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MOLECULAR CHARACTERIZATION OF PROTEIN PHOSPHORYLATION IN PLANT PHOTOSYNTHETIC MEMBRANES

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Front cover:
Picture of spinach as shown in book "Flora vom Deutschland, Österreich und der Schweiz" by Otto Wilhelm Thomé (1885).
Pictures of thale cress as shown in book "Nordens flora" by Carl Axel Magnus Lindman (1905).
Back cover:
A mass spectrometry spectrum of peptides from "shaved" thylakoid membranes.

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Litterarum radices amaras, fructus dulces

Kunskapens rötter är bittra,
men dess frukter ljuva.

- Aristoteles

Till min familj

Olle
Syster och bror med familjer
Mamma och pappa

ABSTRACT

Higher plants cannot move to a more favorable place when the environmental conditions are changing. To adapt to changes in light, temperature and access to water the plants had to evolve special mechanisms at the molecular level. Post-translational modifications of proteins, like phosphorylation, often serve as "on-and-off" switches in regulation of cellular activity and may affect protein-protein interactions. Photosynthesis in higher plants is regulated by reversible protein phosphorylation events, in a unique light- and redox-controlled system. Several biochemical methods are effectively used for characterization of phosphorylated proteins in photosynthetic membranes. Nevertheless, mass spectrometry is the most effective technique when it comes to identification of exact phosphorylation site(s) in the protein sequence, which is the ultimate evidence of protein phosphorylation. The same tandem mass spectrometry analysis identifies other *in vivo* post-translational modifications as well, such as acetylation of the N-terminus of mature protein. To study membrane proteins is a challenging project. In the present work the "shaving" of surface-exposed part of the membrane proteins, where phosphorylation occur, is used. In combination with mass spectrometry, this technique does not require the use of radioactive labeling or antibodies. The present work in spinach and *Arabidopsis thaliana* has identified and characterized several known phosphoproteins, new phosphorylation sites in well-known photosynthetic proteins, as well as two phosphoproteins previously unknown to be present in the photosynthetic membrane. Several photosystem II (PSII) core proteins become phosphorylated in their N-termini (D1, D2, CP43, PsbH), process involved in the regulation of the repair cycle of photo-damaged PSII complexes. The protein-protein interactions between PSII and its light harvesting complex (LHCII) seem to be affected by phosphorylation events in the interface area. In higher plants, phosphorylation sites have been identified in LHCII polypeptides, in one of the proteins (CP29) present in the interface area, as well as in the peripheral TSP9 protein. The TSP9 protein is unique among photosynthetic phosphoproteins, since it is a plant-specific soluble protein that becomes triple-phosphorylated in the middle part of the protein. It is also shown that photosystem I (PSI) is subjected to protein phosphorylation. The extrinsic PSI subunit PsaD becomes phosphorylated in its N-terminus. In addition, the latest characterized subunit of PSI, PsaP, is identified as a phosphoprotein. PsaP is an intrinsic protein assembled on the same side of the PSI complex as LHCII attaches. Several kinases are involved in phosphorylation of photo-synthetic proteins, some more specific to PSII core proteins whereas others recognize LHCII proteins better. The STN8 kinase does not phosphorylate LHCII proteins, but is involved in the phosphorylation of the PSII core proteins D1, D2, CP43 and PsbH. STN8 is light-activated and is also specific in phosphorylation of threonine-4 (Thr-4)

in the PsbH protein, but only after another kinase has phosphorylated Thr-2 first. A common feature of all kinases in plant photosynthetic membranes is the specificity for Thr residues and that the phosphorylation reactions occur in the N-terminal sequence of the proteins, except for the TSP9 protein. Nowadays, research is on the way to solve the complex network of regulation of photosynthetic activity via protein phosphorylation, but far more efforts are needed to get a complete view of the importance of all phosphorylation events and enzymatic specificity.

POPULÄRVETENSKAPLIG SAMMANFATTNING (IN SWEDISH)

Växter kan inte förflytta sig till en mer gynnsam plats när vädret förändras. I stället har de utvecklat andra sätt för att anpassa sig på bästa sätt till årstidsväxlingar, soltillgång, temperaturskiftningar m.m. Proteiner (förr kallat äggviteämnen) är växtens byggstenar och består i sin tur av mindre beståndsdelar, aminosyror. S.k. enzymer (arbetande proteiner) fungerar som små maskiner som är specifika i sitt arbete och behöver få signaler för att veta när de ska vara aktiva. Utan reglering skulle timingen av viktiga reaktioner tappas bort. Vanligt förekommande är att detta sker genom olika förändringar av enzymerna eller proteiner i dess närhet (s.k. posttranslationella modifieringar). Bland dessa modifieringar är protein-fosforylering allra vanligast och består av att en fosfogrupp sätts på proteinet. Ofta fungerar förändringen som en ”av-och-på” knapp för om proteinet skall vara aktivt eller ej, samt påverkar om två/flera proteiner håller ihop med varandra.

Även fotosyntesen i gröna växter styrs via protein-fosforylering, som i sin tur påverkas av bl.a. ljusets kvalitet och kvantitet. Fotosyntesen är den för oss livsviktiga process som förser oss med syre att andas och sker i bl.a. växter och blågröna alger (cyanobakterier). Med hjälp av de gröna klorofyll-molekylerna kan energin i solljuset tas till vara, för att slutligen lagras i form av kolhydrater.

Flera olika biokemiska metoder har framgångsrikt använts för att studera fosforylerade proteiner i de fotosyntetiska membranen. Länge har metoder använts där separering av fotosyntes-proteiner på en gel ingår. För att mäta förekomsten av fosforylerade proteiner används vanligen radioaktivt märkta fosfogrupper eller antikroppar som bara binder till fosforylerade proteiner. Effektivast är dock masspektrometri (MS) för att bestämma var i proteinmolekylen fosfogruppen sitter. Masspektrometri är en bioteknisk metod att väga väldigt små vikter, bl.a. små viktförändringar i proteiner p.g.a. att en fosfogrupp adderats. Att studera membran-proteiner (proteiner som finns i ett dubbellager av fettsyror) är en utmanande uppgift, som innebär många extraproblem jämfört med att studera proteiner som finns i vätskor. I detta arbete har vi använt en metod där man ”rakar” membranen, så att de delar av membranproteinerna som sticker ut på sidorna av membranet lossnar. Man utnyttjar sedan att membranet är tyngre än den omgivande vätskan för att separera de två proverna. Att sedan arbeta med de kortare bortklippta proteinbitarna (s.k. peptider) som finns i vätskan är mycket lättare än att arbeta med hela membranet. De delar av proteinet som finns i membranet är därtill mindre intressanta, eftersom fosforylering sker i lättåtkomliga, utstående delar av proteinerna. Då det finns många olika proteiner som är delaktiga i fotosyntesen, kan det vara svårt att analysera provet direkt där allt finns i en salig blandning. Därför försöker man i många fall rena provet, genom t.ex. den anrikningsmetod som har använts i detta arbete, så att man bara har proteinbitar som är fosforylerade i slutprovet. Dessa fosforylerade peptider har sedan identifierats med masspektrometri.

I denna avhandling har jag studerat proteiner delaktiga i fotosyntesen som kan bli fosforylerade. Som utgångsmaterial har jag använt blad från spenat och ”ogräset” backtrav (*Arabidopsis thaliana)*. Några fosfoproteiner som var kända från andra studier har hittats, liksom att fosfatgruppen binder in till proteiner som man tidigare visste var delaktiga i fotosyntesen, men inte visste var modifierade. Därtill har två nya proteiner identifierats, som vi för första gången kan säga är inblandade i fotosyntesen. Dessa två små proteiner har karakteriserats, i ett försök att förstå deras funktion. Vi har även försökt svara på frågan när proteinet är fosforylerat, om det krävs t.ex. vissa yttre förhållanden (dag/natt, starkt/svagt ljus, låg/vanlig/hög temperatur). Hur stor andel av enskilt protein som är fosforylerat är noggrant reglerat, och styrs av s.k. kinaser och fosfataser, de enzymer som sätter dit respektive plockar bort fosfogruppen från proteinet. Denna reglering är viktig för att fotosyntesen ska ske så effektivt som möjligt under rådande yttre förhållanden. I detta arbete har jag kunnat konstatera att kinaserna inte sätter dit fosfogruppen på vilket protein som helst, utan är specifika. Dels binder de bättre till vissa beståndsdelar av proteinet, nämligen aminosyran treonin, och dels kräver de att aminosyrorna som finns runt omkring ska se ut på ett visst sätt.

Dagens forskning med allt mer avancerad bioteknik tillgänglig, är på väg att lösa det komplexa nätverket som finns för optimal reglering av fotosyntesen via protein-fosforylering. Mycket mer forskning behövs dock för att lyckas få en totalbild av varför en del proteiner blir fosforylerade och hur specifika inblandade enzymer är.

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals (I-V):

I. **Maria Hansson and Alexander V. Vener (2003)**
Identification of three previously unknown *in vivo* protein phosphorylation sites in thylakoid membranes of *Arabidopsis thaliana*
Mol Cell Proteomics 2(8): 550-559

II. **Anastassia Khrouchtchova, Maria Hansson, Virpi Paakkarinen, Julia P. Vainonen, Suping Zhang, Poul Erik Jensen, Henrik Vibe Scheller, Alexander V. Vener, Eva-Mari Aro and Anna Haldrup (2005)**
A previously found thylakoid membrane protein of 14 kDa (TMP14) is a novel subunit of plant photosystem I and is designated PSI-P
FEBS Lett 579(21): 4,808-4,812

III. **Inger Carlberg, Maria Hansson, Thomas Kieselbach, Wolfgang P. Schröder, Bertil Andersson and Alexander V. Vener (2003)**
A novel plant protein undergoing light-induced phosphorylation and release from the photosynthetic thylakoid membranes
Proc Natl Acad Sci U S A 100(2): 757-762

IV. **Maria Hansson, Tiphaine Dupuis, Ragna Strömquist, Bertil Andersson, Alexander V. Vener and Inger Carlberg (2006)**
The mobile thylakoid phosphoprotein TSP9 interacts with the light harvesting complex II and the peripheries of both photosystems
Under revision in J Biol Chem

V. **Julia P. Vainonen, Maria Hansson and Alexander V. Vener (2005)**
STN8 protein kinase in *Arabidopsis thaliana* is specific in phosphorylation of photosystem II core proteins
J Biol Chem 280(39): 33,679-33,686

TABLE OF CONTENTS

ABBREVIATIONS

The most important abbreviations for the understanding of this thesis are listed below:

Ac-	N-terminal acetylation of the protein
ATP	adenosine 5'-triphosphate (energy carrier produced in photosynthesis)
CID	collision-induced dissociation, also called MS/MS
CP	chlorophyll binding protein
DNA	deoxyribonucleic acid (where genes are coded)
ESI	electrospray ionization
IMAC	immobilized metal affinity chromatography
kDa	kilo Dalton (weight unit for proteins)
LC	liquid chromatography
LHC	light harvesting complex
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry, also called CID
m/z	mass over charge ratio (x-axis in MS spectrum)
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form (reducing potential produced in photosynthesis)
OEC	oxygen evolving complex
PsaA-P	subunits of the photosystem I protein complex
PsbA-Z	subunits of the photosystem II protein complex
PSI	photosystem I
PSII	photosystem II
s	phosphorylated serine residue (in the peptide sequence)
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
t	phosphorylated threonine residue (in the peptide sequence)
TMP	thylakoid membrane phosphoprotein
TOF	time of flight (detector in MS)
TSP	thylakoid soluble phosphoprotein

INTRODUCTION

During the last years, there has been an explosion of sequenced genomes of several different organisms, such as human, bacteria and plants. It is obvious that the information from the sequenced genomes is not enough to understand the life of the cell. The post-genomic era is reached and many studies are directed towards proteomics, the knowledge of all the gene products expressed at a specific time and environmental condition, including their post-translational modifications. A single gene can be represented by several isoforms of the protein in the living organism, which additionally implies the importance of studying the living organisms on the protein level.

