafter illuminated for 10s, 1h, 2h or 4h with white light. Chloroplasts were prepared from barely seedlings grown in white light for 4.5 days. Plastid membranes were prepared from the isolated plastids for separation of complexes by CN-PAGE. In each experiment, plastids membranes from two developmental time points were isolated. In order to link the experiments, each experiment contained one overlapping and one novel time point. Five experiments including ten preparations of complexes were necessary to cover the two end points and three internal time points. The gels of all experiments were finally scanned for auto fluorescence directly after electrophoresis of the protein complexes (fig. 3.11).

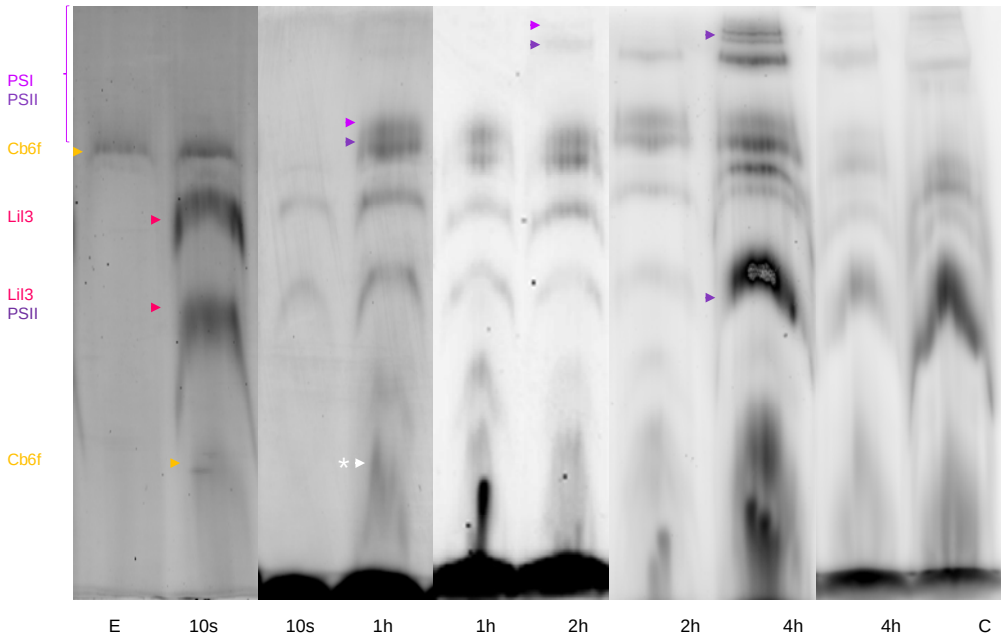


**Figure 3.11: Autofluorescence of chlorophyll binding membrane protein complexes.** Protein complexes were isolated from plastid membranes of dark-grown barley seedlings illuminated for 0s (E), 10s, 1h, 2h and 4h or were isolated from barley seedlings grown in the light C, and scanned for autofluorescence after separation by CN-PAGE. The colours and coloured triangles, light purple, purple, yellow and pink mark complexes of photosystem I, photosystem II, cytochrome $b_6f$ (Cyt $b_6f$), and Lil3, respectively. The complex marked with a white * could not be indentified.

Analysis of the autofluorescence from the native protein complexes revealed overall that the number of autofluorescent protein complexes in the plastid membrane phase increased during deetiolation of the barley seedling (fig. 3.11, E-C). In detail, autofluorescence was identified merely in the Cytb6f complex in etioplast membranes, whereas with illumination of the seedlings the Lil3 complex assembled in 10s plastids. After one hour of illumination, part of the photosystem I- (PSI) and photosystem II -complex (PSII) reaction center complexes accumulated and already after two hours of illumination, high molecular mass PS I and II complexes could be indentified. When the leaves were exposed to white light for four hours (4h), all the photosystem complexes including the high molecular mass supercomplexes and large amounts of the trimeric LHCII complexes were visible. Finally, the number of autofluorescent complexes extracted from chloroplast membranes did not differ from the complexes isolated

from the four hour plastids (4h). However, the total amount of proteins in all of the photosystem complexes was higher in chloroplasts then in plastids after 4h illumination of dark-grown seedlings. This result indicated that the autofluorescent proteins complexes constituting the photosystem machinery in plastid membranes is completely assembled within four hours after the onset of illumination of 4.5 day-old dark-grown seedlings.

In order to link the results of chlorophyll autofluorescence to a binding with protein, it was concluded that a transfer of the proteins from the native gel onto NCM would allow reading out the autofluorescence of the pigments bound to protein and thereafter detect the proteins by gel-blot analysis. Hence, gels scanned for autofluorescence (fig. 3.11) were blotted and the five blots were scanned for auto fluorescence (fig 3.12).

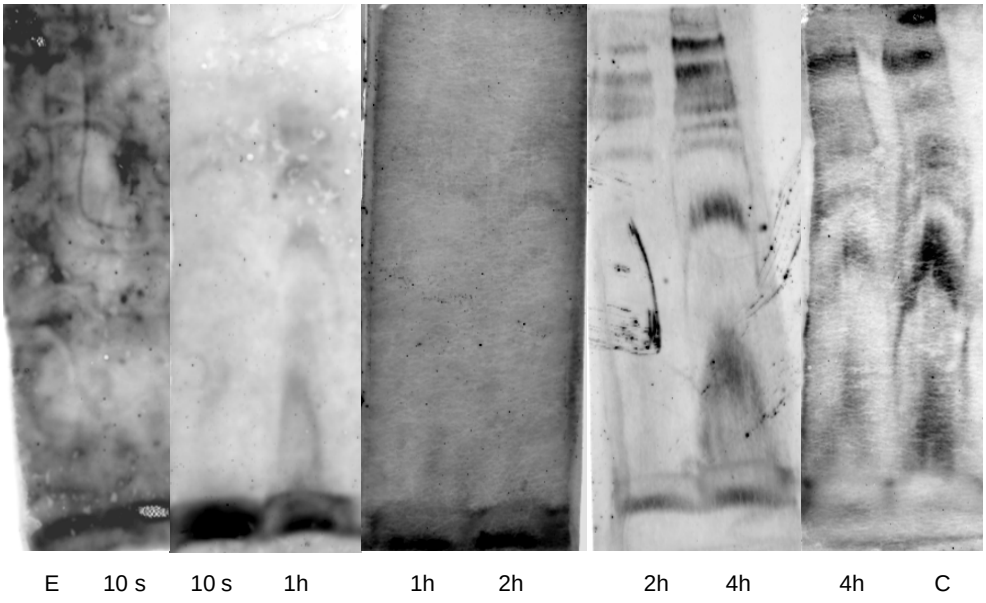


**Figure 3.12: Autofluorescence of chlorophyll binding membrane protein complexes after transfer of protein complexes to nitrocellulose membrane.**

Labelling of experiments as outlined in the legend of figure 3.11.

Autofluorescence read out of the gel-blot was not comparable to the quality of the direct autofluorescence scanning of the native gel. Fluorescence was quenched by high background fluorescence or signals were very faint in the first three experiments between the etiolated and the 2h illuminated developmental stage.

Especially, a weak signal was recorded in the blot with the 10s and the one hour illuminated (1h) plastids. In contrast, the autofluorescence from experiment four and five which characterized the developmental stages between 2h and the chloroplast was well detectable and in part autofluorescence signals were even amplified relative to the native gel scan (fig. 3.12). Especially the autofluorescence signal of the LHCII- and from the complex containing the PSI-LHCI complex in the chloroplast membranes were increased in the gel-blot scans relative to the native gel scans. Although, the gel-blot analysis could not be fully analyzed due to the low degree of autofluorescence in the first three experiments, the gel-blots offered a new possibility for parallel visualization of pigment and protein (fig 3.14).

In order to visualize the location of the Lil3 proteins on the gel-blot, the NCM was incubated with Lil3 antibody. A secondary antibody linked to the fluorescent dye Cy3 was used for fluorescence read out. Hence, after binding of both antibodies the blots could be scanned for the presence of the Cy3 antibody in order to detect the Lil3 complex (fig 3.13).

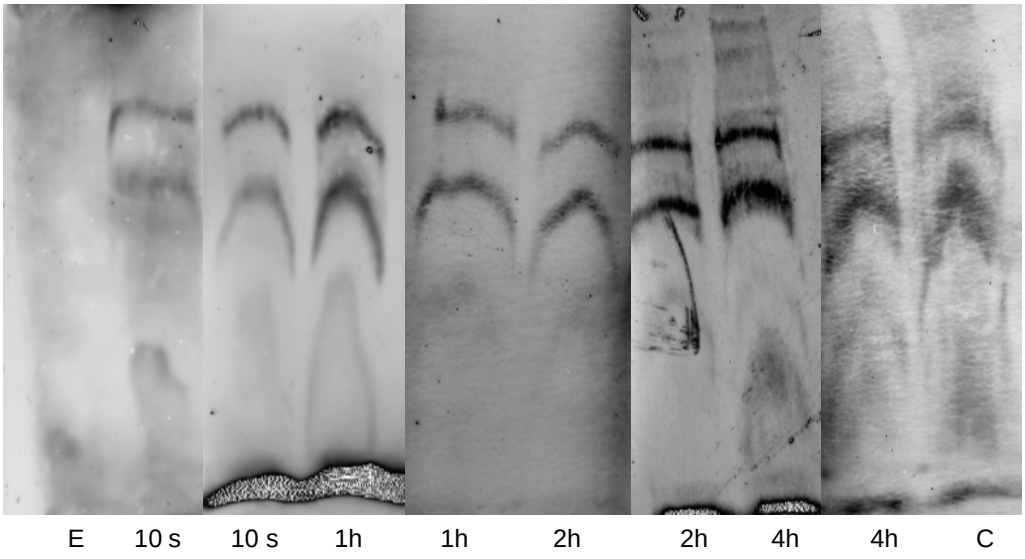


**Figure 3.13: Cy3 based fluorescence of Lil3 complexes during deetiolation of barley seedlings.**

Protein complexes of plastid membranes were separated by CN-PAGE and transferred to NCM by blotting. Labelling of experiments as outlined in the legend of figure 3.11.

Incubation of the gel-blot with the Lil3 antibody resulted in very clear and convincing signals between the 10s and C time points. Here, each of the experiments revealed two major antigenic signals for the Lil3 protein complexes. Both complexes were present at around the same molecular mass in each of the experiments. Also only a low degree of intensity differences were visible within the experiments. The highest degree of differences was detectable between the 10s and 1h time points in the second experiment. Overall the data indicated that the amount of Lil3 in protein is hardly changed throughout the development of the plastid, when the plants have been illuminated. In contrast, the results could not be well interpreted for the developmental time point of the etioplasts (E), since here only a diffuse signal near the running front (RF) of the native gel was recorded. The information from the gel-blot analysis of the etioplast membrane fraction however clearly indicated that no Lil3 protein complexes were detectable at this developmental time point.

Finally, the different stages of greening were overlaid, in order to compare the development of chlorophyll binding complexes to the development of Lil3 complexes (fig 3.14). The gel-blot based experimental setup enabled it to overlay both fluorescence scans. In order to create this image, the Cy3 based and chlorophyll based fluorescence signals were stored with different colours. The antibody based signal from the Cy3-coupled secondary antibody was stored in green and the chlorophyll based auto fluorescence was stored in red. Then both images were mixed. This analysis has the advantage that protein with equal fluorescence intensity in green and red show up as a yellow signal. Since the Cy3 signal was based on the antibody reaction with the Lil3 protein and the chlorophyll fluorescence on a pigment a yellow to orange colour could reveal that the fluorescence from the pigment is originating from a pigment bound to the Lil3 protein.

