ION TRANSPORT IN CHLOROPLASTS WITH ROLE IN REGULATION OF PHOTOSYNTHESIS

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For my family!

"I may not have gone where I intended to go, but I think I have ended up where I needed to be."
Dirk Gently

Ion transport in chloroplasts with role in regulation of photosynthesis

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ABSTRACT

Photosynthesis is the primary energy source of almost all ecosystems on Earth. Oxygenic photosynthesis has appeared approximately 2.4 billion years ago and has since then gradually evolved into the complex process that land plants, algae and cyanobacteria perform today. During the course of evolution, these organisms have also developed mechanisms to improve photosynthetic efficiency, to cope with changes in the environment, and effectively use the available resources.

The environment in which photosynthetic organisms grow contains numerous ionic compounds. These compounds are taken up and used in numerous important processes, including photosynthesis in chloroplasts. As in the rest of the cell, specialized proteins named channels and transporters mediate ion transport across membranes and control ion homeostasis in chloroplasts.

The work presented in this thesis addresses the role in regulation of photosynthesis of ion channels and transporters from the chloroplast inner envelope **(Paper I)**, and the thylakoid membrane **(Paper I to V)** of *Arabidopsis thaliana*. Potassium ion fluxes mediated by the chloroplast K^+/H^+ antiporters KEA1, KEA2 and KEA3 regulate the composition of the proton motive force (PMF) across the thylakoid membrane that activates photoprotective mechanisms (NPQ) **(Paper I)**. In **Paper II**, an Arabidopsis mutant named *pam71* is found disturbed in photosystem II efficiency and the adjustment of PMF, due to altered Ca^{2+} homeostasis in the chloroplast. In **Paper III**, it is shown that the thylakoid phosphate transporter PHT4;1 affects the availability of phosphate for ATP synthesis, and also alters NPQ activation kinetics and PMF composition. A novel thylakoid voltage-dependent chloride channel (VCCN1) is identified in **Paper IV**, and shown to affect PMF and NPQ activation upon illumination and after rapid shifts from low light to high light. In **Paper V** it is shown that the thylakoid chloride channel CLCe contributes to the modulation of PMF as well as to the regulation of electron transfer and state transition.

Taken together, the findings of this thesis bring novel mechanisms of anion and cation transport across the thylakoid membrane and the chloroplast inner envelope with role in regulation of photosynthesis.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text:

I. Kunz HH, Gierth M*, Herdean A*, Satoh-Cruz M, Kramer DM, Spetea C & Schroeder JI (2014) Plastidial transporters KEA1,-2, and-3 are essential for chloroplast osmoregulation, integrity, and pH regulation in Arabidopsis. *Proceedings of the National Academy of Sciences USA* 111: 7480-7485.

II. Schneider A, Herdean A, Steinberger I, Morper A, Labs M, Spetea C & Leister D. The *photosynthesis-affected mutant71* of Arabidopsis influences photosystem II efficiency and the proton motive force composition. *Manuscript.*

III. Karlsson PM*, Herdean A*, Adolfsson L, Beebo A, Nziengui H, Irigoyen S, Ünnep R, Zsiros O, Nagy G, Garab G, Aronsson H, Versaw WK & Spetea C (2015). The Arabidopsis thylakoid transporter PHT4;1 influences phosphate availability for ATP synthesis and plant growth. *The Plant Journal,* doi: 10.1111/tpj.12962**.

IV. Herdean A, Teardo E, Nilsson AK, Pfeil BE, Johansson ON, Ünnep R, Zsiros O, Nagy G, Dana S, Solymosi K, Garab G, Szabó I, Spetea C* & Lundin B*. A voltage-dependent chloride channel fine-tunes photosynthesis in plants. *Manuscript.*

V. Herdean A, Nziengui H, Zsiros O, Garab G, Lundin B & Spetea C. The Arabidopsis thylakoid chloride channel AtCLCe contributes to chloride homeostasis and photosynthetic regulation. *Manuscript*.

Other papers not included in this thesis:

Yin L, Fristedt R, Herdean A, Solymosi K, Bertrand M, Andersson MX, Mamedov F, Vener AV, Schoefs B & Spetea C (2012) Photosystem II function and dynamics in three widely used *Arabidopsis thaliana* accessions. *PloS One* 7: e46206-e46206.

Flood PJ, Yin L, Herdean A, Harbinson J, Aarts MG, & Spetea C (2014) Natural variation in phosphorylation of photosystem II proteins in *Arabidopsis thaliana*: is it caused by genetic variation in the STN kinases? *Philosophical Transactions of the Royal Society B: Biological Sciences* 369: 20130499.

Fristedt R, Herdean A, Blaby-Haas CE, Mamedov F, Merchant SS, Last RL & Lundin B (2015). PHOTOSYSTEM II PROTEIN33, a protein conserved in the plastid lineage, is associated with the chloroplast thylakoid membrane and provides stability to photosystem II supercomplexes in Arabidopsis. *Plant Physiology* 167: 481-492.

* Shared authorship

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LIST OF ABBREVIATIONS

AGI	Arabidopsis gene index
CBB	Calvin Benson Bassham
CLC	Chloride channel
Cyt	Cytochrome
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DIRK	Dark interval relaxation kinetics
ECS	Electrochromic band shift
ETC	Electron transfer chain
ETR	Electron transfer rate
e^-	Electrons
F_0	Minimum fluorescence level at time 0
F_m	Maximum fluorescence level after a saturating pulse
g_H^+	H^+ conductivity of thylakoid ATP synthase
H^+	Protons
KEA	Potassium efflux antiporter
LHC	Light-harvesting complex
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NPQ	Non-photochemical quenching
OEC	Oxygen-evolving complex
pam	Photosynthesis-affected mutant
PC	Plastocyanin
PHT	Phosphate transporter
P_i	Inorganic phosphate
PMF	Proton motive force
PS	Photosystem
WOC	Water-oxidizing complex
VCCN	Voltage-dependent chloride channel
WT	Wild type
Φ (II)	PSII quantum yield
ΔpH	Difference in pH between thylakoid lumen and stroma
$\Delta\Psi$	Difference in electrical potential between thylakoid lumen and stroma
ν_H^+	Steady-state proton flux via ATP synthase

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INTRODUCTION

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1.1 Photosynthesis is the process by which solar energy is absorbed by some living organisms (plants, algae and certain bacteria), converted and stored as chemical energy.

Based on geological evidence photosynthesis-capable organisms are estimated to have appeared more than 3 billion years ago. The first organisms to successfully utilize energy from sunlight were most likely anoxygenic (non-oxygen evolving), and used hydrogen peroxide as the primary electron donor, and before that ferrous iron [1]. The greatest leap in innovation of the photosynthetic mechanism was the transition to oxygenic photosynthesis. The use of water as the primary electron source, and the subsequent release of O_2 changed the composition of the Earth's atmosphere starting approximately 2.4 billion years ago [2]. Oxygenic photosynthesis and its by-product O_2 changed Earth's environment to such an extent that it affected the evolution of life, allowing for organisms to grow in size and complexity (Fig. 1). During millennia photosynthesis evolved and diversified to become an efficient energy-harvesting mechanism in all environments.