Oxygenic photosynthesis

The mammals would not live today if it was not for oxygenic photosynthesis, which provides oxygen and organic matter to living organisms all over the world. Photosynthetic organisms have the capacity to capture the energy of sunlight, convert it to chemical energy and use it to build biomolecules. Eukaryotic plants and algae as well as prokaryotic cyanobacteria perform oxygenic photosynthesis in the thylakoid membrane (photosynthetic membrane). In plants and algae thylakoid membranes are present in the plastid organelle chloroplast. As the schematic model in Figure 1 shows, the chloroplast comprises three separate membranes (outer chloroplast envelope, inner chloroplast envelope and thylakoid membrane) and three soluble compartments (intermembrane space, chloroplast stroma and thylakoid lumen). The light reactions of photosynthesis, which produce reducing power and energy, are performed in thylakoid membranes. In vascular plants and some green algae thylakoid membranes are structurally heterogeneous; they are divided in the appressed regions of grana stacks and the stroma lamellae, non-appressed thylakoid membranes interconnecting the grana stacks (see e.g. (Andersson and Anderson, 1980)). The reactions of carbon fixation (so-called dark reactions of photosynthesis or Calvin cycle), synthesis of

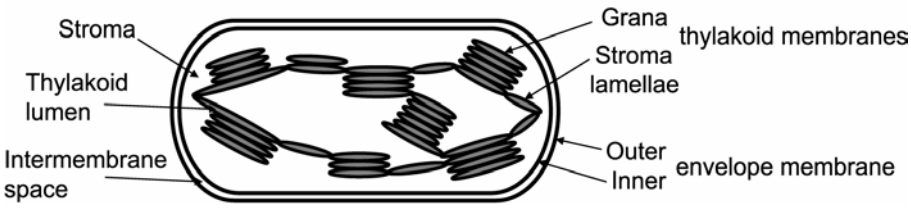

Figure 1. Schematic model of compartments in the chloroplast organelle. Membrane fractions are inner and outer envelope as well as grana and stroma thylakoids. Soluble fractions are stroma, thylakoid lumen and intermembrane space.

chloroplast-encoded proteins and biosynthesis of starch are carried out in the chloroplast stroma. The thylakoid lumen was for a long time believed to be an empty space, but proteomic studies have showed presence of as many as 80 lumenal proteins (Kieselbach and Schröder, 2003). This thesis focuses on the light reactions of photosynthesis and the word photosynthesis will refer to the reactions occurring in thylakoid membranes.

The experimental model systems used in this thesis have been the higher plants spinach *(Spinacia oleracea)* and thale cress *(Arabidopsis thaliana)*. Spinach has been the model plant for decades of photosynthetic research, for instance because of the fast growth of plants to moderately large size. More recently, *Arabidopsis thaliana* has started to be used and is now the main tool for genomic research in plants. The main reason is the completion of the genome sequence in 2000 (The Arabidopsis Genome Initiative, 2000) as the first flowering plant, which gave a start of the post-genomic era in plant research. The ~125 million base pair (125 Mbp) long genome is divided in five chromosomes, as well as mitochondrial and chloroplast DNA, with approximately 26,750 protein coding genes (TAIR6 release November 2005, www.arabidopsis.org). Unfortunately, the small size of the *Arabidopsis* plants can be a limitation for biochemical and immunochemical assays during protein extraction. Growing the plants hydroponically partly circumvents this restriction (Norén *et al.*, 2004).

Protein complexes present in thylakoid membranes

The structural knowledge about the four main protein complexes participating in oxygenic photosynthesis has increased immensely during the last years (Figure 2; reviewed in e.g. (Nelson and Ben-Shem, 2004; Dekker and Boekema, 2005)). Each of these complexes (photosystem I and II, cytochrome b_6f and ATP synthase) consists of multiple protein subunits, and some of them bind pigments and redox cofactors.

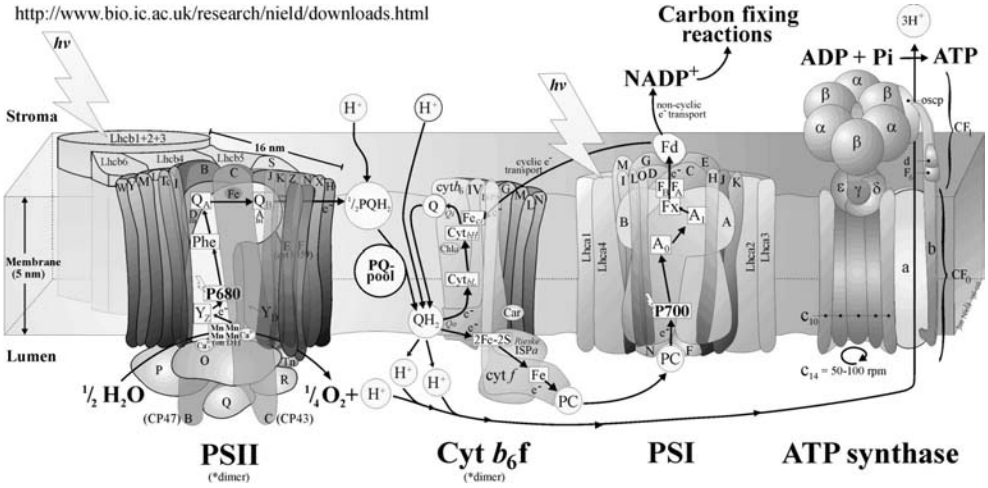

Figure 2. Schematic model of the four main membrane protein complexes involved in oxygenic photosynthesis. Photosystem II (PSII), cytochrome b_6f, photosystem I (PSI) and ATP synthase are shown with structural information on the organization of their subunits in eukaryotes. The flashes illustrate sunlight (*hv*) and the arrows indicate flow of electrons (e^-) and protons (H^+). (by Jon Nield, Imperial College, UK, (Nield, 1997))

The major functions of **photosystem II (PSII)** are photooxidation of water to oxygen and providing electrons for the electron transport chain (Barber and Nield, 2002). The PSII reaction center is composed of the two intrinsic proteins D1 (PsbA) and D2 (PsbD). The reaction center is surrounded by the two large proteins CP43 (PsbC) and CP47 (PsbB), which serve as inner antennae and accept the excitation energy from the peripheral antenna. These four subunits build up the PSII core together with an additional number of small intrinsic subunits (PsbE-F (cyt b559), PsbH-N, PsbR-X, PsbW-Z) (Shi and Schröder, 2004). On the lumenal side of PSII the three extrinsic proteins of the oxygen evolving complex (OEC, protein PsbO, PsbP and PsbQ in plants) are attached (Ferreira *et al.*, 2004). The OEC-proteins stabilize the manganese cluster essential for the water splitting reactions.

To support the PSII core complex with extracted sunlight energy the core complex is surrounded by the peripheral "major" **light harvesting antenna complex (LHCII)** with their non-covalently attached chlorophyll molecules. LHCII is composed of trimers of *lhcb1-3* gene products. LHCII is a pigment-rich complex containing about half of the chlorophyll content and a third of the protein mass of green plant thylakoid membranes. The three inner "minor" antenna proteins, monomers of CP24 (Lhcb6), CP26 (Lhcb5) and CP29 (Lhcb4) are situated between the PSII core and major LHCII (Jansson, 1999; Yakushevska *et al.*, 2001). PSII-LHCII supercomplexes are dimeric in green plants and contain usually 2-4 copies of trimeric LHCII complexes (Barber and Nield, 2002).

The electrons from PSII are used in reduction of the plastoquinone (PQ) pool. Upon illumination plastoquinone becomes reduced (to plastoquinol, PQH_2), detached from PSII complex and subsequently oxidized upon delivery of electrons further to the **cytochrome b_6f complex (cyt b_6f)**. Cyt b_6f is a dimeric membrane protein complex of ~9 subunits and its iron-sulphur cluster is part of the electron transport chain (Zhang *et al.*, 2001; Whitelegge *et al.*, 2002).

The **photosystem I (PSI)** complex accepts electrons from cyt b_6f via the small soluble plastocyanin (PC) protein. In a light-driven electron transfer via ferredoxin (Fd) the electrons are finally used for reduction of $NADP^+$ to form NADPH, which produces intracellular reducing potential for carbon dioxide assimilation in stroma. PSI core in plants consists of two large subunits (PsaA-B), four extrinsic subunits (PsaC-E, PsaN) and a number of intrinsic subunits (PsaF-L, PsaO-P) ((Scheller *et al.*, 2001; Jensen *et al.*, 2003); Paper II). PSI in green plants and green algae consists of monomeric core complexes with monomers of four different light harvesting proteins (LHCI, coded by *lhca1-4* genes), but in photosynthetic cyanobacteria PSI exists as trimers.

The fourth large protein complex in thylakoid membranes is **ATP synthase (ATPase)** (Groth and Pohl, 2001). During the electron flow within PSII and between the two photosystems, a proton gradient is formed across the thylakoid membrane. The transport of protons (H^+) from lumen back to stroma drives the production of energy-rich ATP, performed by ATP synthase.

The distribution of the protein complexes in thylakoid membranes is not homogenous (Andersson and Anderson, 1980; Albertsson *et al.*, 1990). PSII and LHCII reside mainly in the grana, while PSI and ATP synthase with their bulky stromal-exposed parts reside mainly in the stroma lamellae. Cytochrome b_6f is distributed about evenly between the two types of membranes.

Need of adaptation to environmental conditions

The higher plants cannot move to a more favorable place when the environmental conditions change, so they had to evolve molecular mechanisms to adapt to changes in light, temperature and access to water. Light is a necessary driving force for oxygenic photosynthesis, but the light conditions can vary rapidly due to passing clouds, as well as on daily or seasonal basis. In order to optimize reactions in the cell, regulation occurs on both long- and short-term basis. The levels of individual proteins are adjusted in long-term regulation of protein synthesis (transcription and translation level) and degradation. In short-term regulation, the activity of the proteins (enzymatic level) are adjusted by e.g. post-translational modifications. Without regulatory mechanisms, the direction and timing of important cellular activities would be lost. Post-translational modifications often serve as "on-and-off" switches in the regulation of cellular activity or may affect protein-protein interactions. Protein phosphorylation

is one of the most common post-translational modifications and as many as one third of all proteins may be phosphorylated at any time (Hubbard and Cohen, 1993). However, the ratio of phosphorylated to non-phosphorylated protein can be very low at specific physiological conditions. The attachment of a phosphoryl group (HPO_3) occurs mainly to hydroxyl groups of residues (in serine, threonine and tyrosine).

Regulation of photosynthesis is required to ensure efficient capture of light energy under limited light levels, and to provide protection against the potentially damaging effects of excess light. This is partially regulated by reversible protein phosphorylation events, in a unique light- and redox-controlled system (Allen *et al.*, 1981; Aro and Ohad, 2003). A well-known phenomenon is the short-term response to light conditions favoring one of the photosystems, by migration of LHCII from the PSII-rich grana to the PSI-rich stroma and back, known as "state transitions" (Allen, 2003). The light condition when the plastoquinone pool is oxidized (light also favoring PSI excitation) and "all" LHCII is associated with PSII is named 'state 1'. In 'state 2' (under so-called PSII light conditions) part of LHCII is detached from PSII, migrated to stroma lamellae and associated with PSI, which leads to less excitation in PSII. In this way, the distribution of absorbed light energy is balanced between the two photosystems in an optimal way. Phosphorylation of LHCII proteins as regulating factor for the movement of LHCII has been postulated (Allen *et al.*, 1981). State transition is more pronounced in the unicellular green algae *Chlamydomonas reinhardtii*, where up to 80% of LHCII is associated to PSI (Delosme *et al.*, 1996), compared to only 15-20% of LHCII migrating in higher plants (Allen, 1992).