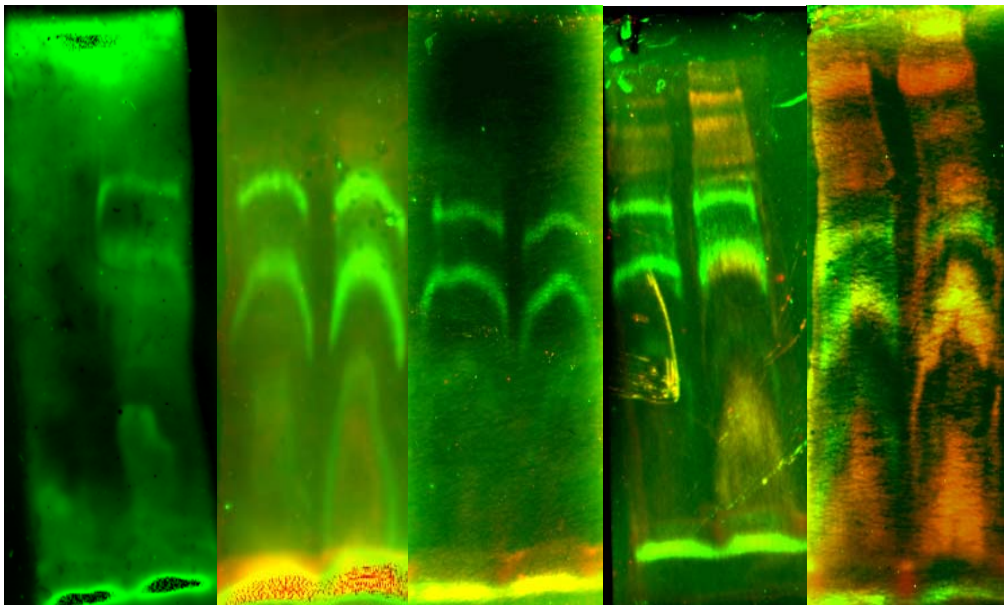


**Figure 3.14: Fluorescence overlay of Lil3 complexes detected by Cy3 labeled antibodies (green colour,) and of chlorophyll-binding auto fluorescent protein complexes (, red colour) from in different stages of deetiolation.**
The figure represents an overlay of fig. 3.12 and 3.13.

The overlay image made visible that the detected auto fluorescence complexes did not match with the detected Lil3 complexes. However, since the chlorophyll based autofluorescence was overlaid by a high background in the first experiments, only the last two experiments for the time points between 2h and the chloroplasts could be interpreted. There was a clear overlap of auto-fluorescence and antibody detectable in the four hour illuminated plastids (4h) and the chloroplast (C) lane (fig 3.14, 4h and C). However this auto fluorescence signal originated from a LHCII complex. The result therefore indicated that the trimeric LHCII complex of chloroplast membranes has the same molecular mass as the lower molecular mass Lil3 complex. Since the auto fluorescent signals could only be detected without doubt in the 2h, 4h and C lane, no final conclusion about the pigment binding of Lil3 complexes was possible in this experiment.

The first experiment was not fully satisfactory for an analysis of the pigment binding by the Lil3 protein. Therefore, the Lil3 complexes were identified in the different stages of deetiolation by antibody based detection in the ECL system by a HRP-linked secondary antibody in a second approach, (fig 3.15).

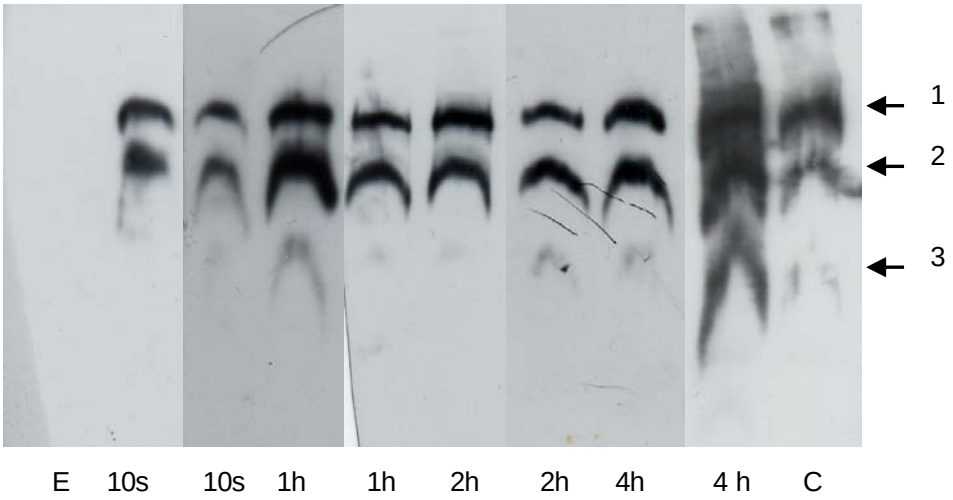


**Figure 3.15: Gel-blot analysis of Lil3 protein complexes by a HRP based ECL system.** Membrane protein extracts from plastids isolated at different stages of deetiolation from barley seedlings were separated in a CN-gel and Lil3 proteins detected by a Lil3 specific antibody. Labelling of experiments as outlined in the legend of figure 3.11.

Development of the primary Lil3 antibody binding to the Lil3 proteins in this experiments by the HRP-secondary antibody corroborated that the primary antibody did not detect any Lil3 complexes in the etioplast (E) (fig 3.15, E). However, in the 10s plastids (10s), the two Lil3 bands with different molecular masses were detectable and both showed about the same signal strength. Again, the signals from both molecular masses were found throughout all illumination stages.

In some of the lanes the signal was more intensive than in other lanes. This was especially the case in the second experiment in which the one hour illuminated plastids (1h) were compared with the 10s plastids. However, the signal was not higher in the control lane for the 1h illumination stage indicating that the increased signal strength in the 1h lanes could be related to an increased amount of plastids applied in this lane.

In this approach, the gel-blot images showing the antibody detection of Lil3 in the HRP based ECL system (figure 3.15), were aligned to the autofluorescence of chlorophyll binding complexes in the clear native gel system (fig 3.11). It was envisioned that a correlation between the assembly of Lil3 complexes and the assembly of chlorophyll in the Lil3 protein complexes should become evident from a comparison of the two different types of experimental analysis strategies. The images of each of the two experiments were therefore aligned on the level of the different deetiolation stages (fig 3.16).

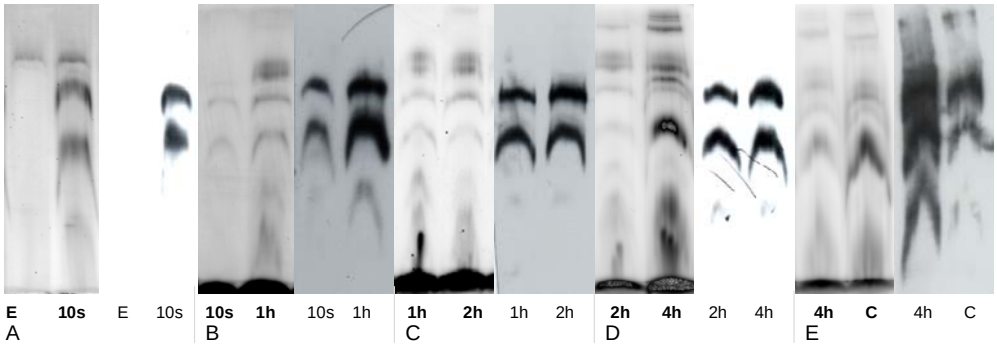


**Figure 3.16: A combination of figure 3.11 and 3.15.**

Figures labelled A-E indicate the plastid membrane complexes isolated from plastids of the different deetiolation stages of barley seedlings. The two **bold letters** on the left **side** in each figure, mark the autofluorecsence scan (figure 3.11). The letters on the right of the figure mark the Lil3/HRP gel-blot analysis (fig. 3.15). **A:** Etioplast and 10s plastids (10s). **B:** 10s plastids and plastids from one hour illuminated seedlings (1h). **C:** plastids from 1h and two hour illuminated seedlings (2h). **D:** plastids from 2h and four hours illuminated seedling (4h). **E:** 4h and Chloroplasts.

Clearly, there was no autofluorescence signal in the Lil3 of the etiolated leaves (E) besides the known autofluorescence signal that corresponded to the Cyst $b_6f$ complex (see sec. 1.1.10 for theory about the deetiolation). However, already in the 10s plastids, the signal of both the Lil3 complex, and of the Cyst $b_6f$ could be detected by autofluorescence analysis. This result was corroborated by the antibody based identification of the Lil3 protein complexes. The result showed that no Lil3 complex was present in etioplasts, whereas even a very short illumination of the leaves was sufficient to induce the assembly of the Lil3 protein complexes (Fig. 3.16). Also, the data clearly showed that the pigment based

autofluorescent and antibody based Lil3 protein signal were precisely located at the same molecular mass after the CN-PAGE. In each of the consecutive time points this result was found again. With the only difference that in the second autofluorescent experiment which linked the 10s plastids to the plastid membrane proteins from 1h illuminated leaves in addition, the photosystem I and photosystem II complexes were detected. After 2h of illumination, more of the photosystem complexes had accumulated and after 4h of illumination the higher assembly states of the photosystem complexes could be detected in the high molecular mass regions after CN-PAGE. The parallel gel-blot analysis however revealed a more or less constant amount of the Lil3 protein complexes with a slight exception after 1h illumination of the leaves. At this time point more Lil3 protein complex appeared to be present than in any of the other developmental time points.

In summary, the experiment as shown in the representative example in figure 3.16 shows a very clear correlation between the Lil3 protein detection by the Lil3 antibody and the auto-fluorescence pigments. I was therefore concluded that the autofluorescent chlorophyll molecules are most likely bound to the Lil3 protein complex.

## 3.7 Dotblot analysis for quantification of Lil3 protein accumulation

For the quantification of Lil3 proteins during the greening of etiolated barley seedlings, it was investigated whether a dotblot analysis of Lil3 proteins utilizing an extraction of the proteins with the detergent SDS was possible in order to simplify the process of sample preparation for protein quantification (fig 3.17).