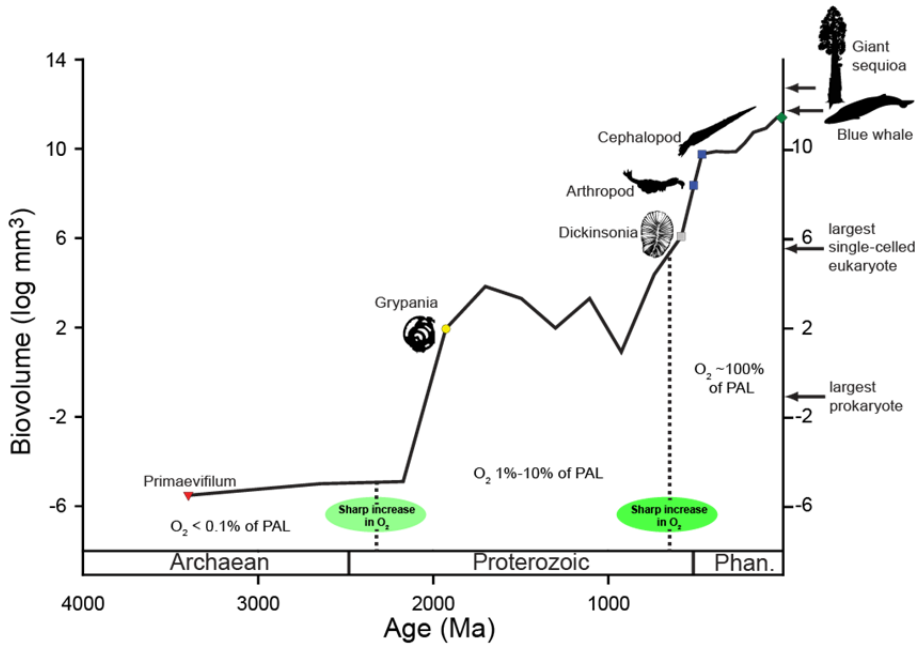

Figure 1. Size of fossils throughout history and the two sharp increases in atmospheric O_2 from photosynthesis. Oxygen is shown as percentage of present atmospheric levels (PAL) during the Archaean, Proterozoic and Phanerozoic geological eons. Adapted from "*Two-phase increase in the maximum size of life over 3.5 billion years reflects biological innovation and environmental opportunity.*" by JL Payne et al. (2009) *Proc Natl Acad Sci USA* **106:** 24-27. Adapted with permission.

Oxygenic photosynthesis is a complex process where many simultaneous mechanisms interact with each other to culminate with the reduction of inorganic carbon, which in turn is converted into organic molecules to build cellular components. Part of the photosynthetic machinery has evolved mechanisms for

efficient light harvesting, oxidation of water, reduction of $NADP^+$ and phosphorylation of ADP; this sum of processes is known as the light-dependent reactions. Another part of the photosynthetic machinery uses ATP and NADPH, to fix CO_2 and synthesize a 3-carbon sugar (triose phosphate), during the so-called light-independent reactions or the Calvin-Benson-Bassham (CBB) cycle. The work presented in this thesis will focus mostly on the machinery that performs the light reactions (**Paper I to V**), with some emphasis on the CBB cycle (**Paper II and III**).

1.2 Photosynthesis in land plants and algae takes place in a specialized organelle called chloroplast. Chloroplasts are plastids that are thought to be the result of an endosymbiotic event between an early eukaryote and cyanobacteria. This endosymbiosis is considered to have taken place after the one yielding mitochondria since all eukaryotes have mitochondria, but not all have chloroplasts.

Two distinct membranes separate the chloroplast stroma from the cytosol: the outer envelope membrane and the inner envelope membrane. The outer envelope is a highly permeable membrane that originated from the eukaryotic host, whereas the inner envelope is less permeable but with a high number of transport proteins, and has originated from the ancestral cyanobacteria. The third membrane system in chloroplasts is the photosynthetic membrane, also called the thylakoid membrane. The thylakoid membrane separates the two solute compartments of the chloroplasts the acidic thylakoid lumen, and the alkaline chloroplast stroma.

A specific organization of the thylakoid membrane (Fig. 2), also called thylakoid ultrastructure, exists in order to maximize light harvesting, energy transfer and repair mechanisms [3]. Thylakoids have two distinct morphological and functional regions called grana and stroma lamellae. The thylakoid membrane contains all the protein complexes performing the light-dependent reactions photosystem II (PSII), cytochrome b_6f (Cyt b_6f), photosystem I (PSI), and ATP synthase. These protein complexes are distributed between the grana and stroma lamellae, with PSII found predominantly in the grana region and Cyt b_6f, PSI and ATP synthase found predominantly in the stroma lamellae (Fig. 2). PSII and PSI are additionally connected to distinct light harvesting protein-pigment complexes, which in turn follow the distribution of PSII and PSI throughout the membrane. Despite that PSII and PSI are spatially separated in thylakoids, in certain situations these two complexes together with their light harvesting antennas can come in close proximity and transfer excitation energy from one complex to the other in a process called spill-over [4]. Furthermore, another similar mechanism that implies changes in thylakoid ultrastructure is state transition [5], a process regulated by phosphorylation of light harvesting antennas as well as anion distribution between chloroplast stroma and thylakoid lumen **(Paper V)**.

The chloroplast stroma is the site of the light-independent reactions (CBB cycle), which despite its name occurs only during the day since they consume ATP and NAPDH generated by the light-dependent reactions. The CBB cycle has 3

phases: carbon fixation, reduction reactions, and ribulose 1,5-biphosphate regeneration.

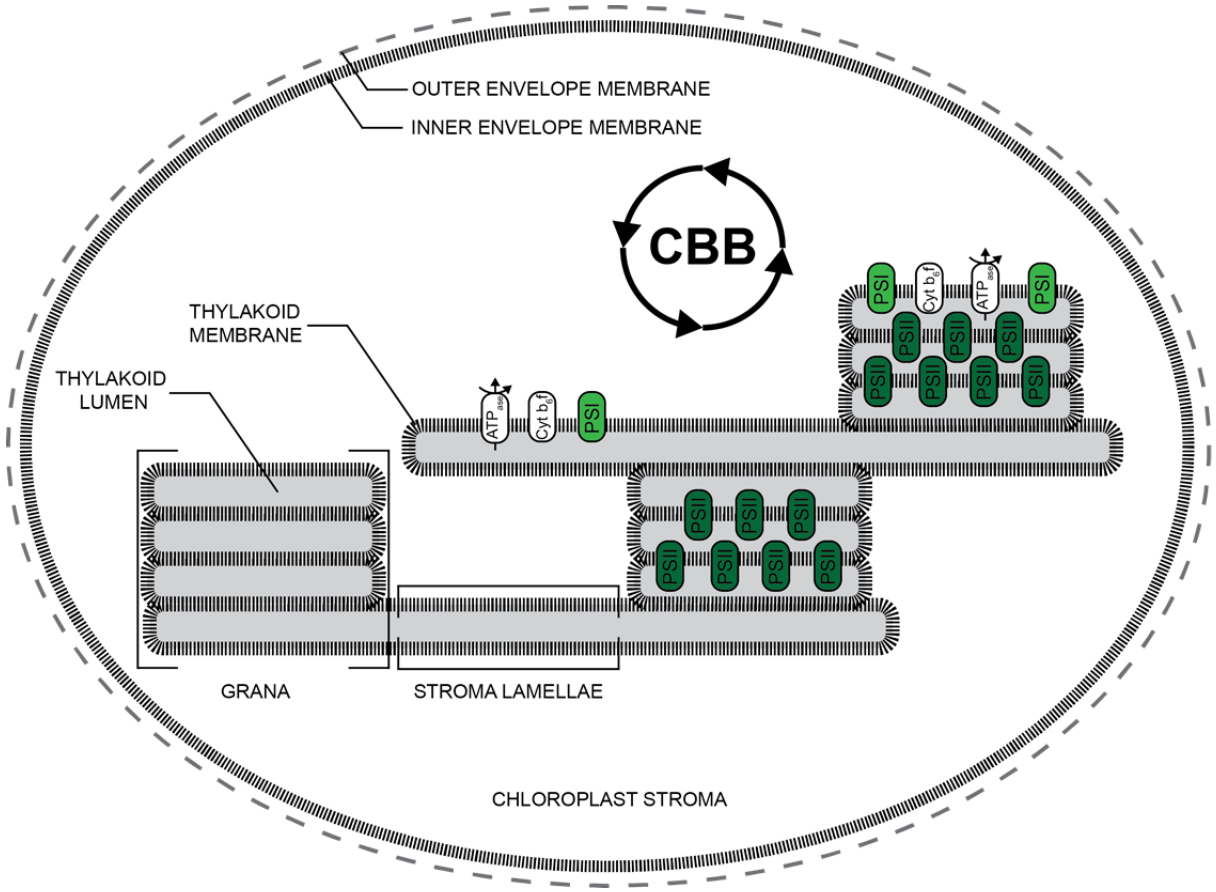

Figure 2. Schematic representation of the chloroplast ultrastructure. Figure presents the chloroplast photosynthetic membrane (thylakoid membrane) organized into grana and stroma lamellae. Distribution of photosynthetic protein complexes between grana stacks (appressed thylakoids) and stroma lamellae (non-appressed thylakoids) is shown, with photosystem II (PSII) found in grana regions and PSI, cytochrome b_6f (Cyt b_6f) and ATP synthase in the stroma lamellae, and to a lesser extent in grana stacks. Chloroplasts have two soluble compartments: thylakoid lumen (grey) and chloroplast stroma (white). In the chloroplast stroma carbon fixation takes place via the CBB cycle, shown with circular black arrows.