AIMS OF THIS THESIS

The main aim of this thesis was systematic molecular characterization of multiple *in vivo* protein phosphorylation events in plant photosynthetic membranes. This included further characterization of known phosphoproteins, as well as identification and characterization of new phosphoproteins. Another aim was to characterize enzymes involved in protein phosphorylation in thylakoid membranes. Methodological levelopments, like optimization of techniques for enrichment of phosphorylated proteins/peptides and application of mass spectrometry for identification of multiple post-translational modifications, were also important aspects of this research work.

METHODS FOR IDENTIFICATION OF PROTEIN PHOSPHORYLATION IN PHOTOSYNTHETIC MEMBRANES

There are some traditional methods for the identification of phosphorylated proteins, used in combination with separation of proteins on a denaturating polyacrylamide gel (SDS-PAGE) (Laemmli, 1970). In more recent years mass spectrometry (MS) has been established as an invaluable tool for identification of novel phosphorylation sites, since MS allows exact identification of modified amino acids in the protein. If the start material is a complex mixture, like the total protein content of thylakoid membranes, the MS analysis is more fruitful in combination with prior enrichment of phosphorylated protein/peptides.

Gel electrophoresis based approach

Radioactive labeling *in vivo* or *in vitro* is the most common technique used for studies of phosphorylation in thylakoid membranes (Figure 3). The first report in 1977 on phosphorylation of thylakoid proteins was after light incubation of pea chloroplasts with radioactive orthophosphate (*in vitro* phosphorylation), followed by protein separation by gel electrophoresis and detection of phosphoproteins on autoradiogram (Bennett, 1977). Also detached leaves were allowed to take up ^{32}P-orthophosphate, to study *in vivo* phosphorylation (Bennett, 1977). Further on, many of the detected phosphoproteins have been identified by other methods. Based on separation of radioactive *in vitro* labeled thylakoid membranes into a soluble and a membrane fraction, the first known thylakoid soluble phosphoprotein, TSP9 (the 12-kDa phosphoprotein), was identified (Figure 3; (Bhalla and Bennett, 1987); Paper III). Advantages in using radioactive phosphate (either as orthophosphate or as ATP) are high sensitivity and well established methodology. The limitation of the technique in detecting endogenous phosphorylation levels can be circumvented in some cases by the labeling *in vivo* (Owens and Ohad, 1982). Endogenous protein phosphorylation levels in chloroplasts/thylakoids from plants may be analyzed with other methods described below.

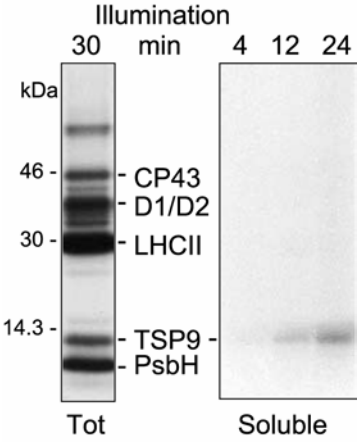

Figure 3. Autoradiogram of ^{32}P-labeled thylakoid membrane proteins separated by SDS-PAGE. Purified thylakoid membranes were incubated with ^{32}P-ATP in light for the indicated time periods. Both total thylakoid membranes (tot) and the proteins released from the membranes (soluble) were analyzed. The bands corresponding to the major thylakoid phosphoproteins, as well as TSP9 protein, are indicated. In the soluble fraction, the TSP9 protein can be detected, but not the integral thylakoid phosphoproteins.

Immunological analysis with **phospho-amino acid specific antibodies** is another way to detect phosphoproteins (Figure 4). This method has an advantage over radioactive labeling experiments in allowing detection of *in vivo* phosphorylation levels of the thylakoid proteins, and can be used to compare phosphorylation levels of an individual protein under different environmental conditions. The use of phospho-threonine antibodies is rather limited, unfortunately, in that the intensity of the immunoresponse with different commercial antibodies differs between various phosphoproteins, and in detection of only the major thylakoid phosphoproteins (see Figure 4; (Aro *et al.*, 2004); Paper V). Phosphorylation of thylakoid proteins on tyrosine residues has also been proposed on the basis of phosphotyrosine antibodies use, but it has not been confirmed by protein identification (Tullberg *et al.*, 1998).

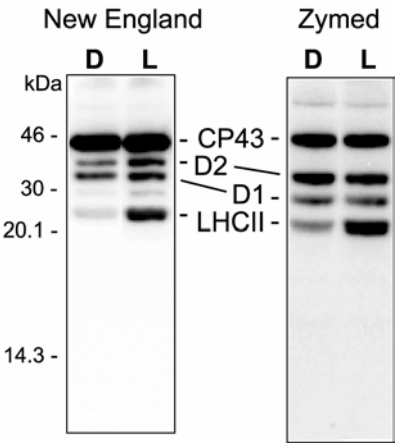

Figure 4. Thylakoid protein phosphorylation studied by immunoblot. Thylakoid membranes were isolated at the end of dark period (D) or after 4 h of light (L), separated by SDS-PAGE and immunoblotted with phosphothreonine antibodies from New England Biolabs or Zymed Laboratories Inc, as indicated. The bands originating from the major thylakoid phosphoproteins are indicated.

Phosphorylated proteins may be detected of **a shift in the electrophoretic mobility** of the individual protein. The phosphorylated forms of the PSII proteins D1, D2 and CP43 have been found to have a slightly slower electrophoretic mobility than the corresponding non-phosphorylated proteins (Callahan *et al.*, 1990; de Vitry *et al.*, 1991; Elich *et al.*, 1992; Rintamäki *et al.*, 1997). The detection using specific antibody against each individual protein is, however, limited to the well-characterized proteins.

To confirm the presence of the phosphorylated form of the protein, thylakoid membranes can **be treated with alkaline phosphatase** prior to electrophoresis. The disappearance of the band corresponding to the putative phosphorylated protein form in the treated sample compared to the untreated, indicates phosphorylation of a specific protein (as in (Depege *et al.*, 2003; Bellafiore *et al.*, 2005)). In this way, several thylakoid protein bands have been postulated to originate from the phosphorylated form of a protein, but identification of the protein and the phosphorylated amino acid is still to be conducted. Another possibility is to use specific antibodies, which recognize exclusively phosphorylated or non-phosphorylated forms of the individual protein (Elich *et al.*, 1992), but this procedure is much more time consuming.

Recent development in the **selective staining of phosphoproteins** in the gel with a specific fluorophore (Pro-Q Diamond dye, (Steinberg *et al.*, 2003)) eliminates the need of comparison of stained gel with immunoblot/autoradiogram. However, as with most gel electrophoresis based methods, this technique needs additional methods for accurate identification of the phosphoproteins. So far this recently developed staining technique has been used successfully for total protein extracts from *Arabidopsis* seeds (Wolschin and Weckwerth, 2005).

A traditional method to sequence a protein is **N-terminal sequencing by Edman degradation** of gel-separated proteins transferred to a membrane. However, sequencing by Edman degradation can not be used for many thylakoid proteins, since N-terminal acetylation (see Table 1 on page 22) blocks the protein degradation. Edman degradation may help in identification of the protein, but usually not in identification of the phosphorylated amino acid if it is not in the N-terminus. When the protein is radioactive labeled before gel-separation, the release of radioactivity during Edman degradation procedure can be detected, like in the identification of phosphorylated Thr-2 in spinach PsbH (Michel and Bennett, 1987). To detect if the phosphorylated amino acid is a serine, threonine or tyrosine residue, complete hydrolysis of individual protein (radioactive labeled), followed by high voltage paper electrophoresis of the individual residues can be used (as in (Bennett, 1977; Bhalla and Bennett, 1987)).

Mass spectrometry

In the late 1980s two different 'soft' ionization techniques were developed for mass spectrometry (MS) analysis, which allowed proteins and peptides to be analyzed without breaking the covalent structure of these molecules. Subsequently the 2002 Nobel prize in Chemistry recognized the fundamental importance of matrix-assisted laser desorption ionization (MALDI) (Tanaka *et al.*, 1988) and electrospray ionization (ESI) (Fenn *et al.*, 1989) for mass spectrometry analysis of biological macromolecules. Further development in the time-of-flight (TOF) and other detectors, as well as mass filters (mainly quadropoles) have increased the sensitivity in MS analysis.

Incorporation of a phosphate group (HPO_3) in the protein sequence **increases the mass of the intact protein by 80 Da**, which can be detected using mass spectrometry. Analysis of the chloroplast grana proteome from spinach and pea by liquid chromatography connected to electrospray ionization mass spectrometry (LC-ESI-MS) has confirmed the phosphorylation of full-length D1, D2, CP43, PsbH (double-phosphorylated) and two LHCII proteins, based on observed +80 Da adducts (Gomez *et al.*, 2002). Three different phosphorylation forms of mature TSP9 were detected by MALDI-TOF (Paper III), as illustrated in Figure 5 by a mass increase of +80, +160 and +240 Da of mature TSP9 protein with a mass of 8,640 Da.

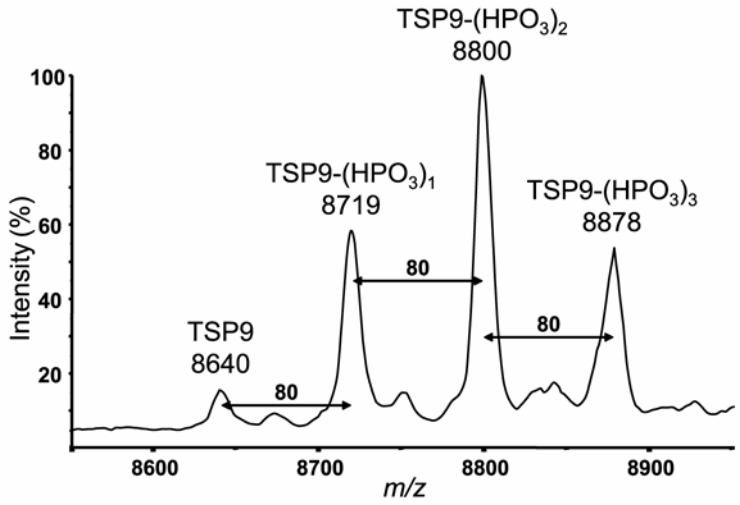

Figure 5. The MALDI-TOF spectrum shows the four isoforms of intact TSP9 protein. The masses (in Da) of non-, mono-, double- and triple-phosphorylated forms of TSP9 are indicated. The mass differences (80 Da) corresponding to phosphate groups are illustrated.

Proteolytic digest of proteins with *de novo* sequencing of phosphorylated peptides by tandem mass spectrometry (MS/MS) is the optimal technique for identification of exact phosphorylation sites. The high-quality fragmentation spectra

allow *de novo* sequencing of the phosphopeptides (Figure 6). It is important to keep in mind that the ultimate evidence for phosphorylation requires identification of the phosphorylated amino acid in the sequence of the corresponding protein. The combination of mass spectrometry analysis with plant genomic sequence information nowadays allows identification of both phosphorylation site(s) and the parent protein, even if the identified phosphopeptide sequence is rather short. Mass spectrometry analysis, using positive mode MS/MS, has been most efficient in mapping of phosphorylation sites in plant thylakoid proteins (see Table 1 on page 22). After the first reports on MS/MS of thylakoid phosphoproteins (Michel *et al.*, 1988; Michel *et al.*, 1991), several additional plant phosphoproteins have now been identified without the need of first purifying the individual protein (summarized in Table 1; (Vener *et al.*, 2001); Paper I, III and V; (Rinalducci *et al.*, 2006)).