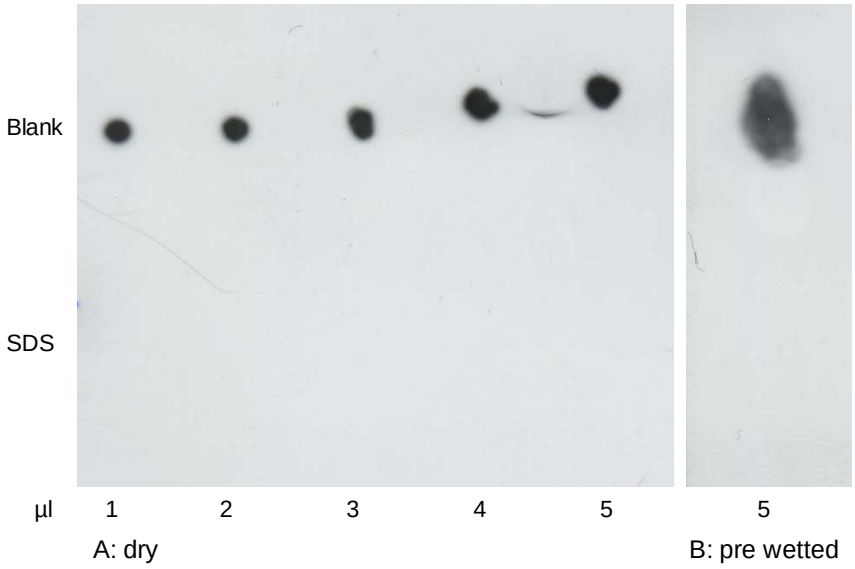


**Figure 3.17: Dotblot analysis of Lil3**

**A:** Dotblot analysis of Lil3 in increasing amounts of chloroplasts. Increasing volumes of a chloroplast suspension was loaded directly onto a nitrocellulose membrane. Chloroplasts were solubilised in SDS (SDS) or no detergent (Blank) was added to the chloroplasts before application of the chloroplast to the membrane. **B:** Dotblot analysis of nitrocellulose membrane prewetted in 1xTBS before application of the samples. Identification of proteins was achieved utilizing the Lil3/HRP antibody system.

SDS samples of isolated chloroplasts were directly applied on the blot (Fig 3.17.A). In the blank sample, no detergent was added. Different volumes dilutions of sample were loaded on the respective dots to test the volume of sample that should be added. The signal was absent in the dots containing SDS- detergent. There was however a clear signal from all the dots of the blank sample which increased slightly with increasing amount of applied sample.

For one of the dotblots, the blot membrane was soaked in 1xTBS before applying the sample, to test if a pre wetted membrane improves the binding capacity for the proteins (fig. 3.17.B).The soaked membrane didn't show any signal for the SDS-sample and in the blank the signal was weaker and smeared out on a larger area than in the blot that wasn't soaked in TBS.

Since the SDS detergent didn't work on the dotblot, samples with other detergents were also tested (fig. 3.18).

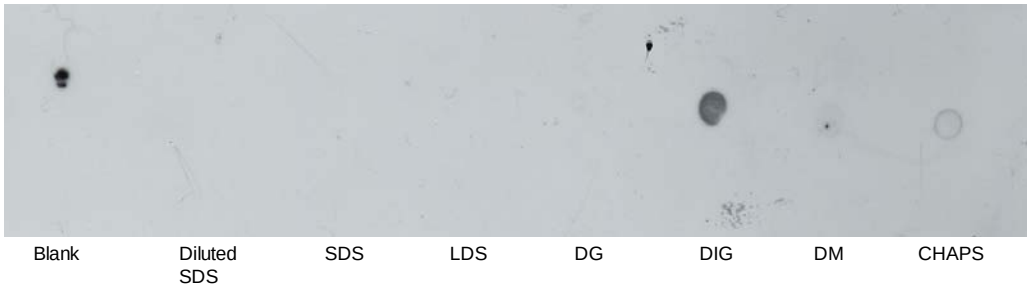


**Figure 3.18**: **Dotblot of leaf proteins solubilised by detergents SDS, LDS, DG, DM and CHAPS.**

Lil3 antibody was used for the detection of proteins.

The addition of a detergent to the plastids was suspected crucial for extraction of membrane proteins and since the Lil3 protein was shown to be exclusively located in the membrane phase solublization by a detergent appeared necessary (see fig. 3.10). There was no signal in the dots loaded with samples containing SDS, diluted SDS, LDS and DG respectively. In the dot solubilised with Digitonin (DIG) only a weak and smeared signal could be detected. In the dot where CHAPS was added, a weak spot was visible however it was not very clear. The DM sample gave the weakest of the three visible detergent dots. In the blank, the antibody signal was clear but in a small area.

## 3.8 Amount of leaves

As the detection of Lil3 proteins after direct application of SDS solubilised proteins on the blot membrane did not work, SDS solubilised ground leafs were run on SDS gels before immuno detection. In order to get the best signal possible for the quantification, the number of leaves that should be used in each sample was tested. Parallels of ten second illuminated (10s) and green (C) leaves were tested, since the amount of at least chlorophyll binding proteins changed between these stages. Samples containing one, two and three leaves respectively were prepared by grinding, and run on SDS gels. A part of the gel was stained by Coomassie (fig. 3.19).

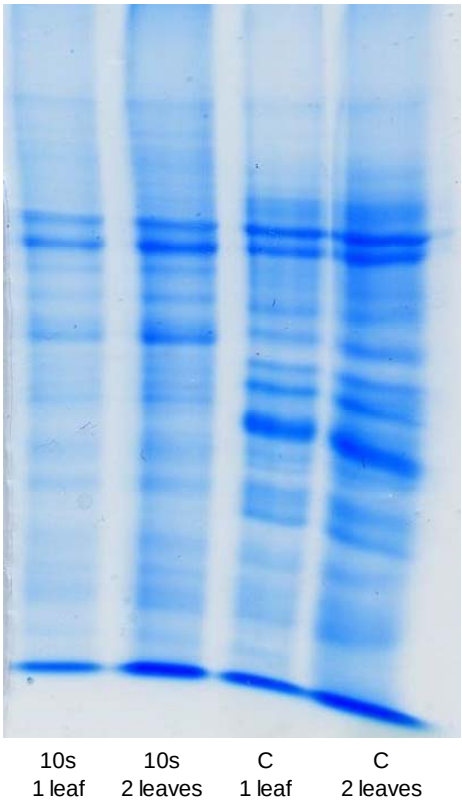


**Figure 3.19: Comassie stained SDS protein gel.**
The lanes are parallels of ten second illuminated (10s) and green (C) leaves. Both leaf types were tested with samples containing one and two leaves.

Bands in wells with two leaves were stained more intensively than the lanes containing one leaf. This was due to a higher protein amount, and could be seen in both the green (C) and the ten second illuminated (10s) leaves. Both lanes of the C had some wider bands than the 10s. This was because there was more protein accumulated in the C than in the 10s.

The SDS gels with the different number of leaves were blotted and the Lil3 was detected by Lil3 antibody. The signal of the antibody was increasing parallel with the increasing amount of plant material in both the green (C) and the ten second illuminated (10s) leaves (fig. 3.20).

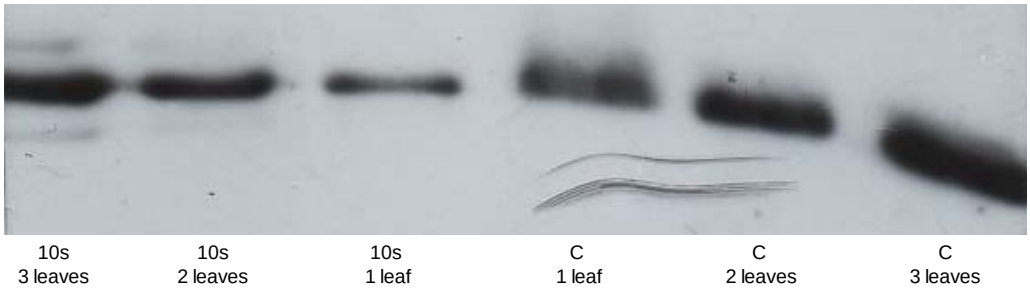


**Figure 3.20: Gel-blot of Lil3 after SDS-PAGE.**

Parallels of ten second illuminated (10s) and green (c) leaves were tested. Both leaf types were run with samples containing one, two and three leaves.

The signal was very weak in the one leaf samples. In the two leaf samples the signal was clearer for both leaf types. However the best signal for both the ten second illuminated (10s) and green (C) leaves was in the sample containing three leaves. Three leaves were therefore used for quantification.

## 3.9 Using frozen leaves for the quantification

For practical reasons it was tested if the leaves could be frozen after harvesting, before the grinding. The frozen leaves were therefore run on an SDS gel together with directly ground leaves. The parallels were of different leaf types; green leaves (C), one hour illuminated (1h), ten second illuminated (10s) etiolated (E). Some SDS gels were stained with Coomassie (fig 3.21).

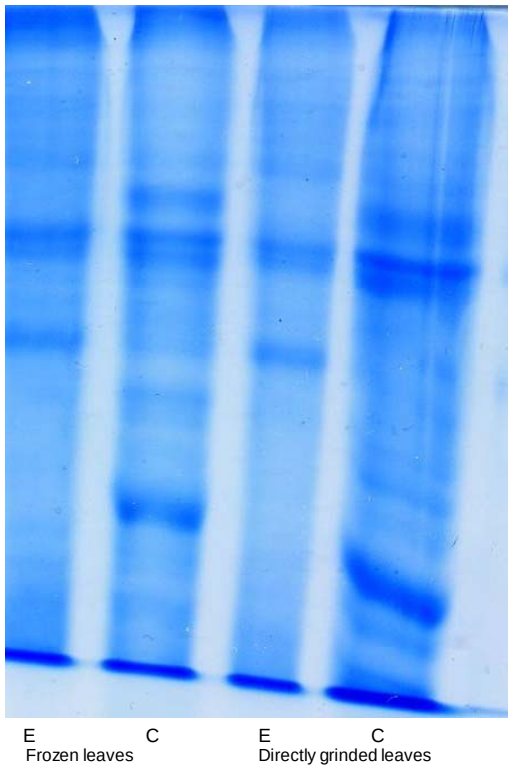


**Figure 3.21: Coomassie stained proteins of green (C) and etiolated (E) leaves on a SDS gel.**
The two lanes on the left side of the figure are the frozen leaves, and the two lanes on the right side are the directly ground leaves.

In the Coomassie stained SDS gel there was no visible difference between the frozen and the directly ground leaves. Here, proteins were also detected by the Lil3 antibody (fig 3.22).

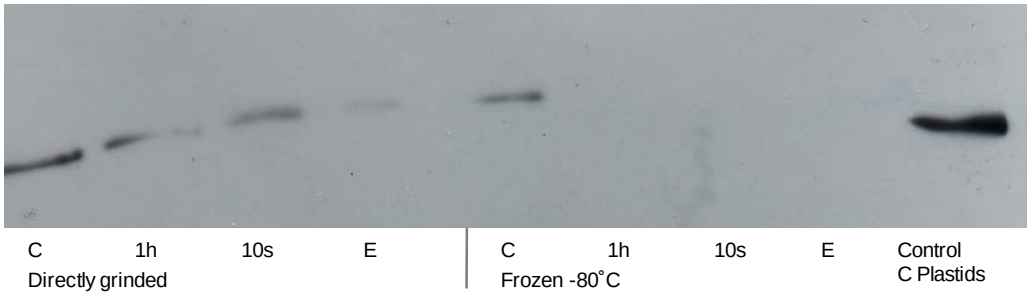


**Figure 3.22: Gel-blot of a SDS gel with directly ground leaves and frozen leaves at different stages of greening.**
The samples were of green (C), one hour illuminated (1h), ten second illuminated (10s) and etiolated (E) leaves. Proteins were detected by Lil3 antibody.

In the lanes containing frozen leaves, the signal was absent, except for the lane containing the green leaves (C). In the directly ground leaves all leaf types gave a signal. The etiolated leaves (E) were hardly visible. The ten second illuminated leaves (10s) was a little clearer but weaker than the one hour illuminated leaves (1h). Signal of the chloroplasts was the clearest, except for the control containing isolated plastids of chloroplasts.