1.3 Light-harvesting antennas capture the energy carried by photons using a special set of photosynthetic pigments. There are three basic classes of pigments used in light harvesting: chlorophylls, carotenoids and phycobilins. Chlorophylls are considered to be the most important, with chlorophyll *a* the most significant one. Chlorophylls are essential because they form a porphyrin ring where electrons (e^-) are free to move. When an e^- in a chlorophyll *a* porphyrin ring receives excitation energy from the antenna, its energy level becomes elevated, and the e^- moves to a different molecule towards the terminal electron acceptor. Carotenoids are usually

red, orange or yellow and are composed of two small six-carbon rings connected by a chain of carbon atoms. Carotenoids transfer excitation energy to chlorophylls and not directly to the electron transfer chain. Phycobilins are only found in Cyanobacteria and Rhodophyta, and unlike the other pigments, these are soluble and not present in membranes. Instead, they form mobile light-harvesting antennas known as phycobilisomes.

Antennas are highly regulated and very diverse among different organisms. By increasing the size of the light-harvesting antenna, an organism is able to increase the absorption cross-section for light without having to build more reaction centers, which would be costly in terms of cellular resources. Additionally, by varying the pigment composition more of the light spectrum can be trapped. Connectivity between antennas (energy collector) and reaction centers (energy sink) is a common regulatory mechanism that organisms use to maximize harvesting efficiency or protect against photo-damage.

Plants have two major light-harvesting complexes, each associated with either PSII (LHCII) or PSI (LHCI). LHCII but not LHCI has been shown to be able to disconnect from PSII and connect to PSI during state transitions [5], hence increasing the PSI antenna size. This process is reversible and is triggered in response to changes in light quality. The dynamics of LHCII movement is regulated by phosphorylation, and subsequently by changes in thylakoid architecture, as well as ion fluxes **(Paper V)**.

1.4 Photosystem II (PSII) is a protein supercomplex that exists as a dimer in vivo and can have in excess of 30 subunits, depending on the organism [6]. It is located in the thylakoid membrane and found predominantly in the grana stacks in land plants (Fig. 2). The core of PSII, defined as the minimum number of proteins required to oxidize water, is highly conserved across all photosynthetic organisms, and it consists of D1, D2, CP43, CP47 and cyt b_{559} subunits [6] (Fig. 3). The oxygen-evolving complex (OEC) or water-oxidizing complex (WOC) is the Mn_4CaO_5 inorganic cluster located at the donor side of PSII that splits 2 H_2O molecules into O_2, 4 H^+ and 4 e^-. The OEC is the primary electron source for the electron transfer chain (ETC). Since its discovery [7-9], the OEC has been subject for many studies to solve the exact mechanism by which it oxidizes water. The original model proposed by Kok and co-workers [8, 9] is still used today and assumes four intermediate steps (S states: S_0 to S_4), *i.e*., four conformational changes of the inorganic cluster that result in the release of O_2. The exact position of the Mn_4CaO_5 atoms in each of the S states as well as its assembly remain unclear today, despite the extensive work to solve it [10-12].

Electrons from the OEC are shuttled to tyrosine Z (Y_z), the primary electron acceptor, an amino acid that is part of the D1 subunit of PSII. From Y_z electrons move to a chlorophyll molecule named P680. P680 channels excitation energy from the light-harvesting antennas and elevates the energy levels of the electron before it moves to pheophytin (Phe), which is another chlorophyll molecule but without the

central Mg^{2+}. Further the electrons move to a quionone molecule $Q_{A,}$ and then to Q_B before transfer to the Cyt b_6f complex by a mobile plastoquinone (PQ) molecule. PQ takes 2 e− from PSII and 2 H^+ from the chloroplast stroma, by this adding 2 more H^+ to the trans-thylakoid proton gradient.

PSII is often the primary target of photodamage, and is also the complex with the highest turnover rate in chloroplasts even in normal growth conditions. PSII function and dynamics are additionally altered when the thylakoid ionic environment is changed. Oversaturation of the chloroplast with K^+ reduces PSII activity **(Paper I)**, and excess of Ca^{2+} in the thylakoid lumen can cause instability and low activity of the OEC **(Paper II)**. Changes in chloroplast anion (Cl^-) homeostasis impacts much less PSII function **(Paper IV, V)**.

1.5 Cytochrome b_6f is the protein complex that links PSI and PSII by oxidizing the lipophilic plastoquinol and reducing the soluble plastocyanin (Fig. 3). It functions as a dimer and can have 8 to 9 subunits depending on the organism [13, 14]. Cyt b_6f mediates the linear and cyclic electron transport that is coupled with H^+ translocation, significantly contributing to the transmembrane H^+ gradient required for ADP phosphorylation. Cyclic electron transport does not generate NADPH, and it involves oxidation of PSI-reduced ferredoxin (Fd) [15] or ferredoxin:$NADP^+$ oxidoreductase (FNR) [16].

1.6 Photosystem I, unlike PSII, is predominantly located in the non-appressed regions of the thylakoid membrane (Fig. 2), and it catalyses a light-dependent electron transport from plastocyanin to ferredoxin (Fig. 3). It consists of up to 14 subunits [17], and has been found in monomeric as well as trimeric form [18, 19]. Light-dependent charge separation occurs in a chlorophyll *a* molecule named P700, which channels excitation energy from the PSI-specific light-harvesting antenna LHCI. Additional light-harvesting antennas (LHCII) can in certain conditions connect to the PSI antenna complexes in response to changes in light quality or intensity, in a process called state transition [20]. Electron transport within PSI starts with P700 that is considered to be the primary electron donor, which in turn takes its electrons from PC. Excited P700 transfers an electron to another chlorophyll molecule termed A0, followed by a phylloquinone termed A1, and then to 3 iron-sulphur centres F_X, F_A and F_B before reaching the PSI terminal electron acceptor ferredoxin. Ferredoxin ultimately transfers electrons to FNR, which will reduce $NADP^+$ to NADPH that is used in the CBB cycle. In contrast to P680 which is considered the strongest biological oxidizing agent, P700 is considered the strongest biological reducing agent.

1.7 ATP synthase is a large protein complex consisting of two distinct parts: the catalytic hydrophilic CF1, and the transmembrane H^+-translocating CF0. CF1 consists of 9 proteins separated in 5 groups (3α, 3β, 1γ, 1δ and 1ε) [21], whereas

CF0 consists of 4 distinct proteins (I, II, III and IV), where protein III is found in 12-14 copies [22, 23]. The ATP synthase is a rotary type of protein that uses the electrochemical H^+ gradient generated by the PSII-Cytb$_6$f-PSI electron transfer chain in order to ultimately phosphorylate ADP into ATP (Fig. 3). The CF0 subunit IV channels H^+ from the thylakoid lumen to the chloroplast stroma and uses the resulting mechanical energy to generate ATP, which is subsequently used in the CBB cycle.