Phosphopeptides undergo characteristic fragmentation when subjected to collision induced dissociation (CID, also called MS/MS), allowing them to be distinguished from non-phosphorylated peptides (Carr *et al.*, 1996; Carr *et al.*, 2005). A phosphoester bond between the phosphoryl group and the peptide is less stable than a peptide bond, which leads to a prominent neutral loss of phosphoric acid (H_3PO_4, mass of

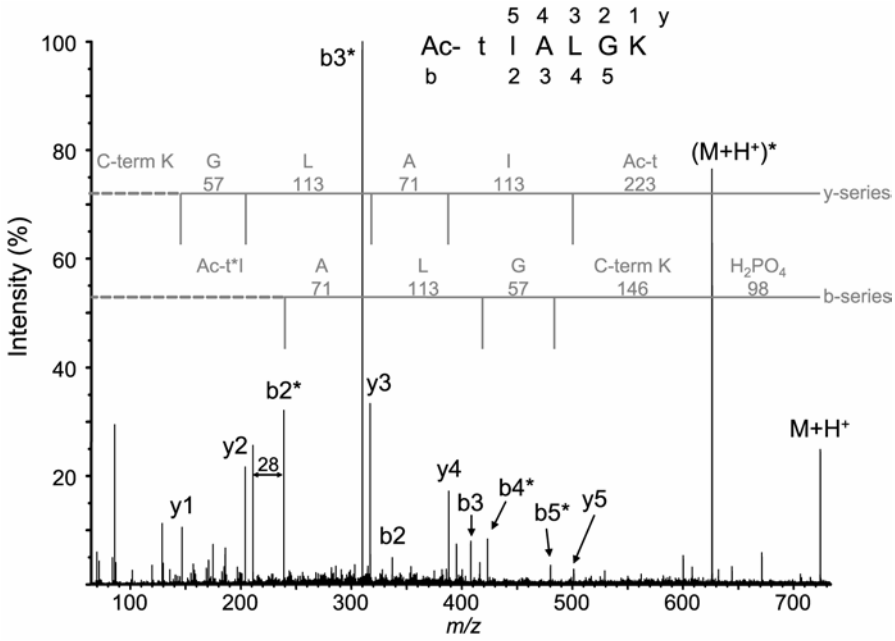

Figure 6. Positive mode MS/MS spectrum of phosphopeptide from *Arabidopsis* D2 protein. The peptide sequence is shown with t indicating phosphorylated threonine residue and Ac- acetylation of N-terminus. The parent peptide ion (*m/z* 724.4) is marked with $M+H^+$. The b (N-terminal) and y (C-terminal) fragment ions are labeled in the spectrum and mass differences between fragment ions in respective series of fragment ions are illustrated in grey. Fragment ions that contained phosphorylated amino acid and underwent the neutral loss of phosphoric acid (H_3PO_4, mass 98 Da) are marked with asterisk (*).

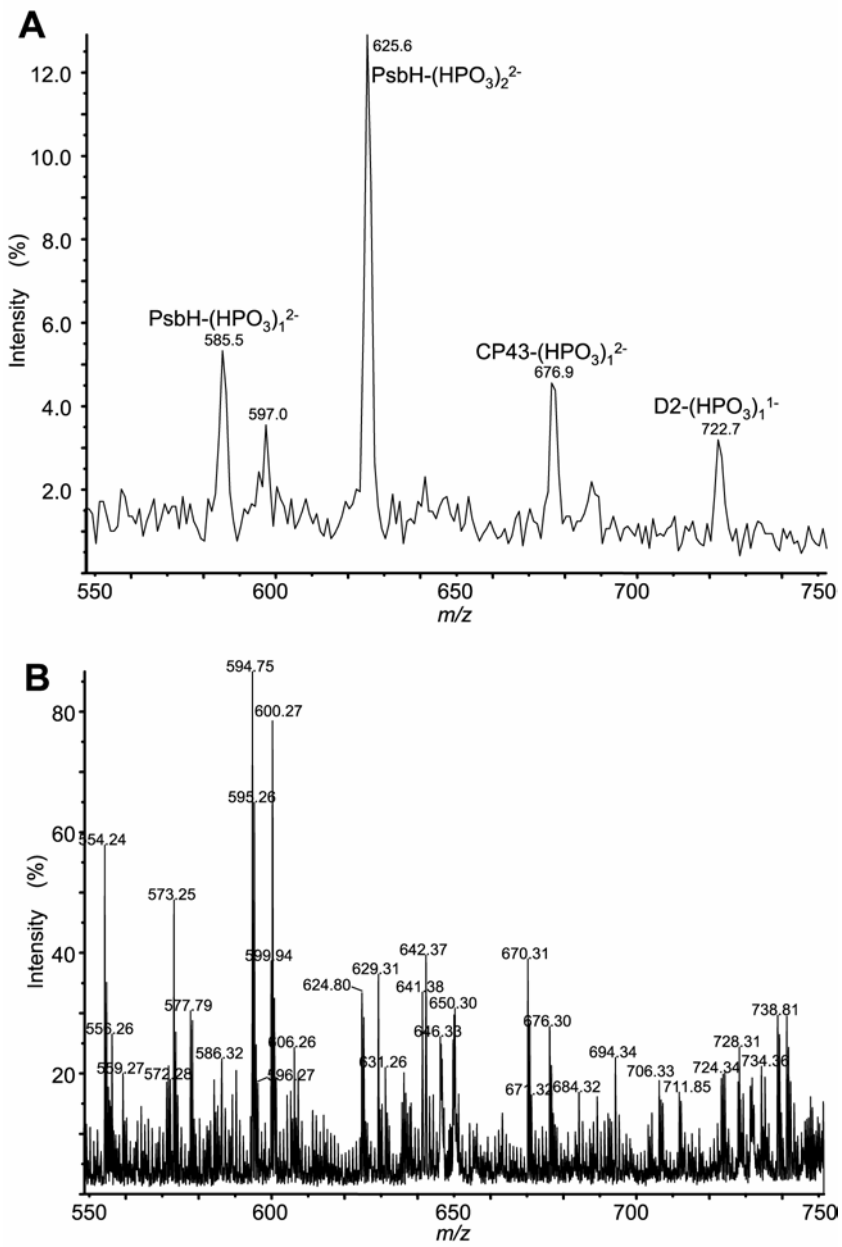

Figure 7. Precursor ion scan in negative mode for marker ion PO_3^- (*m/z* 79) of released peptides collected from shaved thylakoid membranes (A). Peaks originating from known phosphopeptides are marked. (B) Corresponding negative mode MS spectrum shows the complexity of the same sample.

98 Da) from the positively charged peptide ions (Steen *et al.*, 2001; Vener *et al.*, 2001; Shou *et al.*, 2002; Carr *et al.*, 2005). Often abundant peaks corresponding to fragments that have underwent this neutral loss are helpful to identify which residue is phosphorylated, using positive mode MS/MS. The series of y (C-terminal) and b (N-terminal) fragment ions without the neutral loss (originated from non-phosphorylated fragments of the peptide) together with the distinct ions that underwent the neutral loss (originated from phosphorylated peptide fragments; y and b marked with asterisks) in the spectrum allow unambiguous identification of the phosphorylation site(s) (see Figure 6 and Paper I, III and V).

Negative mode **precursor ion scans** for the marker ions at m/z 97 ($H_2PO_4^-$) and 79 (PO_3^-) can be used for selective determination of masses (more correctly corresponding m/z) for threonine or serine phosphorylated peptides (Figure 7; (Carr *et al.*, 1996)), but identification of peptide sequence often needs additional positive mode MS/MS. Anyway, a precursor scan dramatically simplifies the spectrum of masses for phosphorylated peptides among all non-phosphorylated peptides (compare the spectra in Figure 7).

Enrichment of phosphorylated proteins/peptides

In the case of analysis of complex protein mixtures, in which the phosphoproteins are present in only minor amounts, enrichment of phosphorylated peptide/protein before successful analysis with mass spectrometry is often required.

Immobilized metal affinity chromatography (IMAC) is principally based on interaction between the negatively charged phosphate group in the phosphopeptide and the positively charged metal-incorporated column. The phosphorylated peptides or proteins are bound under acidic pH and later eluted with phosphate buffer or higher pH (Andersson and Porath, 1986; Posewitz and Tempst, 1999). A potential downfall of IMAC is that peptides containing many negatively charged residues might also interact. Conversion of carboxylic acids (in C-terminus, in aspartic and glutamic acid) to methyl esters prior to IMAC improve the specific binding of phosphopeptides (Figure 8; (Ficarro *et al.*, 2002); Paper I and V). This improved IMAC protocol helped to identify minor phosphoproteins in thylakoid membranes, when used for the first time in plant phosphoproteomic studies (Paper I).

Metal oxide affinity chromatography (MOAC) used in combination with LC-MS allowed identification of several phosphoproteins from *Arabidopsis* leaf extracts (Wolschin and Weckwerth, 2005). Recently, the successful use of titanium dioxide microcolumns identified seven novel phosphorylation sites in proteins from spinach stromal membranes (Rinalducci *et al.*, 2006). To simplify the complex peptide sample during mass spectrometric analysis, separation of peptides with LC-MS can be used (Vener *et al.*, 2001).

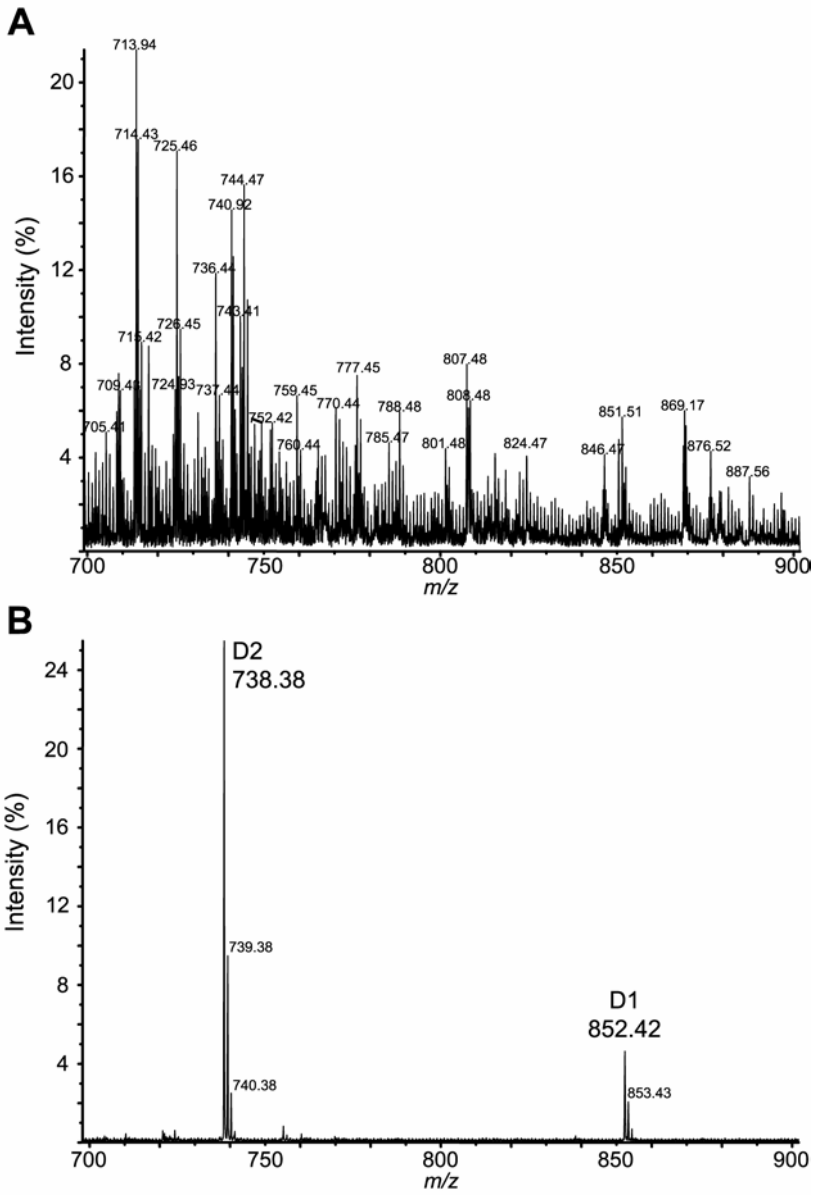

Figure 8. Efficiency of enrichment of phosphopeptides by improved IMAC. Released surface-exposed peptides of thylakoid proteins before (A) and after (B) enrichment of phosphopeptides by improved IMAC. Both positive mode MS spectra (A and B) show zoomed region where D1 and D2 phosphopeptides can be detected.