## 3.10 Weighing leaves

For the quantification of the Lil3, it was important to be sure that one cm of leaf contained the same mass of plant material in each of the leaf types. The yellow etiolated leaves seemed to be more compact, and therefore seemed to weigh more than the green leaves. Therefore the mass of six parallels was measured from leaves exposed to light for one hour (1h) and the green leaves (C) respectively. Every parallel contained ten leaves, each leaf with a length of one cm. Mean values of the measurements were calculated. The Standard deviation was also calculated (fig. 3.23).

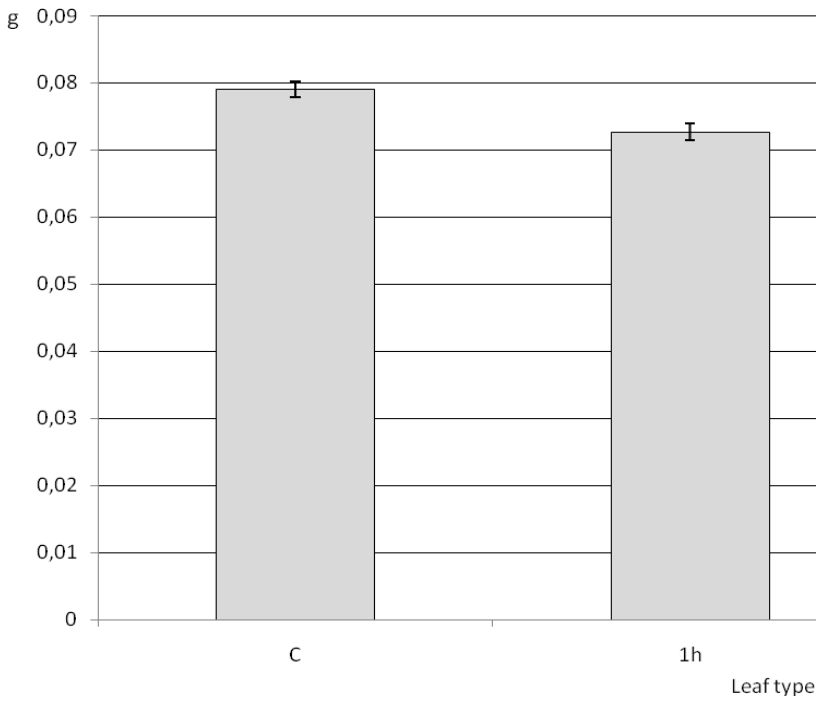


**Figure 3.23: The mass (g) of green (C) and one hour illumiated etiolated (1h) leaves.**
The standard deviation and mean values are based on six paralells.

The mean value were higher in the green (C) leaves compared to the one hour illuminated (1h), with a difference between the mean values to be 0.0064 gram.

## 3.11 Standard curve for quantification

In order to relate the proteins in the quantification back to the amount of plastids and to test how the correlation between an increasing amount of Lil3 protein and the signal intensity of the Lil3 antibody could be described best, a standard curve was prepared. It was made by adding different volumes of sample to SDS gels. The leaves used were illuminated for ten seconds (10s). For the volumes and the corresponding amount of plastids added, see table 3.1.

**Table 3.1:** The amount of plastids corresponding to volume added to each well in figure 3.21 and 3.22.

| **µl of sample** | **Amount of plastids** |
|---|---|
| 0,3125 | $3,47*10^5$ |
| 0,625 | $6,94*10^5$ |
| 1,25 | $1,39*10^6$ |
| 2,5 | $2,78*10^6$ |
| 5 | $5,56*10^6$ |
| 7,5 | $8,33*10^6$ |
| 10 | $1,11*10^7$ |
| 15 | $1,67*10^7$ |

One of the SDS gels in each parallel was Coomassie stained to compare the total protein amount applied to one lane to the signal of the film (fig 3.24).

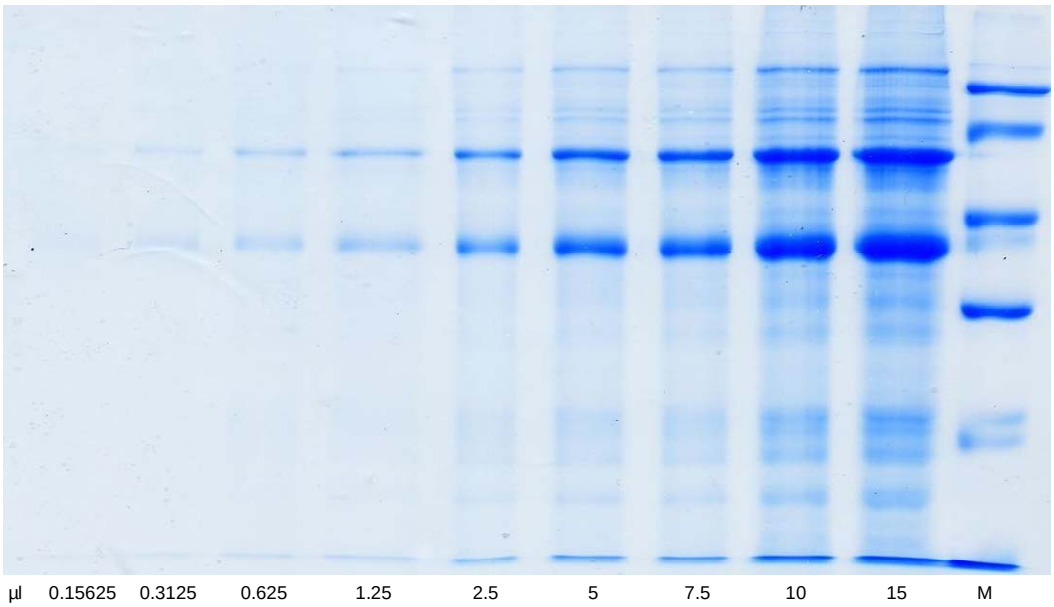


**Figure 3.24: Different amounts of plastids of 10seconds illuminated leaves**

Different amounts of plastids of 10seconds illuminated leaves were loaded on an SDS gel to make a standard curve.The proteins were stained with Coomassie blue.

The protein amount was clearly increasing in each band by the increased amount of plastids added to the wells. In the lane with lowest amount of plastids ($3.47*10^5$ plastids) no bands were seen. The bands were more clear blue for each lane of increasing protein amount. In the last two lanes, many bands of protein were clearly visible.

The Lil3 antibody and the ECL system were used for protein detection and the signals on the films (fig 3.25).

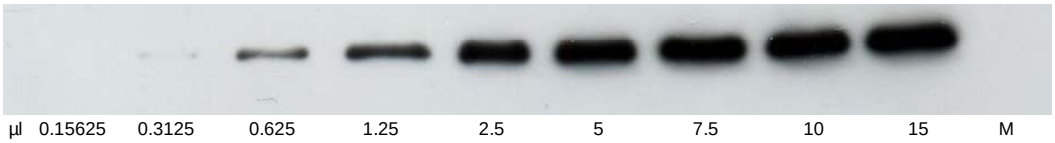


**Figure 3.25: Different amount of plastids of ten seconds illuminated leaves**

Different amount of plastids of ten seconds illuminated leaves, run on an SDS gel to make a standard curve. Detection was by Lil3 antibody.

An inceased amount of Lil3 protein was visible on the films by increasing amount of plastids added to each well. The proteins were hardly visible for the wells with the two lowest amounts of plastids. In the line with $3.47*10^5$ plastids (0.15625 micro litres) no signal could be seen. The next two bands were weak but increasing. Between $2.78*10^6$ and $8.33*10^6$ plastids (2.5-7.5 micro liters) the signal of the bands was very clear. In the lines containing the highest amounts of plastids, the signal was even higher. The amount of plastids was relating to the signal strength of the Lil3 antibody, and thereby amount of Lil3 protein.
In order to make a standard curve, the signals of the films needed a correlating number. This was done by the TINA 2.0 program. A standard curve was then made by transforming the numbers to Microsoft Excel (fig. 3.26).

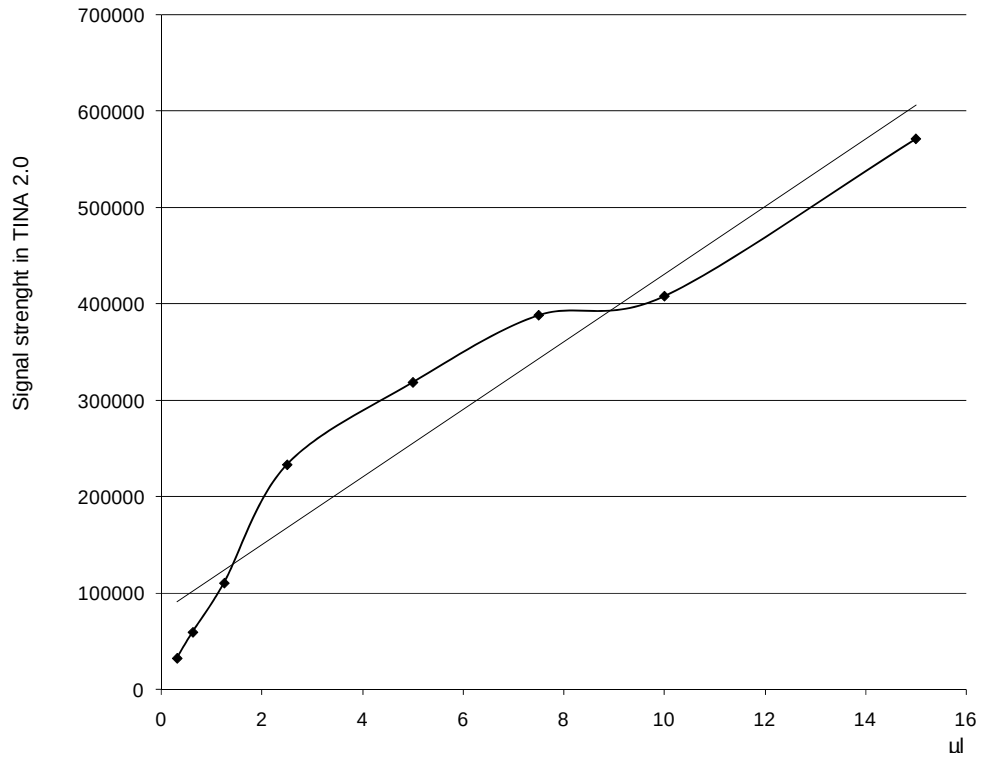


**Figure 3.26: Standard curve for quantification.**
The data were collected in 4 parallels. Signal strength of the antibody was plotted against the volume of proteins added.

There was an increase of signal from the Lil3 proteins when the amount of proteins increased, like could be seen in the films. In the diagram it looked like that there was a linear correlation between the amount of protein and the amount of signal increase therefore a linear correlation for the following quantification was used.

## 3.12 Quantification of Lil3 in plastids

The quantification was first tested with isolated plastids. The plastids from the green (C) and the ten second illuminated (10s) leaves, were isolated and prepared for SDS electrophoresis. Four parallels of both the 10s and the C were run in the gel. The plastids of both groups were of the same isolation. The gel was blotted, and the proteins were detected by Lil3 antibody (fig. 3.27).