Ion transport can have a direct influence on ATP synthase activity by either limiting the supply of inorganic phosphate (Pi) to the catalytic site **(Paper III)**, or by changing the composition of the proton motive force (**Paper IV, V**), which drives ATP synthesis.

Working together PSII, Cyt b$_6$f, PSI and the ATP synthase orchestrate an elegant mechanism that splits H_2O molecules using energy collected from photons, to finally generate ATP and NADPH.

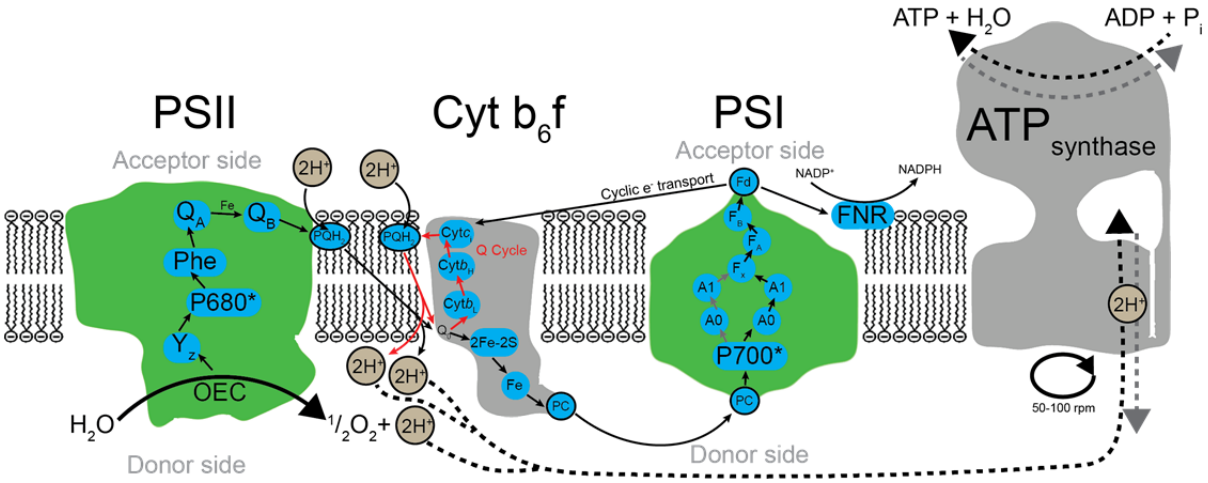

Figure 3. Schematic representation of the electron transfer chain. Small black arrows indicate the linear electron transfer chain starting with the OEC at PSII and ending with FNR at PSI. Red arrows indicate the Q cycle. Grey arrows in PSI indicate an alternative electron transfer pathway. Black dashed arrows indicate H^+-dependent ADP phosphorylation. Grey dashed arrows indicate reversible ATP de-phosphorylation.

1.8 Regulation of photosynthesis on a metabolic level is performed by the circadian clock. Chloroplast gene expression and CO_2 fixation have been shown to be tightly influenced by plant circadian rhythms [24]. The chloroplast has, however, developed additional specialized regulatory mechanisms for fine tuning the photosynthetic efficiency and for response to stress.

Light intensity and composition in natural environments changes quickly and to a large extent during the day, and because of this, plants have developed

photoprotective mechanisms to avoid photodamage, or photoinhibition [25]. Under excess light conditions the primary target of photoinhibition is PSII, whereas under certain conditions (low temperature and low light) PSI is more prone to damage [26]. In response to fast fluctuations in light intensity, photosynthetic organisms have developed similarly fast responses in order to avoid excess excitation energy being channelled from light-harvesting antennas to the reaction centers.

In a relaxed, non-stressful state, light-harvesting antennas are connected to the reaction centers (RCs). When a fast increase in light intensity takes place antennas get disconnected, and by doing this, the RCs become protected from excess excitation energy. The process of antenna quenching is controlled by direct protonation [27] or phosphorylation [5] of LHC, protonation of PsbS [28, 29], and violaxanthin de-epoxidation [30]. Because the thylakoid membrane is extremely protein dense, mobility of LHC is restricted, and hence dynamic and reversible changes in thylakoid membrane architecture occur [31], concomitantly with antenna quenching.

Acidification of the thylakoid lumen is the trigger for most of the photoprotective responses. Ion transporters in turn regulate fast dynamic adjustments of the lumenal pH by import and export of K^+ [32, 33], **Paper I**), and by regulating the P_i availability to ATP synthase **(Paper III)**.

Long-term acclimation of photosynthetic efficiency is another regulatory mechanism, and it involves adjustments of pigment composition and content. Under low light conditions, plants make more chlorophyll to enhance light harvesting and under extended excess light, they reduce chlorophyll content.

1.9 Ion transport proteins belong to three categories with several subcategories in the Transport Classification DataBase (http://www.tcdb.org/, [34]).

1.9.1 Channels and porins constitute the non-energy consuming transport system, and carry solutes down their electrochemical gradient. This class of proteins transport molecules at a very fast rate of 10^7 - 10^8 molecules s^{-1}. Regulation of opening and closing of α-type channels varies depending on the subcellular location and the type of substrate. In chloroplasts, Ca^{2+}- (TPK3 in [33]) and voltage- (VCCN1 in **Paper IV**) gating are common ways of regulating channel activity. CLCe belongs to the chloride channel CLC family ([35, 36] and **Paper V**), but the formal proof of its channel activity as well as its regulatory mechanism is still missing. The β-barrel porins described in the chloroplast to date include exclusively outer envelope proteins responsible for import of amino acids, primary amines, ATP and inorganic pyrophosphate [37].

1.9.2 Secondary transporters require the gradient of a co-transported solute for transport of the compound of interest. This class of proteins include antiporters (transport of two solutes in opposite directions) and symporters (transport of two

solutes in the same direction), and work at a slower rate than channels, 10^2 - 10^4 molecules s^{-1}. Both antiporters and symporters transport down the electrochemical gradient one type of solute and another type against its electrochemical gradient. In chloroplasts, this type of mechanism is used for transport of phosphate (*e.g.,* PHT4;1 in **Paper III**), potassium (e.g., KEAs in **Paper I**), sodium, amino acids and ATP / ADP exchange.