Quantification of the phosphorylation level

A challenging step in phosphorylation analysis is to **quantify the amount of the phosphorylated form of a protein**. A common approach is to use stable isotope *in vitro* labeling to compare control and experimental sample. The peptides can be differentially labeled during the esterification step before enrichment of phosphopeptides by IMAC. Light isotope-labeled peptides from the control and heavy isotope labeled peptides from the experimental sample are mixed 1:1 prior to simultaneous enrichment step and MS analysis (Figure 9A). The difference between the particular peptide from the two samples is the incorporated mass of the methyl group (light-isotope labeling +14 Da, heavy-isotope labeling +17 Da). The difference in the relative amount of phosphorylated peptide in the two samples is determined from the intensities of the pair of peptide peaks in the obtained MS spectrum (Figure 9B). As

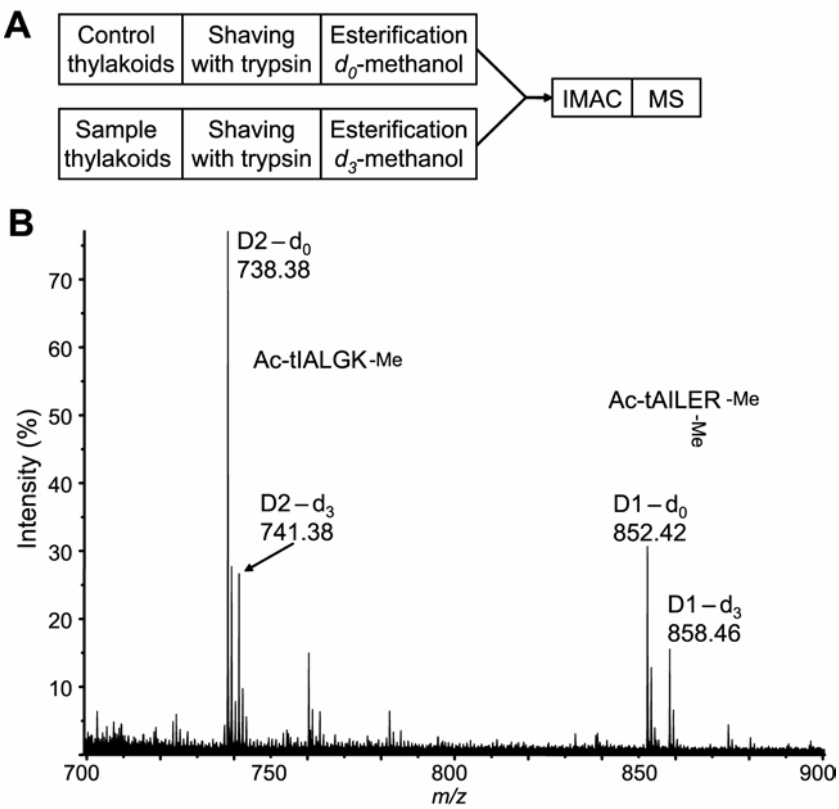

Figure 9. Differentially stable isotope labeling for relative quantification. (A) Scheme of steps used for relative quantification in Paper V. (B) Positive mode MS spectrum zoomed in the region where D1 and D2 phosphopeptides can be detected. Sites of esterification (-Me) are shown in respective peptide sequence. Light isotope (d_0) labeled peptides from wild type were mixed 1:1 with heavy isotope (d_3) labeled peptides from STN8 kinase knockout plant. Reduced phosphorylation levels of phosphopeptides from mutant plants were detected.

internal control the reverse labeling of experimental and control peptides is performed. This relative quantification method can be used to compare phosphorylation levels of thylakoid proteins purified from plants exposed to different environmental conditions. The method has also been used for characterization of protein specificity of individual kinase, by comparison of amount of phosphopeptides in kinase knockout mutant plants with these in wild type plants (Paper V).

Absolute quantification of the phosphorylation level of thylakoid proteins has been obtained by measurements of the ratio of the phosphorylated form compared to non-phosphorylated form of the same protein by densitometric quantification on immunoblots and autoradiograms ((Callahan *et al.*, 1990; Elich *et al.*, 1992; Rintamäki *et al.*, 1996b); Paper II). Quantification of phosphopeptide has been achieved after LC-MS analysis (Vener *et al.*, 2001).

Molecular biology approach

To reveal information about the protein substrates for each individual protein kinase, a reverse genetics approach with kinase-**gene knockout plants** can be helpful ((Depege *et al.*, 2003; Bellafiore *et al.*, 2005); Paper V; (Bonardi *et al.*, 2005)). The use of two independent *Arabidopsis* lines with T-DNA insertions in the *stn8* gene revealed that STN8 protein kinase is specific in phosphorylation of PSII core proteins (Paper V). Mutant lines lacking individual regulatory components for respective kinase/ phosphatase can be used to study regulatory mechanisms.

Site-directed mutagenesis of (potential) phosphorylation sites can be used to investigate the importance of phosphorylated amino acids. Replacing a putative phosphorylation site with an acidic residue (Asp/Glu) can be used to evaluate the importance of the negative charge. Replacing it with another hydroxyl group residue can show substrate specificity of the involved kinase(s). In the green algae *Chlamydomonas reinhardtii* site-directed mutagenesis was used for studies of phosphorylation sites in the D2 (Andronis *et al.*, 1998; Fleischmann and Rochaix, 1999) and PsbH (O'Connor *et al.*, 1998) proteins of PSII complex.

How to study membrane phosphoproteins?

The classical biochemical way to study membrane proteins is purification of individual proteins in the presence of detergent (for solubilization), which is not compatible with direct mass spectrometry analysis. Another way is to use the recently developed "shaving" technique (Vener *et al.*, 2001). The phosphorylated regions of thylakoid phosphoproteins protrude from the outer surface of the membrane, so the

phosphorylation site(s) can be accessed by the corresponding kinase(s) and phosphatase(s). The phosphorylated regions of membrane phosphoproteins can be removed by enzymatic treatment (e.g. trypsinolysis), the so called "shaving" of surface-exposed regions of thylakoid membrane proteins (Figure 10; (Bennett, 1980; Vener *et al.*, 2001); Paper I and V). Comparison of the thylakoid proteins before and after trypsin treatment clearly shows the loss of phosphorylated parts of the proteins into the solution (Figure 10 immunoblot), but transmembrane regions of proteins remain together with the membrane fraction (Figure 10 left). Separation of residual thylakoid membranes and water-soluble surface-exposed peptides is achieved by centrifugation.

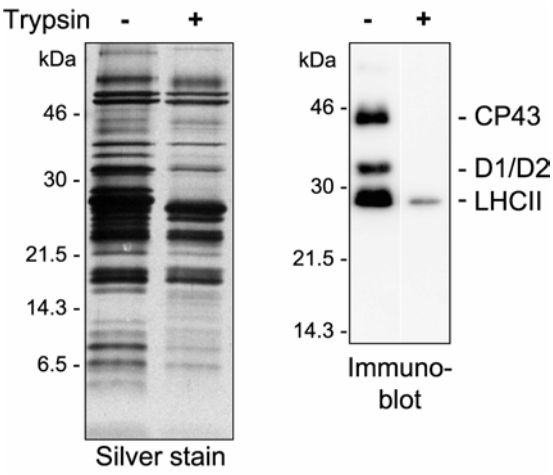

Figure 10. "Shaving" of thylakoid membranes with trypsin results in loss of phosphorylation sites in thylakoid membrane phosphoproteins. Silver stained gel and immunoblot with phosphothreonine antibody from New England Biolabs show thylakoid membranes proteins before (-) and after (+) trypsin treatment.

The complexity of the released surface-exposed peptides of thylakoid proteins is high and the phosphopeptides comprise only 1% of the more than thousand major peptides (Vener *et al.*, 2001). Therefore, the need for enrichment of phosphopeptides by improved IMAC (including methylation of carboxylic groups) was obvious for successful mass spectrometry analysis of phosphopeptides (Paper I and V). This allowed us to identify *in vivo* phosphorylation sites without use of radioactive labeling or antibodies, as well as without prior separation of individual proteins. In addition to the five known phosphorylation sites in PSII core proteins, three previously unknown phosphoproteins were identified in Paper I using the strategy described above (illustrated in Figure 11).

The shaving methodology can circumvent the low success in identification of phosphorylation sites in phosphoproteins after gel electrophoresis. A frequently observed loss of phosphorylated peptides during the in-gel digestion procedure, as well as suppressed ionization of phosphorylated peptides in presence of non-phosphorylated ones during MS analysis, can explain the problem of phosphorylation sites mapping after gel electrophoresis.

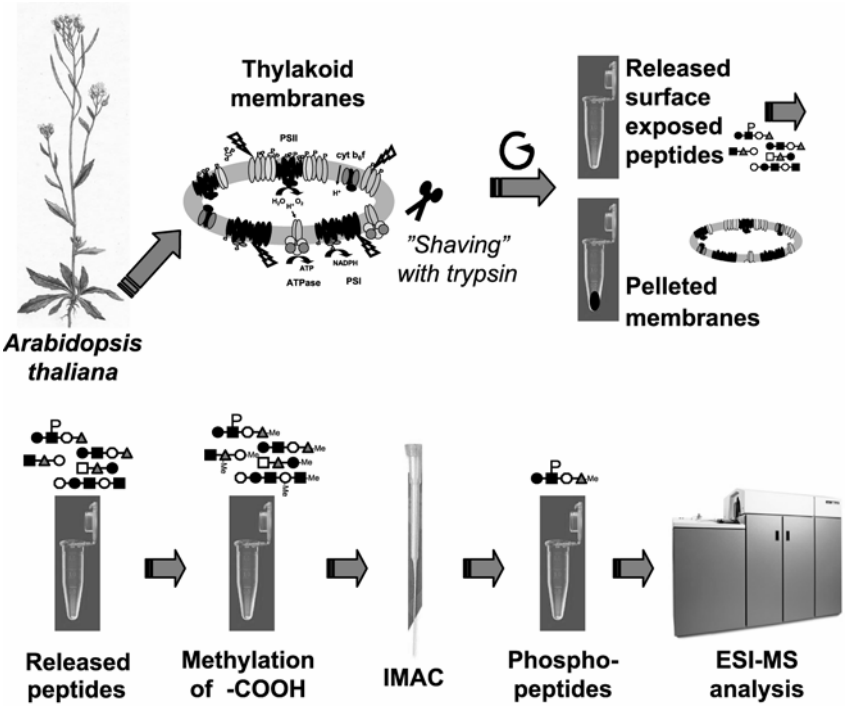

Figure 11. Strategic scheme of phosphopeptide purification steps used in Paper I and V. Thylakoids were purified from *Arabidopsis* leaves and surface-exposed peptides were released by trypsin treatment. Membranous parts of proteins were removed by centrifugation. Methylation of the released peptides was performed before enrichment of phosphopeptides by IMAC column and identification with mass spectrometry.