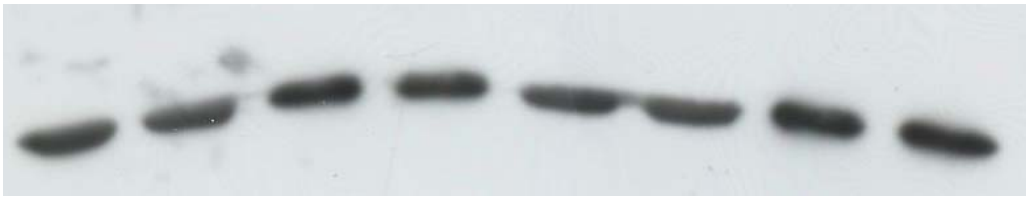


**Figure 3.27: Plastids of green leaves (C) and ten second illuminated leaves (10s) run on SDS gel.**

Protein detection by the Lil3antibody.

The signal seemed a little weaker in the four parallel bands of the chloroplasts, compared to the ten second illuminated plastids. However the difference was not strong. The signals were transferred to Excel (fig. 3.28).

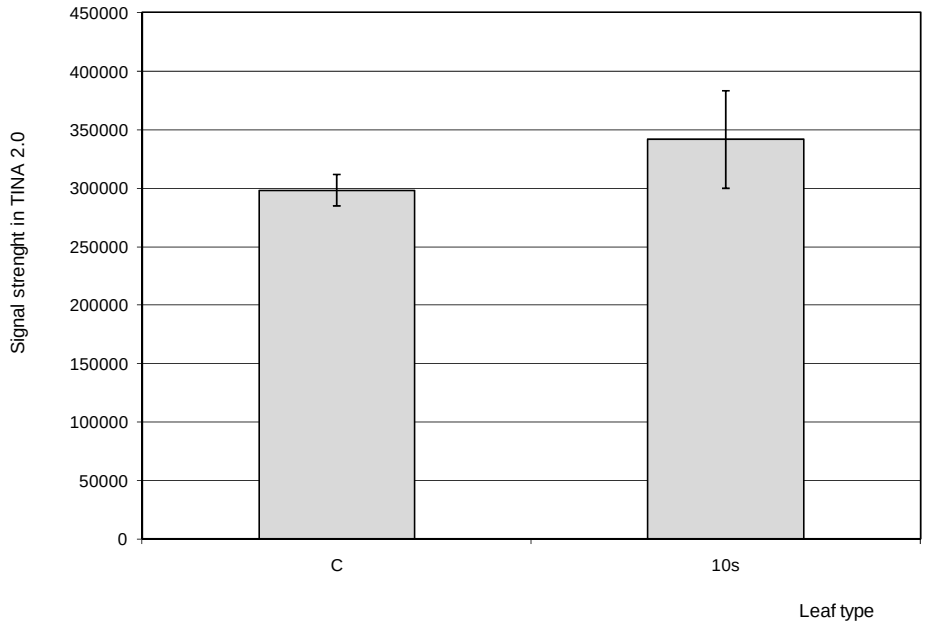


**Figure 3.28: Figure 3.25 illustrated by excel.**
Lil3 antibody signal strength plotted against type of leaf.

The mean value of the green leaves (C) was lower in signal strength compared to the ten seconds illuminated (10s) as seen on the film. Especially for the 10s sample, the standard deviation was high, so in the end there was no significant difference between the leaf types detectable.

## 3.13 Quantification of Lil3 proteins in ground leaves

The amount of Lil3 proteins was quantified in barley during deetiolation. The leaves were exposed to light for hence zero time (etiolated, E), ten seconds (10s), one hour (1h), two hours (2h), four hours (4h) and during the entire growth phase (fully developed chloroplast, C). The leaves were then ground and prepared for SDS electrophoresis. Lil3 proteins were detected by Lil3 antibody. The last time point of one gel was always put on the next gel to make the single gels comparable. For instance the one hour illuminated (1h) leaves were run on the gel with the etiolated leaves (E) and the ten seconds illuminated (10s) leaves. However they were also run on the gel with the two hour (2h) and the four hour (4h) illuminated leaves. The same type of experiment was carried out for the four hour (4h) illuminated leaves (see fig.3.29).


**Figure 3.29: Three exemplary gels used for quantification of the amount of Lil3 in the different stages of deetiolation.**

**1:** Etiolated (E), ten second illuminated (10s) and one hour illuminated (1h) leaves. **2:** One hour illuminated (1h), two hours illuminated (2h) and four hour illuminated (4h) leaves. **3:** Four hour illuminated (4h) and green (c) leaves. After SDS electrophoresis, the proteins were detected by the Lil3 antibody.

The signals on the film were highly divergent from one paralell to another. The signal of the one hour (1h) were higher than the two hour (2h) illuminated leaves in gel number two in the middle. In gel number one, the signal of the 1h was as weak as the etiolated (E) and the ten second (10s) illuminated leaves. The signal of the one of the four hour illuminated leaves (4h) in gel number three by visual inspection appeared to be higher than all of the green leaves (C). However some of the 4h also seemed as weak as the C.

For the different developmental stages, there was a variation in the number of successful parallels. For the E there were 27 parallels, 10s; 25 parallels, 1h; 48 parallels, 2h; 21 parallels 4h; 33 parallels and C; 11 parallels. Film signals were transformed to a number by TINA 2.0 and the summing up and quantification was carried out in Excel. The numbers were presented graphically by Excel (fig. 3.30).

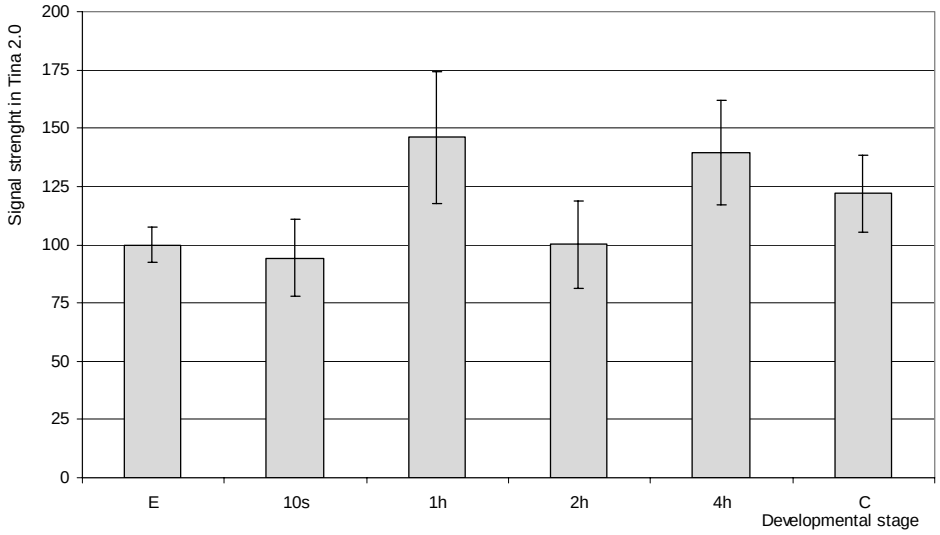


**Figure 3.30: The amount of Lil3 protein during the different stages of greening of the plastids.**

Signal strength was calculated by TINA 2.0 due to signals of the Lil3 antibody. E is the signal of dark grown etiolated leaves. The 10s, 1h, 2h, 4h is dark grown leaves illuminated for ten seconds, one-, two- and four hours. The C is leaves grown in the light. The standard error of deviation is given for each of the developmental time points.

The ten second illuminated (10s) leaves seemed to have the lowest signal strenght corresponding to the Lil3 protein. The signal of the etiolated leaves (E) was a little higher (6 %) than for the 10s. The signal for the two hour illuminated leaves (2h) was almost the same as for the E (1.5 % higher). The signal of the 1 hour illuminated (1h) leaves was around 45 % higher then the E and the 10s. The value for the green leaves (C) was 22 % higher compared to the E. The amount for four hours illuminated leaves (4h) was 39 % higher than the E.

The highest signal of Lil3 protein was hence seen in the one hour illuminated leaves (1h). The highest difference in protein amount was between the etiolated and the one hour illuminated leaves. The 4h and C were also higher than the other groups.

The variations within each group are visible by the standard deviations. The highest standard deviation was seen in the 1h leaves. This goup also had the highest number of paralells. The C however had a low number of parallels and a low standard deviation. The E had a high number of parallels but a low standard deviation compared to the 1h. The other groups (10s, 2h) had a high number of parallels and a high standard deviation like the 1h.

# 4 Discussion

## 4.1 Identifying the Lil3 protein

To identify the Lil3 protein, a combination of immunodetection and mass spectrometry was used. At first, the Lil3 protein had been detected by the Lil3 antibody at a molecular mass around 29 kDa (fig 3.1). However, when Lil3 had been first identified in barley, the full length of the Lil3 proteins had been calculated to have a molecular mass around 25 kDa (Reisinger et al. 2008c). The causes that can bring about such a large deviation in the determination of the proteins molecular mass will have to be investigated in detail and are difficult to explain. It however is important to note that in all molecular mass determinations of proteins in SDS-PAGE, determinations are performed relative to a set of molecular mass standard proteins. If alterations in the molecular mass of these proteins have taken place, no normalization of the data can be performed and hence the two independent findings can no longer be compared. Also, it has to be considered that a high number of factors influence the mobility of proteins within the network of polyacrylamide. Among these, the loading of the molecular mass standard and sample proteins with the charging molecule SDS is the most important factor to influence the mobility of the ionized proteins in the electric field. It is therefore also well conceivable that in the two experiments, loading of the standard or sample proteins with SDS was different, whereby the Lil3 proteins in the two experiments appeared to move at different velocity relative to the molecular mass standard. It was concluded that the main information from this experiment was not the correct determination of the proteins molecular mass, but the immunological identification of the protein among the mixture of plastid proteins from barley plastids that had been separated by SDS-PAGE.

An alternative method for molecular mass determination of proteins is mass spectrometry. Here, the methodology was not employed for determination of the proteins molecular mass but the protein was cut into peptide fragments in order to determine the aminoacid sequence of the peptide fragments. This method was used in order to ensure by a second technology that the protein identified by

the immunological approach is indeed the Lil3 protein. Usually both, an identification of proteins by MS and a verification of the proteins by immunological gel-blot analysis is performed and method complement well to ensure the identity of proteins (Granvogl et al. 2006). For MS analysis, a gel was cut in the area of Lil3 antibody signal (fig 3.2), the proteins were digested into peptides and after extraction from the gel, and peptides were analyzed by mass spectrometry (MS).