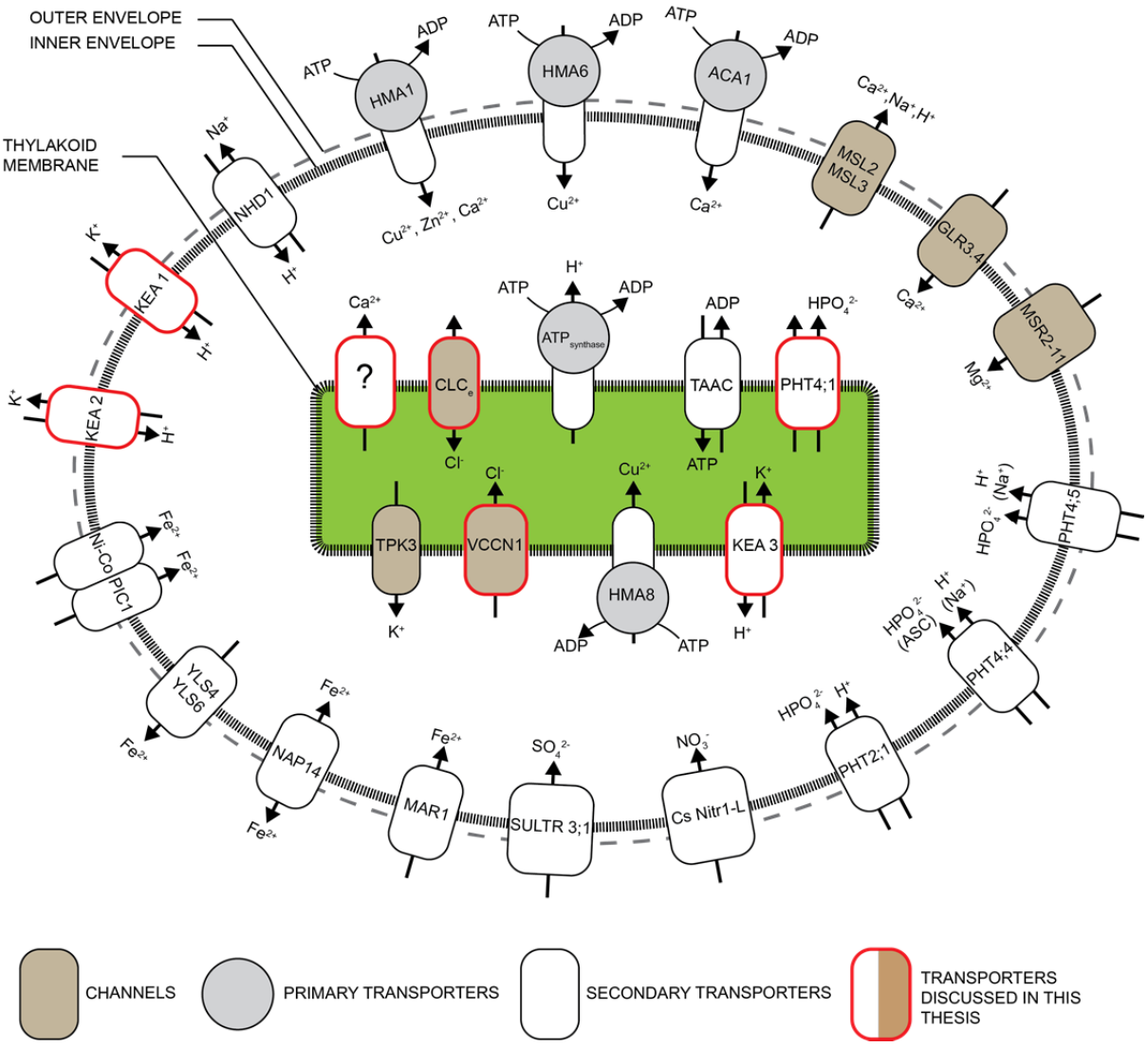

Figure 4. Schematic map of ion transporters from the chloroplast. Transporters located in the thylakoid membrane are found on the stroma lamellae region of thylakoids rather than in the stacked grana (not represented in the figure). The chloroplast outer envelope does not have ion transporters, being easily permeable for most solutes. Adapted from "*Ions channels/transporters and chloroplast regulation."* by G. Finazzi et al. (2014) *Cell Calcium* 58: 86-97. Adapted with permission.

1.9.3 Primary transporters consume energy in the form of ATP or electrochemical gradient to accomplish transport across membranes. This type of transporters is the slowest one with a rate of 10 - 10^3 molecules s^{-1}. Photosynthetic reaction centers, PSII and PSI, are considered in this category due to their role in translocation of e^- across the thylakoid membrane. The ATP synthase is another complex defined as a primary transporter because of its H^+-translocating properties. Cu^{2+} and Zn^{2+} are also transported via ATPases that consume ATP to drive transport against their concentration gradient.

As in the rest of the cell, due to their relative impermeable membranes, chloroplasts have highly specialized transport proteins (Fig. 4, Table 1) for transport of ions across the inner envelope and thylakoid membrane. To date 28 such proteins have been found in *Arabidopsis thaliana* (reviewed in [38]), and an additional channel is identified in this thesis **(Paper IV)**. A Ca^{2+} efflux activity is also found in the thylakoid membrane (**Paper II)**, and it remains to be elucidated which is the protein responsible and to which transport category it belongs.

Protein classification	Protein name	AGI number	Chloroplast location	Substrate	Reference
Channels	TPK3	At4g19160	Thylakoid	K^+	[33]
	CLCe	At4g35440	Thylakoid	Cl^-, NO_3^-?	[35, 36] **Paper V**
	VCCN1	#	Thylakoid	Cl^-, NO_3^-	**Paper IV**
	MSL2	At5g10490	Envelope	Ca^{2+}, Na^+, H^+	[39]
	MSL3	At1g58200	Envelope	Ca^{2+}, Na^+, H^+	[39]
	GLR3.4	At1g05200	Envelope	Ca^{2+}	[40]
	MSR2-11	At5g22830	Envelope	Mg^{2+}	[41]
Secondary transporters	TAAC/ PAPST1	At5g01500	Thylakoid/ envelope	ATP/ADP; PAPS	[42-44]
	KEA3	At4g04850	Thylakoid	K^+	[32, 45], **Paper I**
	PHT4;1	At2g29650	Thylakoid/ envelope	HPO_4^{2-}	[46-49], **Paper III**
	KEA1	At1g01790	Envelope	K^+	[45], **Paper I**
	KEA2	At4g00630	Envelope	K^+	[45, 50], **Paper I**
	PHT2;1	At3g26570	Envelope	HPO_4^{2-}	[51, 52]
	PHT4;4	At4g00370	Envelope	HPO_4^{2-}, ASC	[53, 54]
	PHT4;5	At5g20380	Envelope?	HPO_4^{2-}	[53]
	Cs Nitr1-L	At1g68570	Envelope	NO_3^-	[55]
	SULTR 3;1	At3g51895	Envelope	SO_4^{2-}	[56]
	MAR1	At5g26820	Envelope	Fe^{2+}	[57]
	NAP14	At5g14100	Envelope	Fe^{2+}	[58]
	YLS4	At5g41000	Envelope	Fe^{2+}	[59, 60]
	YLS6	At3g27020	Envelope	Fe^{2+}	[59, 60]
	PIC1	At2g15290	Envelope	Fe^{2+}	[61, 62]
	NiCo	At4g35080	Envelope	Fe^{2+}	[62]
	NHD1	At3g19490	Envelope	Na^+	[63]
Primary transporters	CF_0	AtCg00130	Thylakoid	H^+	[64, 65]
	HMA8	At5g21930	Thylakoid	Cu^{2+}	[48, 66]
	HMA1	At4g37270	Envelope	Cu^{2+}, Zn^{2+}, Ca^{2+}	[67]
	HMA6	At4g33520	Envelope	Cu^{2+}	[66, 68]
	ACA1	At1g27770	Envelope	Ca^{2+}	[69]

Table 1. List of known transporters from the chloroplast. The above list excludes the photosynthetic reaction centers PSII and PSI, due to their principal role in e^- transport rather than ions. # AGI number is only given in **Paper IV**.

SCIENTIFIC AIMS

2

The aim of the work presented in this thesis is to investigate the role of chloroplast ion transport in the context of photosynthesis.

Specific aims:

Paper I To establish the physiological role in Arabidopsis of the envelope-located K^+/H^+ antiporters KEA1 and KEA2, and the thylakoid-located KEA3 for photosynthesis and plant growth.

Paper II To study the phenotype of the Arabidopsis *photosynthesis-affected mutant71* (*pam71*), in relation to Ca^{2+} homeostasis in the chloroplast.

Paper III To investigate the importance in Arabidopsis of the thylakoid-located phosphate transporter PHT4;1 for ATP synthesis and plant growth.

Paper IV To characterize the role in photosynthesis of a newly identified voltage-dependent chloride channel (VCCN1) localized to the Arabidopsis thylakoid membrane

Paper V To further investigate the role of the thylakoid-located chloride channel CLCe in photosynthesis.