Recently Peck and co-workers demonstrated that the combination of trypsin digestion of cytoplasmic face-out vesicles, IMAC (without chemical modification of peptides) and LC-MS/MS is a suitable strategy for large-scale studies of phospho-proteomics in *Arabidopsis* plasma membranes (Nuhse *et al.*, 2003). With this strategy over 300 phosphorylation sites in plasma membrane proteins were identified, which is accessible via the database for functional genomics of plant phosphorylation, PlantsP (Nuhse *et al.*, 2004).

The orientation of membrane proteins can be studied by differential purification of the membrane followed by shaving with trypsin, so called vectorial proteomics (reviewed in (Vener and Strålfors, 2005)). Vectorial proteomics was successful in phosphorylation studies in human fat cells (Aboulaich *et al.*, 2004). The difficulty with purification of pure unbroken thylakoid membranes or inside-out vesicles of thylakoids has so far retained successful application of the vectorial proteomic methodology to thylakoid membrane proteins.

CHARACTERISTICS AND FUNCTIONS OF PROTEIN PHOSPHORYLATION IN PLANT THYLAKOID MEMBRANES

Light-induced protein phosphorylation in plant chloroplast membranes was discovered by Bennett almost 30 years ago (Bennett, 1977). Since then the knowledge about the phosphoproteins has increased, but still the identity and role of all thylakoid phosphoproteins are not known. The ultimate evidence for phosphorylation of a specific protein is the identification of phosphorylated residue(s) in its amino acid sequence. Use of ESI-MS and MS/MS during the recent years has allowed identification of more than ten thylakoid phosphoproteins (see Table 1). Most of them are integral membrane proteins and only two are soluble proteins.

Mass spectrometric *de novo* sequencing of peptides shaved from thylakoid membrane proteins in plants and green algae have, beside the phosphorylation site(s), identified several other post-translational modifications. Vener and co-workers have defined the exact, often incorrectly predicted, cleavage site of transit peptides ((Vener *et al.*, 2001); Paper I), N-terminal acetylation of mature proteins ((Vener *et al.*, 2001); Paper I; (Turkina *et al.*, 2004)) as well as the unique absence of removal of a predicted transit peptide (Turkina *et al.*, 2004). Also deamidation of aspargine residues have been identified in thylakoid integral and soluble phosphoproteins ((Vener *et al.*, 2001); Paper III).

The identities of the major phosphoproteins in thylakoid membranes (D1, D2, CP43, PsbH, and LHCII) have been known for a long time, but the identification of minor phosphoproteins will likely continue in the future. The following time-consuming part of research work is characterization of the phosphoproteins and the functional importance of their phosphorylation.

The discovery of multiple-phosphorylated thylakoid proteins has emerged in recent years. The double-phosphorylated PsbH was the first thylakoid protein identified to become more than mono-phosphorylated (Vener *et al.*, 2001). Today three triple-phosphorylated spinach thylakoid proteins are known (TSP9, CP43 and Lhcb1), and in green algae as many as seven phosphorylation sites have been identified in CP29 protein (Turkina *et al.*, 2004; Kargul *et al.*, 2005; Turkina *et al.*, 2006).

Table 1. Identified phosphorylation sites in plant thylakoid phosphoproteins. The superscript numbers in the peptide sequences correspond to amino acid positions in the mature protein, Ac- designates acetylated N-terminus (of mature protein). Asterisk (*) after reference indicate other method than ESI-MS/MS was used for identification of the phosphorylation site.

Protein	Plant	Phosphopeptide	Reference
D1 (PsbA)	*Arabidopsis*	Ac-t^{1}AILER	(Vener *et al.*, 2001)
	spinach	Ac-t^{1}AILERR	(Michel *et al.*, 1988)
D2 (PsbD)	*Arabidopsis*	Ac-t^{1}IALGK	(Vener *et al.*, 2001)
	spinach	Ac-t^{1}IAVGK	(Michel *et al.*, 1988)
CP43 (PsbC)	*Arabidopsis*	Ac-t^{1}LFNGTLALAGR	(Vener *et al.*, 2001)
	spinach	Ac-t^{1}LFNGTLTLAGR	(Michel *et al.*, 1988)
		NGTLt^{8}LAGRDQETTGF	(Rinalducci *et al.*, 2006)
		SPt^{332}GEVIFGGETM	(Rinalducci *et al.*, 2006)
PsbH	*Arabidopsis*	At^{2}GTVEDSSR	(Vener *et al.*, 2001)
		At^{2}Gt^{4}VEDSSR	(Vener *et al.*, 2001)
	spinach	At^{2}GTVESSSR	(Michel and Bennett, 1987)*
LHCII (Lhcb1)	*Arabidopsis*	Ac-RKt^{3}VAKPK	(Vener *et al.*, 2001)
	spinach	Ac-RKt^{3}AGKPKT	(Michel *et al.*, 1991)
		Ac-RKt^{3}AGKPKN	(Michel *et al.*, 1991)
		Ac-RKs^{3}AGKPKN	(Michel *et al.*, 1991)
		t^{9}VQSSSPWYGPDR	(Rinalducci *et al.*, 2006)
		NVSSGs^{14}PWYGPDR	(Rinalducci *et al.*, 2006)
LHCII (Lhcb2)	*Arabidopsis*	Ac-RRt^{3}VK	(Paper V)
	spinach	Ac-RRt^{3}VKSAPQ	(Michel *et al.*, 1991)
CP29 (Lhcb4)	*Arabidopsis*	Ac-RFGFGt^{5}K	(Paper I)
	maize	AGGIIGt^{83}RFE	(Testi *et al.*, 1996)*
PsaD	*Arabidopsis*	Ekt^{3}DSSAAAAAAPATK	(Paper I)
PsaP (TMP14)	*Arabidopsis*	ATtEVGEAPATTTEAETTE	(Paper I)
TSP9	Spinach	SSGSt^{46}SGK	(Paper III)
		GGt^{53}TSGK	(Paper III)
		KGt^{60}VSIPSK	(Paper III)
Rieske Fe-S (PetC)	Spinach	At^{2}SIPADNVPDMQK	(Rinalducci *et al.*, 2006)

Photosystem II phosphoproteins

Ironically, light that is needed to drive photosynthesis is also the main cause of damages to the photosynthetic apparatus, with photosystem II as main target. When the rate of damage and inactivation of the PSII complex, which increase with increasing light intensities, exceeds that of PSII reparation, photoinhibition of PSII occur (reviewed in (Aro *et al.*, 1993; Andersson and Aro, 2001)). Specially the **PSII reaction center protein D1** (coded by the *psbA* gene) is damaged and inactivated, and has the highest turnover rate of all chloroplast proteins (Mattoo *et al.*, 1981; Andersson and Aro, 2001). The phosphorylation in the N-terminus of D1 seems not to directly influence the PSII photoinactivation, but it is crucial for the repair cycle of D1 (Rintamäki and Aro, 2001). After inactivation, the damaged PSII monomer migrates from grana to stroma regions of thylakoid membranes, the PSII complex is partially disassembled, the worn-out D1 protein is degraded and replaced by a newly synthesized D1 copy, and at last the PSII core complex migrates back to the stacked grana thylakoids and assembles to functional PSII-LHCII dimers (Andersson and Aro, 2001). The exchange of the D1 protein requires degradation of damaged D1, which only occurs to the dephosphorylated form (Koivuniemi *et al.*, 1995; Rintamäki *et al.*, 1996a). The phosphorylated form of D1 was found to be virtually resistant to degradation. This strict regulation of D1 degradation is of importance for high efficiency in the D1 turnover process. If the D1 protein would be degraded before a new copy of the protein is available, the whole PSII complex might dissociate leading to degradation of other subunits. At high light intensities, where the photoinhibition rate is high, as much as 70-85% of D1 can become phosphorylated, corresponding to the proportion of the protein in appressed thylakoid membranes (Ebbert and Godde, 1996; Rintamäki *et al.*, 1996a).

Among the PSII core proteins, also the **PSII reaction center protein D2** (*psbD* gene product), **PSII core antenna protein CP43** (gene *psbC*) and the **9-kDa phosphoprotein PsbH** undergo N-terminal phosphorylation (see Table 1). These three proteins are integral subunits with their N-terminal facing the stromal side of thylakoid membranes. PsbH differs from the other PSII core phosphoproteins in *Arabidopsis* by having two phosphorylation sites, Thr-2 and Thr-4. In contrast to the other PSII core protein phosphorylation sites, Thr-4 in PsbH undergoes rapid light-activated phosphorylation (Vener *et al.*, 2001) performed by the STN8 kinase (Paper V). The importance of reversible phosphorylation of D2, CP43 and PsbH is not clear, but the dephosphorylation has been found to be involved in partial disassembly of PSII monomers/dimers, as part of the D1 turnover process (Baena-Gonzalez *et al.*, 1999). However, the reversible phosphorylation as essential for PSII repair in *Arabidopsis* has been questioned (Bonardi *et al.*, 2005). The STN8 kinase was shown to be required for phosphorylation of PSII core proteins, and D1 turnover was indistinguishable in plants lacking STN8 kinase compared to wild type plants (Bonardi

et al., 2005). This absolute requirement of STN8 kinase does not agree anyhow with other results, which show that STN8 only partly phosphorylate PSII core proteins (Paper V).

The *in vivo* phosphorylation levels of D1, D2, CP43 and PsbH under normal growth conditions of *Arabidopsis* plants is higher during daytime (25-50%), but the proteins remain phosphorylated even in dark-adapted plants, with exception of Thr-4 phosphorylation in PsbH (see Figure 4 on page 10; (Vener *et al.*, 2001); Paper V). The reversible phosphorylation of PSII core proteins is also involved in response to heat stress, since increased temperature activate a thylakoid phosphatase (Rokka *et al.*, 2000; Vener *et al.*, 2001), as discussed in the next chapter. Also in green algae D1, D2, CP43 and PsbH become phosphorylated in their N-termini (Delepelaire, 1984; Turkina *et al.*, 2006), which indicate a possible similar regulation via protein phosphorylation in green algae.

The ***psbT*** gene product of 4.0 kDa was found as a putative phosphoprotein by LC-MS analysis of intact grana thylakoid proteins, in which mature pea PsbT protein was detected by a mass increase by 80 Da (mass of phosphoryl group) (Gomez *et al.*, 2002). The identity of the phosphorylation site as well as the molecular function remain to be evaluated.

Light harvesting complex II phosphoproteins

The major light harvesting complex of PSII (LHCII) corresponds to the most heavily phosphorylated protein bands among the phosphoproteins on gel electrophoresis separated thylakoid proteins after illumination (see Figure 3 and 4 on page 10). The phosphorylation levels of LHCII proteins were found to be very low in the dark and increased dramatically in low light (Rintamäki *et al.*, 1997). At higher light intensities the LHCII phosphorylation levels were found to decrease, as a consequence of enough light to drive both photosystems without state transitions (Rintamäki *et al.*, 1997). The phosphorylation of major **LHCII** (mainly **Lhcb1 and Lhcb2** proteins) seems to be important for migration of LHCII from grana to stroma regions during state transition. It has been proven that STN7 protein kinase involved in phosphorylation of LHCII in *Arabidopsis* is essential for state transitions (Bellafiore *et al.*, 2005). However, the original paradigm of mobile LHCII bound to PSII in dephosphorylated form and to PSI in phosphorylated form has to be modified (Snyders and Kohorn, 2001; Zhang and Scheller, 2004). Probably the phosphorylation-induced conformation change of LHCII N-terminus (Zer *et al.*, 1999) is of more importance than the phosphorylation event itself for the interaction with the two photosystem complexes. In addition to the main phosphorylation site on Thr-3 in Lhcb1, also Thr-9 and Ser-14 in its N-terminus can become phosphorylated, which makes Lhcb1 a triple-phosphorylated protein in spinach (Michel *et al.*, 1991;

Rinalducci *et al.*, 2006). In green algae LHCII has been shown to become double-phosphorylated (Turkina *et al.*, 2006).