Usually, two sets of experiments are preformed in the mass spectrometer. First, an MS spectrum of the peptide mixture is recorded and thereafter one of the peptide is selected by a first mass filter in order to selectively break down this peptide into even smaller peptide fragments (fig 3.3 and 3.4). The different fragments are then aligned with the help of computer algorithms to determine the sequence of aminoacids in the different peptide fragments. Then databases can be searched to align the found sequence back to any other aminoacid sequence already determined in other research groups. However, a number of factors may affect this procedure. The preparation of the peptide from a low amount of protein is the most likely to generate protein loss. In the case of the Lil3 protein, the amount of protein in the gel for mass spectrometry identification is critical. However, the detection of the Lil3 protein was based on antibody detection of the protein and amplification of the signal. Especially, the antibody/protein interaction was amplified. With the help of an enzymatic reaction linked to a secondary antibody, a luminescence based amplification of the protein is achieved. Luminescence then leads to a high number of silver grains deposited in the negative film. If the gel-blot membrane is exposed to the negative film for a long time, a low amount of protein on the gel-blot will appear as a strong band. This can easily be misinterpreted as if there would have been a high amount of protein on the gel-blot. In summary, the gel-blot experiment indicated that a high amount of Lil3 protein was present in the polyacrylamide gel. However, for mass spectrometry based aminoacid de novo sequencing work, none of these methods applied relevant. Here, the protein was treated directly in the amount it was present in the gel. Hence, it was not unexpected that the signals obtained in the mass spectra were of low quality. In contrast to the high degree of peptide losses

that had to be expected in addition during the sample preparation, a very nice de novo sequence could be obtained for one of the Lil3 peptides. Hereby, it was an advantage that the identical peptide had already been identified in a previous work from Lil3 of barley (Reisinger et al. 2008c), the exact mass to charge ratio of the peptides were known, and hence fragmentation analysis could be performed with high precision.

Since the antibody was constructed to bind a specific antigenic peptide sequence selected from within the Lil3 protein, at least this part of the Lil3 protein was expected to be identified by the antibody. The question however was also if the polyclonal antibody was monospecific for the Lil3 protein. In the short time illuminated dark-grown seedlings, the Lil3 was found to be the only protein detected at a molecular mass of 25 kDa (Reisinger et al. 2008c). Also in this work the only signal detected in the gel-blots after SDS-PAGE was a protein band in about the correct molecular range (fig 1.1). Based on the parallel identification of one Lil3 peptide we concluded that the Lil3 antibody was monospecific for and only detecting the Lil3 protein. We however already speculated here, whether the antibody detected the original peptide sequence in a denatured state or a reacted against a folded state of the protein sequence. Both reactions were in principal possible, since the denatured proteins separated via SDS-PAGE were transferred to a nitrocellulose membrane after separation by SDS-PAGE had been completed and it is known that a partly renaturation of the proteins takes place during this gel-blot transfer. Hence, the antibody/protein interaction could have well been observed by an antibody binding a renatured Lil3 protein. However, in both cases –either reacting against a denatured or renatured Lil3 protein- the reaction appeared monospecific for the Lil3 protein.

## 4.2 Testing the Lil3 antibody

Antibodies have been widely used for identification and quantification of proteins, to study their location, structure, and function. In plants, examples for studies were antibodies have been used has been in the quantification of LHCII during greening (Mathis and Burkey 1987), the role of Chlorophyll in stabilizing apoprotein (Müller and Eichacker 1999) and the binding of FNR to the PSI

(Andersen et al. 1992). In quantification studies, antibodies have also been used. Successful quantification of decease marker proteins in human cells (Yamada et al. 2009) or quantification of proteins in bacteria may also be mentioned as an example (Gehring et al. 2006). Here, a Lil3 antibody was used for the identification and selective quantification of the Lil3 protein.

In order to characterize the binding specificities of the antibody/Lil3 protein interaction, dilutions series of the polyclonal antibody were generated (Fig 3.6). This type of experiments allows selecting a specific dilution of the concentrated antibody stock. The dilution factor is the critical element for determination of the degree of signal amplification that can be achieved in the experiments. If a too high dilution factor is chosen for the experiments, the concentration of antibodies relative to the amount of protein immobilized on the nitrocellulose will be too low. Then the antibody based signal can not follow an increase in the amount of protein and the signal readout will be saturated. On the reverse, a too low dilution factor will over saturate the amount of protein present of the gel-blot and cross-reactions of the antibody with other less specific proteins on the gel-blot will be detected. Therefore a dilution factor was selected to enable a monospecific and non-saturated detection for a medium amount of protein. As basis for selection of a defined amount of protein, the amount of plastids was selected (methods). In practical terms, the highest dilutions were not used because the signal output was simply too weak. Among the lower dilutions, the signal was clearly overexposed. We therefore selected an intermediary dilution to pick up the changes among the Lil3 proteins.

Experiments conducted with this antibody dilution were then tested for binding of the Lil3 protein in the different developmental states. In these experiments, it became evident that the antibody could detect not only proteins after denaturation in SDS-PAGE. Also, gel-blots conducted after separation of the Lil3 protein in the native state could be detected well (fig 3.7). Two bands were detected in the native state. To ensure specific binding of the primary antibody, also the secondary antibody was tested. This was a remarkable finding and enabled the setup of experiments to quantify the Lil3 protein in the native and denatured state.

However, when the reactivity of the Lil3 antibody was tested against native Lil3 proteins that were isolated by blue native and clear native PAGE, a difference in the quality of the Lil3 antibody signal was noted. The signal after CN-PAGE was increased relative to the blue native (BN) gel and this was also found for the second dimension SDS gels (fig 3.9). In the gel-blot after BN-PAGE, the proteins were in general detected at a lower molecular mass range relative to the CN-PAGE and also in the 2D CN/SDS-PAGE. This could indicate that the strength of the antibody/Lil3 protein interaction was decreased by BN-PAGE. However, it had already been described that a higher number of complexes were present after solubilisation with digitonin compared to dodecylmaltoside (DM) in BN-PAGE (Reisinger and Eichacker, 2007).

A low amount of LDS is used to charge the complexes in CN-PAGE relative to a high amount of Coomassie for charging the protein complexes in BN-PAGE. This could indicate that solubilisation of the protein complexes rather then the antibody/Lil3 interaction has been the cause for the differences found in the separation of complexes in the two native gel systems. Especially, the decrease in molecular mass of the protein complexes in the first dimension gel indicated that the degree of solubilisation of the protein complexes was increased in the BN-PAGE separation whereby most of the Lil3 protein structure was released. Alternatively, the protein complexes could move at a slower velocity in CN-PAGE since less LDS has been added to the sample. The lower degree of solubilisation could also lead to an increased amount of the lipids that remained bound to the complexes, giving them a higher molecular mass/size and hence lower mobility in the gel. These factors could also explain why the complexes appeared at higher molecular mass range in the CN- compared to BN-PAGE. In contrast to the Lil3 complexes, the $cytb_6f$ complexes were found at the same molecular mass in BN and CN gels. So despite the differences in the mobility of the Lil3 complexes, the methods are fully comparable for separation of the other membrane integral protein complexes. It was therefore concluded that CN-PAGE is the better technology to preserver and separate the Lil3 protein complexes for immunologic analysis of Lil3. In the CN-PAGE, two clear bands were resolved

and also a signal in the lower molecular mass range could be resolve for the studies.

## 4.3 Lil3, a membrane protein

Lil3 belong to the family of LHC proteins including proteins like the ELIPS and PsbS. All LHC proteins share a `generic LHC motif` that code (Jansson 1999). Lil3 was therefore predicted to be a membrane protein. Lil3 was also thought to be associated with the PSII protein (Jansson 1999) which is also located in the thylakoid membranes of plastids. Data obtained for the location of the Lil3 protein corroborated that the Lil3 protein is membrane integral. No signal was detected in the stroma part of the plastids (fig 3.10) and there was only one antibody based signal in the membrane part where the Lil3 signal was found and also the protein could be indentified by mass spectrometry (fig. 3.1). It is therefore concluded that the Lil3 is only present in the membrane part of the plastid. An association of the Lil3 protein with PSII was suggested. However, according to this work, the Lil3 protein is already present in 10s plastids. At this time point, no PSII protein complexes have been detected. It therefore needs a more thorough investigation to clarify whether the Lil3 protein could be part of a PSII complex either early or late during development of the PSII complex in the thylakoid membrane.

## 4.4 Assembly of chlorophyll binding complexes during deetiolation

During deetiolation, of etiolated barley seedlings in white light, synthesis of chlorophyll is induced in the plastid and a complete restructurization of the plastid is initiated. Especially the inner membrane systems of these two development states of the plastid are characterized by a completely different pigment and protein composition. One of the proteins with a central function for binding of the de novo synthesized chlorophyll in this biogenetic process could be the Lil3 protein. It was therefore the concept of this work to quantify the Lil3 protein. In the direction of this concept, the question was addressed, how much of the protein is present at the different time points during the development of chloroplasts from etioplasts. The native CN-PAGE appeared as the most important tool in this

context to differentiate between a binding of chlorophyll to the Lil3 proteins and all the other chlorophyll binding protein complexes of the photosynthetic machinery. In the etioplast stage of development, only autofluorescence from the Cyt $b_6f$ complex could be recorded (fig.3.11). However, already after ten second of illumination, the Lil3 protein complexes and after one hour of illumination the chlorophyll binding complexes of PSI/II were detected. One hour later, the PSI LHCI complex is detectable in the autofluorescent gel-blot. After one hour of development, photoactive PSI and after two hours of illumination reduced Ferredoxin has been identified (Ohashi et al. 1989). Both sets of data are therefore in good agreement. However, how the assembly of the chlorophyll molecules with this photosystem protein complex is regulated is still a miracle (Eichacker, personal communication).

The autofluorescent identification of the photosystem complexes occurred via the chlorophyll bound to the protein. Although, the molecular mass of the protein complexes clearly correlated well with the corresponding complexes isolated in chloroplasts, identification was still indirect. However direct proof for the presence of the proteins in the protein complexes can be achieved by binding of an antibody. In the case of the Lil3 protein complexes this was achieved by binding of Lil3 antibodies linked with a Cy3 coupled secondary antibody. The method has been introduced by Reisinger (2008a; Reisinger et al. 2008c) to detect plastid proteins of barley in 2D native-/SDS PAGE. Here, the overlay with the auto fluorescence scan for the presence of chlorophyll provided the first evidence that this method could enable a characterization of both protein and pigment by fluorescence. However, optimization of the gel-blot transfer will be necessary to ensure a low background for detection of the very weak autofluorescence from the photosystem complexes at the early developmental states. At the later stages of development, the challenge will be to titrate the amount of membrane protein loaded per lane onto the electrophoretic system since here the high chlorophyll autofluorescent signal is much stronger then the weak fluorescent signals originating e.g. from the Lil3 protein complexes. (Fig 3.11-3.16).

## 4.5 Testing methods for quantification

The dotblot is a simplified way of the immunodetection. In the dotblot the sample is directly applied to binding onto the nitrocellulose membrane (fig 3.17.A). The signal of the Lil3 identification which originates from the luminescence of the HRP linked secondary antibodies was detected on a negative film. A quantification of Lil3 could then be achieved by point wise readout of the negative film image. Primary antibody dilution and plastid concentration were the central determinants to ensure that the signals on the negative film could be related back to the dotblot. On the basis of high specific antibody detection, the advantage of a dotblot over a separation of the proteins by SDS-PAGE clearly lies in the speed to obtain meaningful results. However, in the experiments conducted, the antibody detection did not work properly when the sample extraction was performed in the presence of SDS (fig 3.17.A). Also prewetting of the NCM did not overcome the detection problems (fig 3.17.B). Dotblots have been used e.g. to analyze phosphopetides (Wang et al. 2009) and Psbs mRNA in spinach (Funk et al. 1995). In these approaches, no detergents had been present in the samples. It therefore will have to be tested in later experiments whether a detergent free analysis could be established. An alternative approach could be to bind the Lil3 antibody to a solid support, like a protein A linked to a sepharose bead. Then the protein-A could be bound to the Fc arm of the Lil3 antibody. The detergent could then be added and the Lil3 protein could be retained in a column after washing off of the other parts. The Lil3 protein could then be quantified in a SDS gel via staining or blotting. This method would however require a lot of antibody to ensure that all the proteins have been retained in the column. Since we suspected the detergent to be important for complete extraction of all the Lil3 from the membrane, it was decided to perform the quantification of Lil3 by SDS dependent total leaf extraction and separation of the membrane proteins in SDS gels.