METHODOLOGY

3

3.1 *Arabidopsis thaliana* as a model plant. *Arabidopsis thaliana* is a small flowering plant native to Eurasia, and it is part of the Brassicaceae family. The first scientific publication about this plant dates back to 1907 and was published by Laibach [70], who characterized its chromosomes. Later on (1943) he published another article about Arabidopsis, pointing out the advantages of working with this plant [71]. In 1965 the first *Arabidopsis* conference was held, and in 1986 the first transformation with *Agrobacterium tumefaciens* was reported [72] as well as the first gene sequence [73]. In 2000 the complete sequence of its genome was published [74], and the potential of T-DNA insertion knockout mutants was demonstrated as early as 1999 [75]. Several institutions have in the past years created T-DNA insertion populations with the aim of providing knockout mutants for all of its 27,000 genes.

Some of the most important advantages of working with *Arabidopsis thaliana* are the following: relatively small genome (135 Mbp); the first plant genome completely sequenced; small size of the plant and short life cycle, making it suitable for transformation with *Agrobacterium tumefaciens*; and the availability of an extensive literature of more than 100 years of research.

3.2 Functional genomics as approach to study transporters. Due to current advances in whole genome sequencing and the fast development of the growing field of bioinformatics, identification of putative genes coding for transporters or channels has become a matter of data mining. In order to identify and characterize new chloroplast-localized transporters, the first step is to check for sequence similarity with known transporters against a database of genes that code for predicted membrane proteins (*e.g.,* http://aramemnon.uni-koeln.de/); it is unlikely for a transporter or channel not to have any transmembrane regions. Secondly, an analysis of the transit peptide sequence will indicate the probability of the chosen sequence to code for a protein localized to the chloroplast. Transit peptides act as targeting sequences that guide the newly-synthesized protein to the appropriate organelle. These sequences vary greatly in length, composition and organization [76]. However, there are readily available algorithms that analyse and predict the destination of the transit peptide (*e.g.*, TargetP, ChloroP, iPSort, WoLF-PSort, MultiLoc).

Once a suitable candidate gene is identified, its subcellular localization needs to be confirmed by a microscope approach using green fluorescence protein (GFP) transformed plants, and western blot (WB) using GFP- or candidate protein specific antibody. Confirmation of correct localization of the protein is done by isolating pure chloroplast inner envelope membranes, as well as thylakoid membranes against which the specific antibodies are used in WB analysis (Fig. 5). In the absence of a candidate protein specific antibody, the expression profile of the candidate gene (method used in **Paper IV**) needs to be analysed to exclude the possibility of the gene not being expressed in chloroplasts but in other plastids in different parts of the plants (*e.g.*, flowers, roots etc.).

Establishing the transporter function is of crucial importance in order to further investigate the physiological role of the protein. Heterologous expression of the native gene and electrophysiological characterization can confirm the substrate specificity and properties of the newly identified ion transporter (method used in **Paper IV**).

Furthermore, a reverse genetics approach is taken to validate the physiological role (method used in **Papers I to V**). Unlike forward genetics that is used to answer the question "What is the genotype of the observed altered phenotype?", reverse genetics aims at answering the question "What is the phenotype of a specifically altered gene?". Because of the currently available bioinformatics tools and the extensive T-DNA insertion libraries, reverse genetics is a very efficient approach to study uncharacterized genes. Knowing the substrate of a novel ion transporter protein, as well as its subcellular location, specific experiments can be designed to investigate the role of the protein in chloroplast metabolism and photosynthesis.

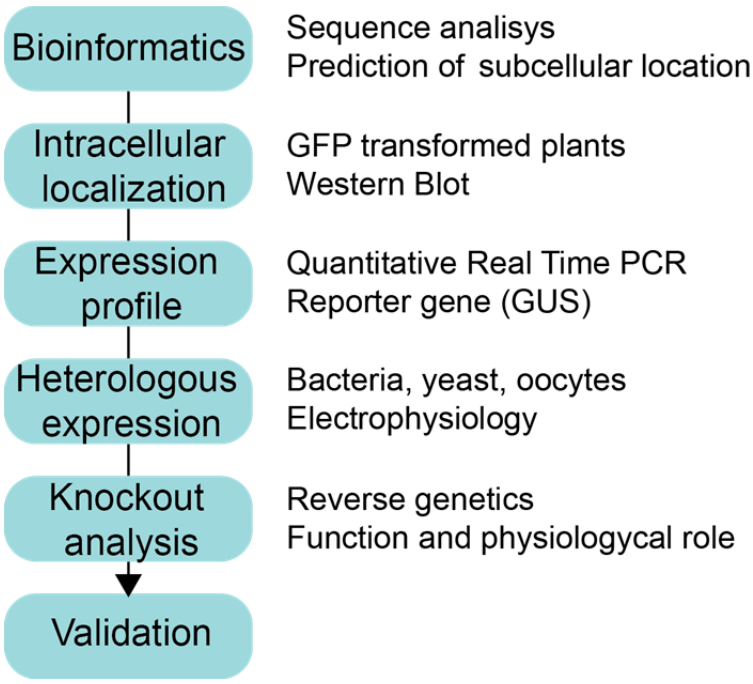

Figure 5. Schematic representation of in silico and experimental approaches used in functional genomics.

An alternative approach to identification of candidate genes from genomic databases is screening of chloroplast proteomics databases. Proteomics has been proven as a successful approach for identification of a large number of chloroplast proteins [48, 77-81]. Nevertheless, due to low abundance of transporter and channel proteins, especially in the thylakoid membrane, where relative to photosynthetic proteins, they are predicted to be found in 10^{7}-10^{14} fold lower quantities, proteomics approaches may not always be sensitive enough to detect such proteins.

3.3 Chlorophyll fluorescence as approach to study photosynthesis. Chlorophyll *a* fluorescence, also known as the Kautsky effect, was discovered in 1931 by Hans Kautsky, who observed chlorophyll *a* fluorescence coming from leafs with the naked eye [82]. Based on his observations, he later proposed that there are two reactions taking place during illumination, presumably charge separation in PSII and PSI [83, 84]. The fluorescence signal is now thought to arise from an energetic back-reaction of the PSII core chlorophyll P680 that returns part of the excitation energy and is re-emitted as light with a peak wavelength at 735 nm. Chlorophyll *a* fluorescence is also emitted from PSI and contributes significantly to measurements of minimum fluorescence (F_0), however it does not vary during illumination [85, 86]. Chlorophyll *b* in vivo does not emit fluorescence because it transfers the excitation energy with 100% efficiency to chlorophyll *a*.

Since its discovery, chlorophyll *a* fluorescence has been extensively used as a non-invasive way to describe the function of the photosynthetic machinery. Various methods and equipment have been since developed to make this approach widely available.

3.3.1 Fast kinetics of fluorescence induction curves, also known as *OJIP* curves, are fluorescence transients that are measured upon illumination with a saturating light pulse of a photosynthetic organism following a period of dark adaptation (method used in **Paper II and V**).