The introduction of negative charges, upon phosphorylation, may affect the protein-protein interactions in PSII-LHCII supercomplexes. In the area of the interaction of major LHCII with PSII complex, the minor light harvesting proteins CP24, CP26 and **CP29 (Lhcb4)** are positioned (Yakushevska *et al.*, 2003). Phosphorylation on Thr-6 in CP29, isoform Lhcb4.2, has been identified during normal growth conditions of *Arabidopsis* (Paper I). Phosphorylation on Thr-83 in maize CP29 has been associated with the resistance to cold stress during high light (Bergantino *et al.*, 1995; Testi *et al.*, 1996). In green algae phosphorylation of both CP26 and CP29 on one and seven residues, respectively, has been identified (Turkina *et al.*, 2006).

It is probable that the phosphorylation of both major and minor LHCII proteins affect the protein interactions in PSII-LHCII supercomplexes. The introductions of highly charged and bulky moieties into specific regions of the proteins on the outer surface of thylakoid membranes can be sufficient to overcome the attractive forces otherwise holding PSII-LHCII together. This is the proposed function of the multiple-phosphorylated CP29 protein in green algae *Chlamydomonas* (Turkina *et al.*, 2006). The regulation of PSII-LHCII interactions in plants probably results from a combination of phosphorylation of CP29, LHCII and the plant-specific TSP9 protein (Paper IV). Both TSP9 in plants and phosphorylated CP29 in green algae have been found to interact with either PSII or PSI (Paper IV, (Kargul *et al.*, 2005; Takahashi *et al.*, 2006)), probably as part of the protein movements during state transitions.

Photosystem I phosphoproteins

For a long time photosystem I was thought not to be regulated by reversible phosphorylation, because PSI phosphoproteins resisted identification. Recently, two PSI subunits have been showed to become phosphorylated (Paper I and II), which extends involvement of protein phosphorylation in photosynthetic membranes beyond PSII and LHCII. Like the PSII and most LHCII phosphoproteins, the PSI subunits become phosphorylated on threonine residues (Paper I, see Table 1), further strengthening the theory of strict threonine-specificity of thylakoid protein kinases (discussed in e.g. (Allen, 1992; Vener, 2005)).

The extrinsic subunit **PsaD** required for proper assembly and stability of the PSI complex (Haldrup *et al.*, 2003), undergoes phosphorylation on the first threonine in the N-terminus of mature protein (Paper I). PsaD is a hydrophilic protein of about 18 kDa and has only a few elements of secondary structure and no stable three-dimensional structure in solution (Antonkine *et al.*, 2003). When PsaD is assembled in the PSI complex, it forms a well defined three-dimensional structure with three

β-sheets (Fromme *et al.*, 2001; Antonkine *et al.*, 2003). The three extrinsic PSI subunits PsaC-E form a "stromal ridge" of PSI involved in the docking of ferredoxin, an electron carrier at the stromal side of photosynthetic membranes (Fromme *et al.*, 2001). The phosphorylation of PsaD may be involved in regulation of PSI stability, of structural changes in PsaD or of ferredoxin reduction by PSI complex (due to its control position at the electron acceptor side of PSI).

The second found phosphoprotein in PSI complex is the newly identified subunit **PsaP**, first called **TMP14** for Thylakoid Membrane Phosphoprotein of 14 kDa (Paper I and II). This small integral protein with two transmembrane regions was identified as a phosphorylated protein localized in *Arabidopsis* thylakoid membranes by use of the shaving approach illustrated in Figure 11 (Paper I). Further localization studies by different purification methods, both blue native/SDS-PAGE and sucrose gradient fractionation, showed that TMP14 is associated exclusively with PSI and not with other protein complexes in plant thylakoid membranes (Paper II). Therefore, the protein was stated to be a novel plant PSI subunit and consequently named PsaP (Paper II). PsaP might be associated with PSI in the region where PsaL, PsaO and PsaH subunits are situated in the PSI complex (Paper II), in the area where also LHCII attach to PSI (Jensen *et al.*, 2003). Homologous genes to *Arabidopsis psaP* gene have been found in other photosynthetic organisms; higher plants as well as cyanobacteria (Paper I). The phosphorylation level of ~25% of total PsaP is not affected by different light conditions in state transitions (Paper II), which makes the functional importance of its *in vivo* phosphorylation an open question. The exact function of PsaP as a PSI subunit still needs to be elucidated, since PsaP contains no sequence homology to classified protein domains.

Other integral thylakoid phosphoproteins

Phosphorylation studies with radioactive labeling of plant thylakoid membranes occasionally detect protein bands coinciding with the location of the α- and β-subunits of **ATP synthase**. Identification of any phosphorylation site in thylakoid ATP synthase subunits has so far failed and a possible explanation of detected bands can be tight binding of ATP molecules to these components (Owens and Ohad, 1982). The plasma membrane H^+-ATP synthase has been identified to become phosphorylated in *Arabidopsis thaliana* (Nuhse *et al.*, 2003).

Recently the first phosphorylation site in plant proteins from the cytochrome b_6f complex was identified. The spinach **Rieske Fe-S protein** (ISP, PetC subunit) was found to be phosphorylated in the N-terminus of the mature protein (Rinalducci *et al.*, 2006). The cytochrome b_6f complex has earlier been reported to become phosphorylated on subunit V (PetO) upon state transitions in green alga

Chlamydomonas reinhardtii (Hamel *et al.*, 2000). However, phosphorylation of subunit V has not yet been confirmed by mapping of the phosphorylation site(s) in this protein.

Associated thylakoid phosphoproteins

Already 20 years ago a phosphorylated protein with an electrophoretic mobility of 12 kDa was detected in spinach thylakoid membranes (Bennett *et al.*, 1987), but this soluble protein had for a long time resisted identification (Lindahl *et al.*, 1995). Recently this protein has been characterized as a thylakoid associated phosphoprotein with a molecular weight of 8,640 Da and named Thylakoid Soluble Phosphoprotein of 9 kDa (**TSP9**) (Paper III). Mass spectrometry analysis revealed the existence of non-, mono-, double- and triple-phosphorylated forms of TSP9 (Figure 5 on page 12) and phosphorylation of three distinct threonine residues in the central part of the protein (Paper III). This was the first thylakoid protein identified to undergo triple-phosphorylation, as well as be phosphorylated in the mid-region of the protein, not in the N-terminal part as in the membrane phosphoproteins (Table 1). The general characteristics of the redox-controlled phosphorylation of TSP9, as well as for its dephosphorylation and sensitivity to inhibitors, closely follows those observed for LHCII proteins, but not those of PSII core phosphoproteins (Bennett *et al.*, 1987; Lindahl *et al.*, 1995; Carlberg *et al.*, 1999). Light-induced phosphorylation associates with a partial release of phosphorylated TSP9 from thylakoid membranes (Figure 3 on page 10; (Bhalla and Bennett, 1987); Paper III). Thus, a possible function of TSP9 as a protein signaling between the chloroplast redox-potential and nuclear gene expression has been suggested (Paper III). The basic region in C-terminal part of TSP9 (total isoelectric point, pI, of 9.8) may act as a DNA-binding domain. TSP9 seem to be a plant-specific protein, since homologous genes can be found in neither green algae nor cyanobacteria, but in 49 different plant species (Paper III and IV). TSP9 is present in both grana and stroma lamellae of thylakoid membranes, but mainly in the grana regions rich in PSII and LHCII complexes (Paper IV). Further investigations were performed to characterize interacting proteins using subfractionation of thylakoid membranes, immunoblots with protein specific antibody and crosslinking of thylakoid membranes combined with mass spectrometry analysis (Paper IV). These analyses have revealed close contact of TSP9 with major LHCII proteins, as well as with the minor antenna proteins CP26 and CP29 (Paper IV). Furthermore, TSP9 was identified in a complex containing PSI proteins, the subunits PsaE, PsaF and PsaL (Paper IV). Therefore phosphorylation of TSP9 can be the plant-specific mechanism regulating the interaction of LHCII with the two photosystems during state transitions, which in green algae is performed via multiple-phosphorylation of CP29 protein (Turkina *et al.*, 2006).

No phosphorylation sites have been identified in plant lumenal proteins, but phosphorylation has been suggested in different subunits of the oxygen evolving complex (OEC) attached to PSII on the lumenal side. OEC23 (PsbP) has been detected as a substrate for a wall-associated kinase (WAK1) present in plasma membranes in *Arabidopsis* (Yang *et al.*, 2003). OEE3 (OEC16, PsbQ) has been identified as a phosphoprotein in green algae, during a phosphoproteomic study in *Chlamydomonas* cultures grown in the presence of phosphatase inhibitors (Wagner *et al.*, 2006). In the same study also the lumenal protein plastocyanin associated with thylakoid membranes and the peptidyl-prolyl isomerase FKBP16-8 in chloroplast lumen were identified as green algae phosphoproteins. Phosphorylation in a lumen-exposed loop of the integral CP43 protein is the first phosphorylation site identified on the lumenal side of plant thylakoid membranes (Rinalducci *et al.*, 2006).

ENZYMES CATALYZING THYLAKOID PROTEIN PHOSPHORYLATION IN PLANTS

To get a complete picture of thylakoid protein phosphorylation, one needs to understand the enzymes involved in the regulation of phosphorylation levels: the protein kinases attaching the phosphoryl group to specific residues and the protein phosphatases removing it. More than 1100 genes encode for protein kinases in the genome of *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000), which is twice the amount of kinases in human (~550 kinases, *Homo sapiens,* (Venter *et al.*, 2001)). This high amount of kinases implicates the importance of cellular regulation via reversible phosphorylation required for adaptation to environmental conditions in plants. The amount of phosphatases is on the other hand not so different between these species; ~110 phosphatase genes in *Arabidopsis* (Kerk *et al.*, 2002) and ~100 in human (Venter *et al.*, 2001), probably reflecting less substrate specificity of phosphatases compared to kinases.

In eukaryotic cells, the protein kinases and phosphatases mainly act on serine, threonine and tyrosine residues, with serine phosphorylation most abundant and tyrosine phosphorylation relatively rare. In thylakoid membranes almost all of the proteins become phosphorylated on threonine residues (see Table 1), which implicate a relatively strict threonine specificity of thylakoid kinases. This is an unusual feature for eukaryotic Ser/Thr protein kinases. Several of the kinases/phosphatases in chloroplasts are suggested to have transmembrane regions spanning the thylakoid membrane and the enzyme domains exposed on the stromal side of thylakoid membranes (see e.g. (Allen, 1992; Vener, 2005)). This is in agreement with the phosphorylation sites facing the stroma in the integral thylakoid phosphoproteins (Bennett, 1980).

The original paradigm of thylakoid proteins being non-phosphorylated in dark and phosphorylated in light, when the thylakoid kinases are activated by the photosynthetic electron flow (Allen *et al.*, 1981; Allen, 1992), has to be modified. Several of the PSII proteins are phosphorylated even after long dark-incubations of plants, even though to a less degree compared to light-incubated plants (Figure 4 on page 10; (Vener *et al.*, 2001)).