For the quantification of Lil3 proteins, first the number of leaves to yield a well detectable signal by the Lil3 antibody was tested (fig 3.19 and 3.20). Leaves were cut a centimeter from the top. A number of three leaves enabled to achieve reproducible and clear results (fig 3.20). In other experiments, a defined amount

of plastids (Müller and Eichacker 1999) or a certain mass of proteins (Mathis and Burkey 1987) have been used to form a basis for the determination of the total amount of protein. In these experiments a certain number of leaves were used. All seedlings were measured and cut the same way. Unfortunately, freezing of the leaves prior to protein extraction did not yield consistent results, since hardly any antibody binding could be established with the extracts isolated from the frozen leaf material after thawing (fig 3.22).

However, in other approaches, frozen samples have been widely used for quantification of proteins in barley (Eichacker et al. 1990; Holtorf and Apel 1995, 1996; Mathis and Burkey 1987). There was no problem with the signal of the antibody after freezing of the sample in these experiments. In contrast to the grinding of material in liquid nitrogen, in these experiments leaves were frozen directly at minus 80 degrees before grinding. After freezing, the leaves were ground in TMK buffer and then the membranes were solubilised. It therefore appeared that the type of freezing affected solubilisation of the proteins, or the detection by the antibody. In order to avoid signal losses freshly grinded leaves were used throughout the experiments to enable quantification of the Lil3 protein. As an additional basis for quantification, the mass of the leaves was measured in addition to the leaf number and an equal leaf mass was used as starting material (fig 3.23). The leaf mass appeared as a useful basis for the protein determination, since the structural compositions of the etiolated and the green leaves were completely different. While etiolated leaves were more round and compact, green leaves were more extended and flat. However, the mass differences between the different types of leaves was found to be low and not considered significant for the quantification.

## 4.6 Standard curve for quantification

Standard curves are in general the means to quantify a signal output per a variable unit. For example, the amount of LHCII per thylakoid was quantified after thylakoid isolation from barley and other species (Mathis and Burkey 1987). In this work, a standard curve was set up to correlate the Lil3 signal on the negative film to the increasing amount of Lil3 protein present per plastid (fig 3.24 and

3.25). Hereby, the amount of Lil3 protein can be related back to the amount of plastids present in the leaf. However, since here not the amount of plastids but leaf number was the basis, the amount of Lil3 proteins could not be directly related back to the amount of chloroplasts. As an alternative, the dry weight of the leaves before or after grinding could have been used. Then the antibody based Lil3 signal could be related to the increasing mass in a standard curve. According to the results, the standard curve showed that there was a direct correlation between the amount of Lil3 proteins present in the ground leaves, and the signal strength of the Lil3 antibody in the negative film (fig 3.26). In other words, by increasing number of Lil3 proteins an increase of the antibody signal could be seen on the film.

## 4.7 Quantification of proteins in plastids

In order to see if there was a correlation between the amount of Lil3 protein in ground leaves and plastids, the quantification was tested with proteins from 10s plastids and from chloroplasts. (fig 3.27). Although the single signal data implied that a higher amount of Lil3 was present in the 10s plastids relative to the chloroplasts, standard deviation calculations indicate that the difference was not significant (fig 3.28). It was also noted that in this experiment all parallel test were taken from the same isolation of plastids. Hence, an error in the determination of the plastid number would therefore influence all measurements. It is therefore suggested that the experiments will be repeated with a higher number of independent plastid isolations to strengthen the validity of the data.

Proteins have been successfully quantified with antibodies in earlier experiments. In this quantification experiments, the protein amount was measured by the signal strength of the antibody on a film. Quantification experiments were performed by Mathis and Burkey (1987) and (1994) where LHCII proteins increased during greening. The proteins were detected by HRP coupled to secondary antibody and compared to a standard curve against known amounts of LHCII. Also, PORA was shown to increase and PORB was shown to be constant during illumination of dark grown barley seedlings (Holtorf et al. 1995). Also here, the amount of proteins was analyzed by the signal strength of the second

antibody. However in these experiments the goal was to see the development of protein during the night/day cycle.

## 4.8 Quantification of Lil3 proteins in ground leaves

As a final test to characterize the kinetic changes of the Lil3 protein during leaf development, SDS-gels were prepared from extracted leaves for each of the time points. Gel-blot analysis however still revealed a high variability in the amount of Lil3 protein per time point. It was therefore not possible to immediately see the outcome of the experiment within one of the single experiments by eye (fig. 3.29). However, when all the signals were assembled in a graph (figure 3.30), an increase of Lil3 proteins between etioplast (E) and the four hour illuminated (4) leaves and the chloroplast (C) were found. The 4h values were on average 39 percent higher than the E values. In C the corresponding values were 22 percent higher than the E values. However, the standard deviations in all time points were high. In the two hour illuminated leaves (2h) the mean values were only 1.5 percent higher than the E values and in the 10s illuminated leaves the mean values were even six percent lower than the E values. All data were outnumbered by the one hour illuminated leaves (1h). Here, mean values were 45 percent higher than in the E values.

Overall it is concluded that antibody based Lil3 signals indicate an increase between the E and the 1h, 4h and C values. This would indicate that the amount of Lil3 increases during the later stages of illumination. However, data are in conflict with the 2h values were the amount of Lil3 protein decreases. According to the standard deviations in all of the groups this maximum at 1h appeared not significant. Hence, an overlap between the standard deviations in all time points appeared to indicate that the amount of Lil3 does not change during development of the green leaf and parallel biogenesis of the photosynthetic machinery.

Earlier experiments (Eichacker, personal communication) show that during the first 15 minutes of deetiolation, the amount of chlorophyll exceeds the amount of chlorophyll binding proteins of PSI/II by a factor of 15-35-fold. The amount of apoproteins (P700, D1/2 and CP43/47) in barley was measured by increased

synthesis of chlorophyll a by Eichacker et al (1996a; Eichacker et al. 1990). The amount of the apoproteins increased to a point, and then it was stabilized, as the proteins were saturated by chlorophyll (1996a; Eichacker et al. 1990). The apoproteins were measured by the scanning for radiolabel incorporated in the proteins. This was done to find out if the apoproteins were stabilized by chlorophyll a, because it was known that the transcripts of these proteins were present in the dark (Eichacker et al. 1990). Since the information about the synthesis of Lil3 protein is limited it will be a challenge to investigate the de novo Lil3 synthesis. However, it should be possible to directly measure the synthesis via the increase in the signal strength of the Lil3 antibody and thereby quantify the synthesis and accumulation of the protein, since the antibody was tested to be specific for the Lil3 protein (section 4.1). Lil3 was proposed to have a function as a storage/transport complex for stabilizing and delivery of chlorophyll to other chlorophyll binding complex (Reisinger et al. 2008c). Hence, the high request for binding of chlorophyll at the beginning of green could explain why the amount of Lil3 shows a maximum after one hour of illumination. However, the protein could also have a role in delivering protochlorophyllide to the chlorophyll synthase (Reisinger et al. 2008c).

Lil3 is believed to be a storage/transport complex for delivery of chlorophyll to the chlorophyll proteins during assembly. Hence, the function would be a binding of the chlorophyll released from chlorophyll synthase and a delivery to the different pigment binding complexes (Reisinger et al. 2008c). If the Lil3 protein would increase or decrease parallel with the demand of the different developmental states of the cells for the delivery of chlorophyll, this could explain the variations within the groups. However, it will require more experiments to figure out whether this concept is correct or whether the Lil3 protein has a different function during for the development of the plant cell.

## 4.9 Future experiments

Future experiments will have to make sure that all samples are prepared under exactly the same conditions and leaves will have to be extracted differently to

ensure complete extraction e.g. the use of liquid nitrogen for fixing the cell material should be test thoroughly. A loss of antibody reactivity had been noted for the frozen leaf material. New experiments should figure out why this is the case.

Gel electrophoresis was found to be one of the causes for a high experimental variation. It is suspected that this was mainly the result of different amounts of volume retained in the sample wells during the gel-loading process. Gel loading will therefore be a matter of independent investigation. The different time points of light exposure should also be run on the same gel, to compare the kinetic time points more easily. This would allow running only 50% less experiments and hence more parallel experiments could be conducted. If a good solution can be found for storage of the different sample fractions, it would be also useful to assemble all of the plastid isolations for the different developmental time points and load them on one gel.

In order to understand the development of the Lil3 proteins it could be interesting to find out more about the composition of the proteins in the different Lil3 complexes. This could give an idea about how the protein works. Experiments should include work like the determination of lipophilic/hydrophilic properties and of the subunits from the protein complex. The complex should also be tested for its capacity to act as binding sites to other proteins within the plastid membrane. If possible, the structure of the protein and of the protein complex should be determined.

# 5 References

Adamska, I., M. Roobol-Boza, M. Lindahl, and B. Andersson. 1999. Isolation of pigment-binding early light inducible proteins from pea. *European Journal of Biochem.* 260:453-460.

Albertsson, P. A., E. Andreasson, and P. Svensson. 1990. The domain organization of the plant thylakoid membrane. *Federation of European Biochemical Societies* 273 (1,2):36-40.

Andersen, B., H. V. Scheller, and M. B. Lindberg. 1992. The PSI-E subunit of photosystem I binds ferredoxin: NADP' oxidoreductase. *Federation of European Biochemical Societies* 311 (2):169-173.

Apel, K., H. J. Santel, R. H.J, and H. Falk. 1980. The Protochlorophyllide Holochrome of Barley. *Federation of European Biochemical Societies* 111:251-258.

Ben-Shem, A., F. Frolow, and N. Nelson. 2003. Crystal structure of plant photosystem I. *Nature Publishing Group* 426:630-635.

Blum, H., H. Beier, and H. J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8 (2):93-109.

Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry* 72:248-254.

Brandstveit, T., J. A. Broen, S. A. Hella, K. Nes, D. Sandli, and B. Viken. 2004. *Kulturplantene*. Edited by O. Landbruksforlaget.

Buchanan, B. B., and Y. Balmer. 2005. Redox regulation: A broadening horizon. *Annual review of plant biology* 56:187-220.

Campbell, N. A., and J. B. Reece. 2002. *Biology*. 6th ed. San Francisco: Benjamin Cummings,.

Cramer, W. A., and W. L. Butlera. 1967. Light-induced absorbance changes of two cytochrome b components in the electron-transport system of spinach chloroplasts *Elsevier B.V.*

Dekker, J. P., and E. J. Boekema. 2005. Supramolecular organization of thylakoid membrane proteins in green plants. *Biochimica et Biophysica Acta* 1706:12-39.

Dreyfuss, B. W., and J. P. Thornber. 1994. Assembly of the light-Harvesting Complexes (LHCs) of Photosystem II. *Plant Physiology* 106:829-839.