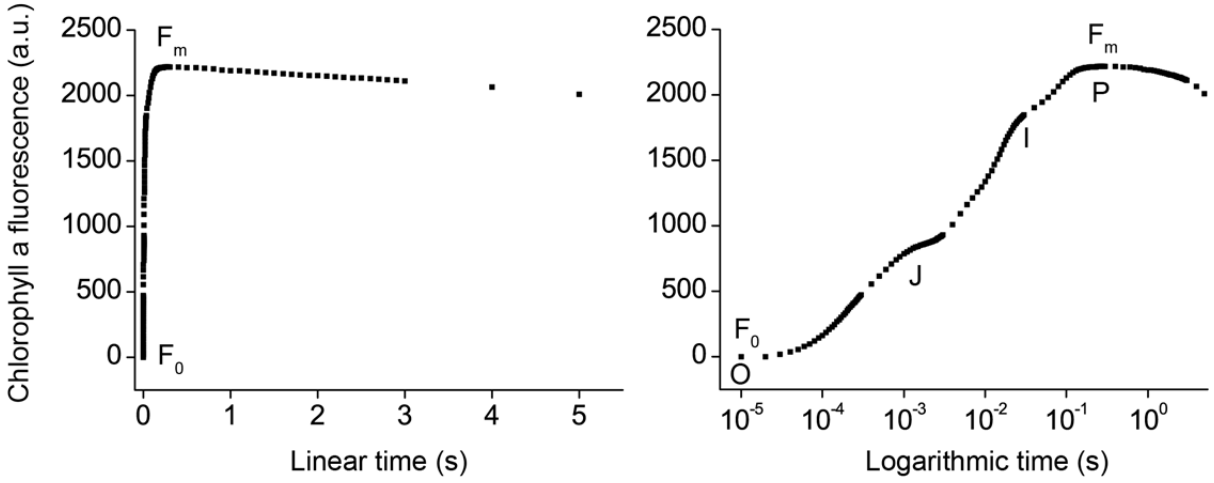

Figure 6. Typical chlorophyll *a* induction transients. *Left panel* represents an induction curve of a 15 min dark-adapted *Arabidopsis thaliana* leaf plotted on a linear scale. F_0 and F_m are here clearly distinguishable. *Right panel* shows the same measurement plotted on a logarithmic time scale. Apart from F_0 and F_m, sometimes termed *O* (origin) and *P* (peak), the additional *J* and *I* intermediate peaks are observable.

Possibly the most widely used parameter derived from induction curves is F_v/F_m, which is calculated as $(F_m-F_0)/F_m$, and it is generally thought that it indicates PSII efficiency or PSII quantum yield. However, recent experimental evidence suggests that fast conformational changes within PSII take place upon illumination and may account for up to 30% of the fluorescence rise, challenging the notion that F_v/F_m reflects a true PSII quantum yield [87].

From a typical fluorescence induction curve (Fig. 6) additional parameters can be derived in what is known as the JIP Test, providing detailed structural, and functional information about the photosynthetic apparatus, especially PSII [86, 88].

3.3.2 Pulse Amplitude Modulated fluorescence (PAM) is another method that has been later developed [89], and unlike F_v/F_m it derives its information over longer time scales (minutes to hours) by determining the F_m' values, i.e. light-adapted maximum fluorescence (method used in **Papers I to V**).

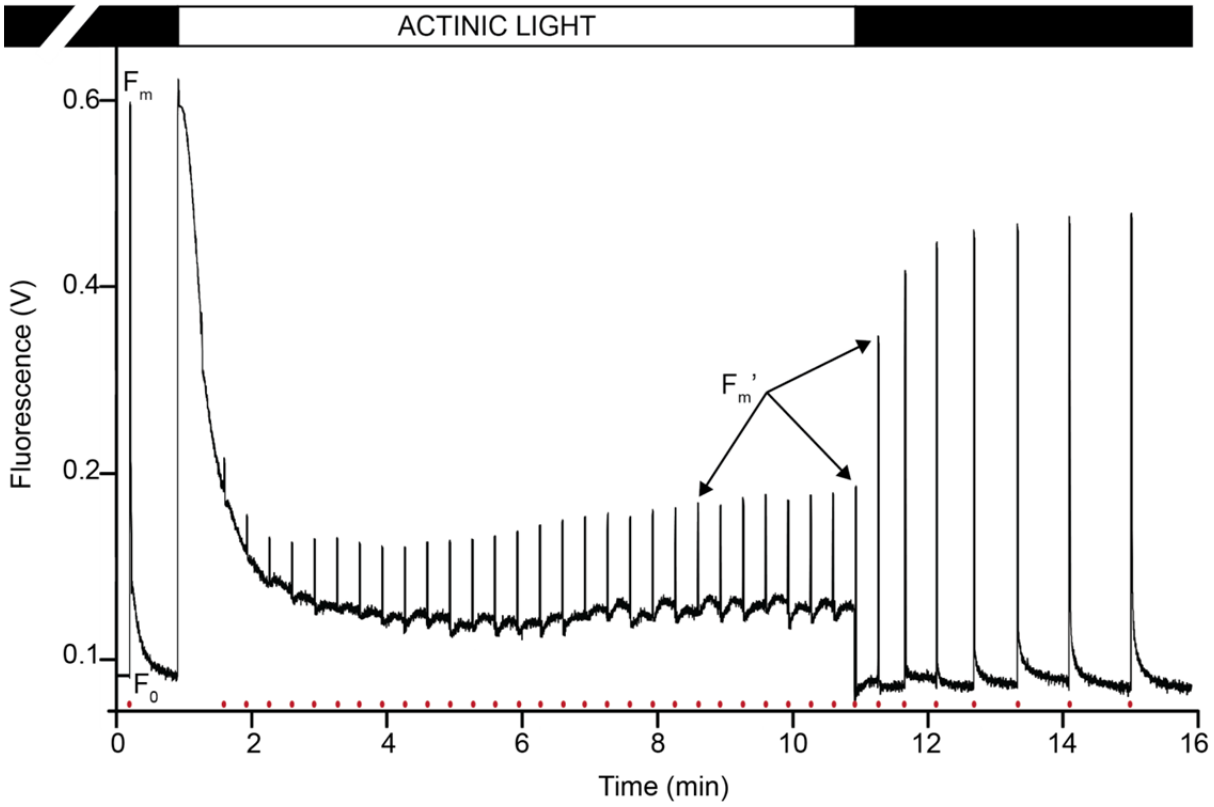

Figure 7. Typical recording of PAM chlorophyll *a* fluorescence. Plotted data show a continuous recording of the fluorescence signal of an *Arabidopsis thaliana* leaf that has been dark adapted for 30 min prior to the measurement. The measurement starts with determination of F_0 and F_m by exposing the leaf to a brief 800-ms saturating pulse (SP) of 5,000 μmol photons m^{-2} s^{-1} actinic red light. After the initial SP the leaf is left without illumination for 1 min to allow the fluorescence signal to reach F_0 levels again, after which the leaf is continuously illuminated with 600 μmol photons m^{-2} s^{-1} actinic red light. During illumination and the subsequent dark

adaptation starting at min 11, the leaf is exposed to pre-set saturating pulses to determine F_m' values. Red dots next to the time axis indicate when the saturating pulses have been applied.

From a typical PAM fluorescence measurement (Fig. 7), several useful parameters can be derived [90]:

- PSII quantum yield (Φ (II))

Φ (II) = (F_m'/F)/F_m'

F = Steady-state fluorescence value prior to the saturation pulse

- Electron Transfer Rate at PSII (ETR II) [91, 92]:

ETR (II) = Φ (II) x PAR x 0.84 x 0.5

PAR = Photosynthetic Active Radiation is the light intensity that the leaf is exposed to during measurement
0.84 = Percentage of absorbed light (84%) [92]
0.5 = Fraction of the absorbed light distributed to PSI and PSII, respectively. This value assumes that 50% light is absorbed by PSII and 50% by PSI

- Non-Photochemical Quenching (NPQ) [90]:

NPQ = (F_m-F_m')/F_m'

- Additional parameters such as quantum yield of regulated energy dissipation (Y(NPQ)), quantum yield of nonregulated energy dissipation (Y(NO)), coefficient of nonphotochemical quenching (qN), coefficient of photochemical quenching ((qP)-based on the “puddle” antenna model, and (qL)- based on the “lake” antenna model) can be calculated using the same data [93].

Φ (II) is a parameter that is used to monitor changes in PSII photochemistry in different experimental setups over longer time intervals. This is a useful indicator when studying mutants that directly or indirectly affect PSII.

ETR (II) represents a measure of the linear electron flow through PSII and is determined as μmol e^- m^{-2} s^{-1}. Accurate determination of ETR values may become problematic when comparing wild type (WT) to mutant plants that differ in pigment or photosynthetic proteins composition because it will alter the two assumed parameters for light absorption (0.84) and distribution (0.5). These parameters may vary between young and old plants, and between plant species. For best estimation of ETR, it is recommended to determine the leaf absorption and PSII/PSI distribution.