An increase in the phosphorylation level of a specific protein, induced for example by light, can be either an effect of up-regulation of the kinase activity or a down-regulation of the phosphatase activity, as well as a combination of both. The present lack of detailed knowledge about the kinases and the phosphatases in thylakoid membranes and their interactions makes it very difficult to determine the factors leading to changes in the steady-state phosphorylation levels of thylakoid proteins. Even though extensive proteomics work on different parts of the chloroplasts, no kinases or phosphatases have been identified on the proteomic level, see the Plastid Proteome database (PPDB, (Friso *et al.*, 2004)).

Protein kinases

The properties of thylakoid protein kinases have been studied for a long time. Different behaviors in phosphorylation of the PSII core proteins and the LHCII proteins, with the 12-kDa phosphoprotein (nowadays identified as TSP9) following the behavior of LHCII, have been found (Bennett, 1980; Bennett *et al.*, 1987; Carlberg *et al.*, 1999). The specificity of different kinases against the minor phosphoproteins recently identified still remains to be evaluated. The identity of the kinases remained unknown for long, and at present there are only five candidate genes in *Arabidopsis thaliana* for membrane kinases that can phosphorylate thylakoid proteins. The family of three TAK kinases seem to be more specific for LHCII proteins (Snyders and Kohorn, 1999, 2001). In green algae *Chlamydomonas reinhardtii* the Stt7 kinase was found to be a LHCII kinase, and two homologous genes were found in the *Arabidopsis* genome (Depege *et al.*, 2003). These kinases are presently known as STN7 kinase phosphorylating LHCII proteins (Bellafiore *et al.*, 2005) and STN8 kinase specific to PSII core phosphoproteins (Paper V; (Bonardi *et al.*, 2005)). A 64-kDa membrane protein that co-purified with PSII in spinach was found to phosphorylate both PSII and LHCII proteins (Race and Hind, 1996), but the identity of this protein kinase is still unknown. The kinases are suggested to act in a redox-regulated kinase-cascade (Snyders and Kohorn, 2001; Depege *et al.*, 2003; Bellafiore *et al.*, 2005), so more thylakoid kinases will probably become identified in the future. Only isolation and sequencing of these proteins or identification of the genes encoding them will make it possible to unambiguously reveal their localizations, substrate specificities and regulatory mechanisms.

The recent identification of a phosphorylation site in a lumen-exposed loop of the CP43 protein (Rinalducci *et al.*, 2006), gives a new view of thylakoid phosphorylation. Other identified phosphorylation sites in PSII and LHCII proteins are in their N-terminal parts exposed on the stromal side of thylakoid membranes, and the identified soluble phosphoproteins PsaD and TSP9 are attached to thylakoid membranes on the stromal side. The existence of a lumenal kinase or an integral

kinase with enzymatic domain facing lumen may be confirmed in the future. Nucleotide-dependent processes have been detected in the thylakoid lumen implying the existence of nucleotides (ATP) in this chloroplast compartment (Spetea *et al.*, 2004). Phosphorylation of lumenal proteins or lumen-exposed part of integral proteins occurring in the chloroplast stroma before import to lumen is another possibility. However, the artifact of phosphorylation of proteins from the lumen during fractionation of the plant cells cannot be ruled out as well.

PSII kinase(s)

The major phosphorylation sites in PSII proteins are at their N-terminal threonine residue, which also undergo amino-acetylation (Table 1). This may be a characteristic of the specificity of the **STN8 kinase** identified in *Arabidopsis thaliana* ((Depege *et al.*, 2003); Paper V; (Bonardi *et al.*, 2005)). The use of a reverse genetic approach, knock-out mutant lines where *stn8* gene is disrupted, has revealed important insights in the specificity of STN8 kinase ((Bellafiore *et al.*, 2005); Paper V; (Bonardi *et al.*, 2005)). The phosphorylation levels of the PSII core proteins purified from mutant plants were reduced both in light- and dark-adapted plants: D1 to 50-60%, D2 to 30-40% and PsbH Thr-2 to 70-80% of the phosphorylation levels in wild type plants (Paper V). This implicates STN8 as one kinase, but not the only one, responsible for phosphorylation of PSII proteins. However, Bonardi and co-workers found the STN8 kinase to be exclusively required for phosphorylation of PSII core proteins (Bonardi *et al.*, 2005), in studies using the same knockout mutant lines. In the work of Bonardi, the phosphorylation levels of PSII core proteins were studied using a phospho-threonine antibody (Bonardi *et al.*, 2005), while in our work immunoblots (with two different antibodies) were used in combination with relative quantification by mass spectrometry (Paper V). The complementary methodology may explain the foundings of not complete absence of phosphorylation in PSII core proteins in plants lacking STN8 kinase (Paper V). Using the relative quantification methodology (see Figure 9 on page 17), a distinct absence of phosphorylation of Thr-4 in PsbH was found in *stn8* mutant plants, as well as in dark-adapted wild type plants (Paper V). Phosphorylation of Thr-4 in wild type requires both light and prior phosphorylation of Thr-2 (Vener *et al.*, 2001), which indicate that STN8 is a light-activated kinase phosphorylating Thr-4 in PsbH only after another kinase has phosphorylated Thr-2 first. The phosphorylation levels of two peptides that can correspond to LHCII proteins encoded by seven different genes in *Arabidopsis* were not changed in *stn8* mutant plants (Paper V), which shows that STN8 kinase is not involved in the phosphorylation of LHCII proteins. Most probably the selectivity towards the very N-terminal residues of D1, D2, CP43 and PsbH can be explained by the specific structure of the STN8 kinase domain (Paper V).

LHCII kinase(s)

There are some gene candidates for kinases able to phosphorylate the major LHCII proteins. Phosphorylation of N-terminal Thr-3 surrounded by basic amino acids occurs in most LHCII proteins, so the recognition sequence seem to be Ac-$(R/K)_2tX_n(R/K)_n$ for the LHCII specific kinases, even if acetylation is not required for their phosphorylation (Michel *et al.*, 1991). The LHCII kinases also seem to be active towards TSP9, since the phosphorylation patterns of TSP9 in a redox-controlled manner follows those of LHCII proteins (Bennett *et al.*, 1987; Lindahl *et al.*, 1995; Carlberg *et al.*, 1999).

The Ser/Thr protein **kinase STN7** contains an N-terminal chloroplast targeting signal and a potential transmembrane domain (Depege *et al.*, 2003). STN7 was found essential for phosphorylation of LHCII and photosynthetic state transitions (Depege *et al.*, 2003; Bellafiore *et al.*, 2005). The plant growth was somewhat impaired under changing light conditions, but not under constant growth light conditions, which implicate the importance of state transitions in a natural environment where plants are subjected to light fluctuations (Bellafiore *et al.*, 2005). Both *Arabidopsis* STN7 and the homolog Stt7 kinase in *Chlamydomonas* have been suggested to be phosphorylated, due to the shift in electrophoretic mobility of the protein after treatment of thylakoid membranes with a phosphatase (Depege *et al.*, 2003; Bellafiore *et al.*, 2005).

Three protein kinases present in thylakoid membranes were identified in *Arabidopsis thaliana* by use of the N-terminal sequence of LHCII proteins as selection target, and were named **TAK1-3** (thylakoid-associated kinase) (Snyders and Kohorn, 1999). The TAKs were found to be able to phosphorylate LHCII as well as PSII core proteins, and were later also found to be required for state transitions (Snyders and Kohorn, 1999, 2001). Immunoblots with phosphothreonine and phosphoserine antiserum have implicated phosphorylation of the three TAK kinases (Snyders and Kohorn, 1999).

Protein phosphatases

As in the case of thylakoid kinases, the presence of several phosphatases is likely. No gene or protein sequence has so far been identified for the enzymes involved in dephosphorylation of thylakoid proteins, even if a number of both integral and soluble chloroplast protein phosphatases have been studied (Bennett, 1980; Carlberg and Andersson, 1996; Hammer *et al.*, 1997; Vener *et al.*, 1998).

Most studied is the **heat-shock induced phosphatase** specific towards the PSII core phosphoproteins. The very N-terminal acetylated and phosphorylated Thr-1 in mature D1, D2 and CP43 proteins (Ac-t…, see Table 1) was suggested to be important for the specificity of this phosphatase (Rokka *et al.*, 2000; Vener *et al.*, 2001). The phosphorylation sites in PsbH show principal sequence differences compared to

the other PSII core proteins, probably influencing the substrate specificity. This phosphatase was found to be activated upon elevated temperatures and regulated by the lumenal cyclophilin-like peptidyl-prolyl isomerase TLP40 (Fulgosi *et al.*, 1998; Rokka *et al.*, 2000). Heat-shock induced activation of the phosphatase coincided with the temperature-induced release of the TLP40 protein from the thylakoid membranes into thylakoid lumen (Rokka *et al.*, 2000).

The soluble protein phosphatases isolated from chloroplasts have been shown to be more efficient in dephosphorylation of LHCII proteins (Sun *et al.*, 1993; Hammer *et al.*, 1997).

CONCLUDING REMARKS

Several different biochemical methods are effectively used for studies of protein phosphorylation in thylakoid membranes. Nevertheless, mass spectrometry is the most suitable technique for identification of exact phosphorylation site(s), providing the ultimate evidence of protein phosphorylation. The same mass spectrometry analysis often identifies other *in vivo* post-translational modifications, besides phosphorylation.

The "shaving" technique is extremely fruitful for characterization of membrane phosphoproteins, since it eliminates the transmembrane parts of proteins and the need of detergents. Moreover, in combination with mass spectrometry this technique does not require the use of radioactive labeling or antibodies.

Elevated knowledge about protein phosphorylation events in plant thylakoid membranes contribute to increased understanding of regulatory mechanisms of photosynthesis. The major phosphoproteins have been known for a long time, but the minor phosphopeptides in thylakoid membranes are now on the way to be characterized.

The phosphorylation studies in this thesis have identified and characterized several known phosphoproteins (D1, D2, CP43, PsbH, Lhcb1), new phosphorylation sites in well-known thylakoid proteins (CP29, Lhcb2, PsaD) as well as two phosphoproteins earlier unknown as thylakoid proteins (TSP9, PsaP). Most of the identified plant thylakoid phosphoproteins are membrane proteins, but the first two soluble phosphoproteins (TSP9, PsaD) have now been identified. The newly found thylakoid phosphoproteins have extended the involvement of reversible protein phosphorylation in photosynthetic membranes beyond the PSII and LHCII protein complexes.

There are several protein kinases involved in thylakoid protein phosphorylation in plants, and they are specific towards threonine residue. The substrate specificity of STN8 protein kinase towards the exact phosphorylation residues in thylakoid proteins has been revealed, and the explanation of the specificity differences for STN8 and STN7 kinases was suggested.

Future perspectives

Molecular characterization of thylakoid protein phosphorylation should reveal the functional role of this process and the mechanisms for regulation of photosynthesis. Characterization of *in vivo* phosphorylation levels of thylakoid proteins is needed, as well as understanding the importance of reversible phosphorylation of specific proteins. One way to address these questions is to study the changes in phosphorylation levels of specific proteins in plants exposed to different environmental conditions.

Using knockout mutant plants the importance of each phosphoprotein can be studied, especially of interest if the protein is without known function or contains no conventional functional domain. Use of recombinant proteins and site-directed mutations of phosphorylation sites can also give further information about the protein and the phosphorylation site(s).

Further investigations about the enzymes involved in phosphorylation of thylakoid proteins are needed to obtain a complete view on the regulation of phosphorylation of thylakoid proteins and their functional roles.

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