Eichacker, L. A., M. Helfrich, W. Ru¨ diger, and B. Mu¨ ller. 1996a. Stabilization of Chlorophyll a-binding Apoproteins P700, CP47, CP43, D2, and D1 by Chlorophyll a or Zn-pheophytin a*. *The Journal Of Biological Chemistry* 271 (50):32174-32179.

Eichacker, L. A., M. Helfrich, W. Rüdiger, and B. Müller. 1996b. Stabilization of Chlorophyll a-binding Apoproteins P700, CP47, CP43, D2, and D1 by Chlorophyll a or Zn-pheophytin a*. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 271 (50):32174-32179.

Eichacker, L. A., and V. Reisinger. 2007. *Blue native PAGE for the analysis of membrane protein complexes, basic principles*.

Eichacker, L. A., J. Soll, L. P., W. Rudiger, R. R. Klein, and J. E. Mullet. 1990. In Vitro Synthesis of Chlorophyll A in the Dark Triggers Accumulation of Chlorophyll A Apoproteins in Barley Etioplasts. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 265 (23):13566-13571.

Funk, C., I. Adamska, B. R. Green, B. Andersson, and G. Rengers. 1995. The Nuclear-encoded Chlorophyll-binding Photosystem II-S Protein Is Stable in the Absence of Pigments*. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 270 (50):30141-30147.

Garavito, M. R., and S. Ferguson-Miller. 2001. Detergents as Tools in Membrane Biochemistry. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 276 (35):32403-32406.

Gehring, A. G., D. M. Albin, A. K. Bhunia, S. A. Reed, S. I. Tu, and J. Uknalis. 2006. Antibody microarray detection of Escherichia coli O157 : H7: Quantification, assay limitations, and capture efficiency. *Analytical Chemistry* 78 (18):6601-6607.

Granvogl, B., P. Gruber, and L. A. Eichacker. 2007. Standardisation of rapid in-gel digestion by mass spectrometry. *WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim* 7:642-654.

Granvogl, G., V. Reisinger, and L. A. Eichacker. 2006. Mapping the proteome of thylakoid membranes by de novo sequencing of intermembrane peptide domains. *Proteomics* 6 (12):3681-3695.

Griffiths, W. T. 1978. Reconstitution of Chlorophyilide Formation by Isolated Etioplast Membranes. *Biochem. J.* 174:681-692.

Holtorf, H., and K. Apel. 1995. The regulation of NADPH-protoehlorophyllide oxidoreductases A and B in green barley plants kept under a diurnal light/dark cycle. *Planta Springer-Verlag* 199:289-295.

———. 1996. The regulation of NADPH-protoehlorophyllide oxidoreductases A and B in green barley plants kept under a diurnal light/dark cycle. *Planta Springer-Verlag* 199:289-295.

Holtorf, H., S. Reinbothe, C. Reinbothe, B. Bereza, and K. Apel. 1995. Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (Hordeum vulgare L.). *Proceedings of the National Academy of Sciences of the United States of America* 92 (8):3254-3258.

Jagendorf, A. T., and E. Uribe. 1966. ATP formation caused by acid-base transition of spinach chloroplasts. *Biochemistry* 55:170-177.

Jansson, S. 1999. A guide to the Lhc genes and their relatives in Arabidopsis. *Elsevier Science* 4 (6):236-240.

Jensen, P. E., A. Haldrup, L. Rosgaard, and H. V. Scheller. 2003. Molecular dissection of photosystem I in higher plants: topology, structure and function. *Plant Biochemistry Laboratory, Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*.

Kanervo, E., M. Singh, M. Suorsa, V. Paakkarinen, E. Aro, N. Battchikova, and E. M. Aro. 2008. Expression of Protein Complexes and Individual Proteins

Upon Transition of Etioplasts to Chloroplasts in Pea *Plant Cell Physiol.* 49 (3):396-410.

Klein, R. R., and J. E. Mullet. 1986. Regulation of chloroplast-encoded chlorophyll-binding protein translation during higher plant chloroplast biogenesis. *J. Biol. Chem.* 261 (24):11138-11145.

Klimmek, F., A. Sjødin, C. Noutsos, D. Leister, and S. Jansson. 2006. Abundantly and Rarely Expressed Lhc Protein Genes Exhibit Distinct Regulation Patterns in Plants. *Plant Physiology* 140:793-804.

Kurisu, G., H. Zhang, J. L. Smith, and W. A. Cramer. 2003. Structure of the cytochrome b6f complex of oxygenic photosynthesis: tuning the cavity. *Science* 302 (5647):1009-1014.

Lammli. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lopez-Juez, E., and K. A. Pyke. 2005. Plastids unleashed: their development and their integration in plant development. *The International Journal of Developmental Biology* 49:557-577.

Lushy, A., L. Verchovsky, and R. Nechushtai. 2002. The Stable Assembly of Newly Synthesized PsaE into the Photosystem I Complex Occurring via the Exchange Mechanism Is Facilitated by Electrostatic Interactions. *Biochemistry* 41 (37):11192-11199.

Mathis, N. M., and K. O. Burkey. 1987. Regulation of Light-Harvesting Chlorophyll Protein Biosynthesis in Greening Seedlings. *Plant Physiology* 85:971-977.

McCarty, R. E., Y. Evron, and E. A. Johnson. 2000. The Chloroplast ATP Synthase: A rotary enzyme? *Annual Review of Plant Physiology and Plant Molecular Biology* 51:83-109.

McCarty, R. E., J. S. Fuhrman, and Y. Tsuchia. 1971. Effects of Adenine Nucleotides on Hydrogen-Ion Transport in Chloroplasts. *Proc. Nat. Acad. Sci.* 68 (10):2522-2526.

Minagawa, J., and Y. Takahashi. 2004. Structure, function and assembly of Photosystem II and its light harvesting proteins. *Photosynthesis Research* 82:241-263.

Minai, L., A. Fish, M. Darash-Yahana, L. Verchovsky, and R. Nechushtai. 2001. The Assembly of the PsaD Subunit into the Membranal Photosystem I Complex Occurs via an Exchange Mechanism. *Biochemistry* 40 (43):12754–12760.

Muhlethaler, K., and A. Frey-Wyssling. 1959. Entwicklung und Struktur der Proplastiden. *THE JOURNAL OF BIOPHYSICAL AND BIOCHEMICAL CYTOLOGY* 6 (3):507-520.

Müller, B., and L. A. Eichacker. 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. *The Plant Cell* 11:2365-2377.

Nelson, N., and A. Ben-Shem. 2004. The Complex Architecture Of Oxygenic Photosynthesis. *NATURE REVIEWS, MOLECULAR CELL BIOLOGY* 5:1-12.

Nelson, N., and C. F. Yocum. 2006. Structure and Function of Photosystems I and II. *The Annual Review of Plant Biology* 57:521-565.

Neuhoff, V., N. Arold, and E. W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9 (6):255-262.

Ohashi, K., A. Tanaka, and H. Tsuji. 1989. Formation of the Photosynthetic Electron Transport System during the Early Phase of Greening in Barley Leaves. *Plant Physiology* 91:409-414.

Oster, U., R. Tanaka, A. Tanaka, and W. Ru¨ diger. 2000. Cloning and functional expression of the gene encoding the key enzyme for the chlorophyll b biosynthesis (CAO) from *Arabidopsis thaliana*. *The Plant Journal* 21 (3):305-310.

Raven, P. H., R. F. Evert, and S. E. Eichhorn. 2003. *Biology of Plants*. Sixth ed. New York: W.H. Freeman and Company.

Reisinger, V., and L. A. Eichacker. 2007. How to analyze Protein Complexes by 2D Blue Native SDS-PAGE. *Practical Proteomics* 1:6-15.

———. 2008. Solubilisation of membrane protein complexes for blue native PAGE. *Journal of Proteomics* 71:277-283.

Reisinger, V., A. P. Hertle, M. Plo¨ scher, and L. A. Eichacker. 2008a. Cytochrome $b_6f$ is a dimeric protochlorophyll a binding complex in etioplasts. *Federation of European Biochemical Societies* 275:1018-1024.

Reisinger, V., A. P. Hertle, M. Ploscher, and L. A. Eichacker. 2008b. Cytochrome $b_6f$ is a dimeric protochlorophyll a binding complex in etioplasts. *Federation of European Biochemical Societies* 275:1018-1024.

Reisinger, V., M. Plo¨ scher, and L. A. Eichacker. 2008c. Lil3 assembles as chlorophyll-binding protein complex during deetiolation. *Federation of European Biochemical Societies* 582:1547-1551.

Rudiger, W., J. Benz, and C. Guthoff. 1980. Detection and Partial Characterization of Activity of Chlorophyll Synthetase in Etioplast Membranes. *European Journal of Biochem.* 109:193-200.

Scheller, H. V., P. E. Jensen, A. Haldrup, C. Lunde, and J. Knoetzel. 2001. Role of subunits in eukaryotic Photosystem I. *Elsevier B.V.* 1507:41-60.

Schägger, H., W. A. Cramer, and v. J. G. 1994. Analysis of Molecular Masses and Oligomeric States of Protein Complexes by Blue Native Electrophoresis and Isolation of Membrane Protein Complexes by Two-Dimensional Native Electrophoresis *Analytical Biochemistry* 217 (2):220-230.

Schägger, H., and H. V. Jagow. 1991. Blue Native Electrophoresis for Isolation of Membrane Protein Complexes in Enzymatically Active Form. *Analytical Biochemistry* 199:223-231.

Skoog, D. A., F. J. Holler, and R. C. Stanley. 2007. *Principles of Instrumental Analysis*. 6th ed: Thomson Brooks/Cole, Canada.

Stroebel, D., Y. Choquet, J. L. Popot, and D. Picot. 2003. An atypical haem in the cytochrome b(6)f complex. *Nature* 426 (6965):413-418.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Nat. Acad. Sci.* 76 (9):4350-4354.

von Wettstein, D., S. Gough, and G. Kannangara. 1995. Chlorophyll Biosynthesis. *The Plant Cell* 7:1039-1057.

Wang, Z. L., G. F. Donga, S. Singh, H. Steen, and J. X. Li. 2009. A simple and effective method for detecting phosphopeptides for phosphoproteomic analysis. *Journal of Proteomics* 72 (5):831-835.

Weigel, M., C. Varotto, P. Pesaresi, G. Finazzi, F. Rappaport, F. Salamini, and D. Leister. 2003. Plastocyanin Is Indispensable for Photosynthetic Electron Flow in *Arabidopsis thaliana*. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 278 (33):31286-31289.

Westermeier, R., N. Barnes, S. Gronau-Czybulka, C. Habeck, H. Schach, H. Schickle, C. Seymour, G. Thesseling, and P. Wiesner. 1997. Electrophoresis in Practice, Second edition. *A Wiley Company*.

Whatley, F. R., K. Tagawa, and D. I. Arnon. 1963. Separation of the light and dark reactions in electron transfer during photosynthesis. *Biochemistry* 49.

Wilm, M., A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379:466-469.

Wittig, I., and H. Schägger. 2005. Advantages and limitations of clear-native PAGE. *Proteomics* 5:4338–4346.

Yamada, M., P. Mao, J. P. Fu, and J. Y. Han. 2009. Rapid Quantification of Disease-Marker Proteins Using Continuous-Flow Immunoseparation in a Nanosieve Fluidic Device. *Analytical Chemistry* 81 (16):7067-7074.

# 6 List of figures