NPQ monitors the apparent heat loss from PSII, in other words dissipation of excess energy as heat. Plants can absorb and utilize a certain amount of energy before light reaches values where it becomes harmful. In order to protect themselves from photo-damage, chloroplasts dissipate the excess energy as heat [94]. Several mechanisms work together to dissipate excess energy as a photoprotective mechanism and are included in NPQ.

NPQ = qE + qZ + qM + qT + qI

qE is the fast energy dependent component, relies on ∆pH, zeaxanthin and PsbS, and is formed within 10-200 s [95, 96].

qZ, also called zeaxanthin-dependent quencher, is a slow inducible component that uses ∆pH for de-epoxidation of violaxanthin into zeaxanthin, which is the pigment responsible for the quenching mechanism. It takes approximately 10-30 min for this mechanism to reach maximum values [95].

qM is a chloroplast photorelocation or avoidance mechanism where chloroplasts physically rearrange themselves within the cell to shade each other from excess light [97].

qT is the state transition component that is important under certain light conditions, specifically under low light, and is dependent on Stn7 –mediated phosphorylation of LHCII [5]. State transition can be studied as a separate mechanism in itself using specially designed protocols that monitor steady state chlorophyll *a* fluorescence under low light and changes in F_m' amplitude under the same conditions [98].

qI is the photoinhibition component, and is ascribed to the photoinhibitory damage of PSII reaction centers. This component forms slowly, persists for several hours after illumination has stopped, and is highly dependent on the PSII repair mechanism [99].

3.4 ElectroChromic band Shift (ECS) at 515 nm was first discovered by Duysens in 1954 [100], and was later demonstrated that it provides a linear measure of the electric potential across the thylakoid membrane [101]. Because the primary electron donor and the electron acceptors of the photosynthetic machinery are located on opposite sides of the thylakoid membrane, as well as the H^+ translocating activity of the Q cycle*,* an electrochemical H^+ gradient is generated across the thylakoid membrane, also termed as proton motive force (PMF). The electrochemical gradient has two components: the pH gradient (∆pH) and the electrical potential gradient ($\Delta\Psi$). Photosynthetic pigments, mainly carotenoids and chlorophyll *b,* in the presence of the generated electric field, change their absorption levels and give rise to ECS at 515 nm [102, 103].

There are two general approaches of collecting data using the ECS method, one using short flashes and recording the 515 signal on a ms time scale (Figure 8), and another one recording the signal during longer illumination intervals (seconds to

minutes), as well as transitions from light to dark (Figure 9) (method used in **Paper I to V**).

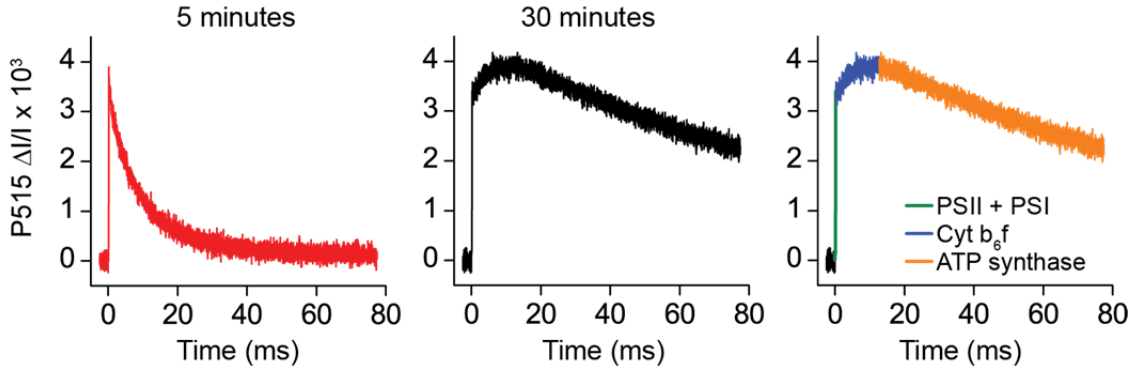

Figure 8. Single turnover flash-induced ECS. 5 min (left panel) or 30 min (middle panel) dark-adapted *Arabidopsis thaliana* leaf exposed to a 5-μs flash of red actinic light of 200,000 μmol photons m^{-2} s^{-1}. Right panel shows the information that the ECS signal contains about the electron transfer chain and ATP synthase.

The types of information that can be obtained using short flashes are the following:

- PSII and PSI simultaneous charge separation; this information is gained from the amplitude of the ECS signal upon exposure to a saturating flash (Figure 8, right panel, marked with green colour). Using DCMU, the contribution of each separate reaction center can be established.
- Electron transfer through Cyt b_6f; this information is found in the second slow rise seen in the 30 min dark-adapted leaf in the time range 0 to ~20 ms (Figure 8, right panel, marked with blue colour). In short dark-adapted samples, this phase is no longer visible (Figure 8, left panel).
- Membrane conductivity or H^+ leakage mainly through ATP synthase; this information corresponds to the decay of the ECS signal starting from ~20 ms in the long dark-adapted sample (Figure 8, right panel, marked with orange) or immediately after the ECS rise after a short dark-adaptation (Figure 8, left panel). The fast decay of the signal in the measurement of the 5-min dark-adapted leaf indicates that ATP synthase activates quickly and can dissipate the H^+ gradient, whereas in the 30-min dark-adapted leaf this process is significantly slower.

Longer recordings of the ECS signal provide distinct type of information from the short ECS recordings. A dark interval relaxation kinetics (DIRK) analysis of the ECS signal [104] can give information about the following aspects:

- Relative values for the total PMF. After illumination is stopped, the ECS signal quickly decays until it reaches a minimum value. The difference between the steady state during illumination and the new

minimum value reached immediately after actinic light is switched off corresponds to the total PMF, marked with red arrow (Figure 9). This is also termed as ECS_T.

- ATP synthase H^+ conductivity (g_{H^+}). When illumination is stopped, the OEC no longer generates H^+, and the PQ pool stops its H^+ transport from chloroplast stroma to thylakoid lumen. Concomitantly, ATP synthase will continue to function until it completely consumes its driving force (PMF). This process takes < 1 s and can be calculated as $g_{H^+}=1/\tau$, where τ is the half time of the signal decay (Figure 9 inset).
- PMF partitioning into ΔpH (ECS_{INV}) and $\Delta\Psi$ (ECS_{SS}) (Figure 9 orange and blue arrows).
- Relative value of steady-state H^+ flux (ν_{H^+}). H^+ flux is calculated as ν_{H^+} = PMF × g_{H^+} and linearly correlates with the photosynthetic linear electron flow [105]. ν_{H^+} can be considered a quantitative measure of ATP synthase activity, whereas g_{H^+} a qualitative one.

Due to changes in pigment composition and light scattering during longer illumination intervals, recordings of the 515 nm signal requires a deconvolution protocol. When using the WALZ DUAL PAM-100, deconvolution is performed automatically by simultaneous recording at 550 nm wavelength, and the signal is directly displayed de-convoluted as P515 (P515 $\Delta I/Ix10^3$ = 550-515 nm) [102]. Additional deconvolution can be done by simultaneously recording light scattering at 535 nm, which has been recently shown to contribute more than expected to the 515 nm signal [106]. The final wavelength signal will be plotted as 550-515-535 nm. Furthermore, an additional normalization protocol is used for the PMF value, in order to compensate for small differences that may occur between biological replicates. For this purpose, a short 5-μs flash-induced ECS (ECS_{ST}) recording is made for each leaf prior to every measurement, and the amplitude of the ECS_{ST} is used to normalize the PMF values between replicates. Corrected PMF = measured PMF × ($maxECS_{ST}$ / ECS_{ST}), where $maxECS_{ST}$ is the highest ECS_{ST} from all the replicates, and ECS_{ST} is the ECS_{ST} corresponding to the PMF that is being normalized.

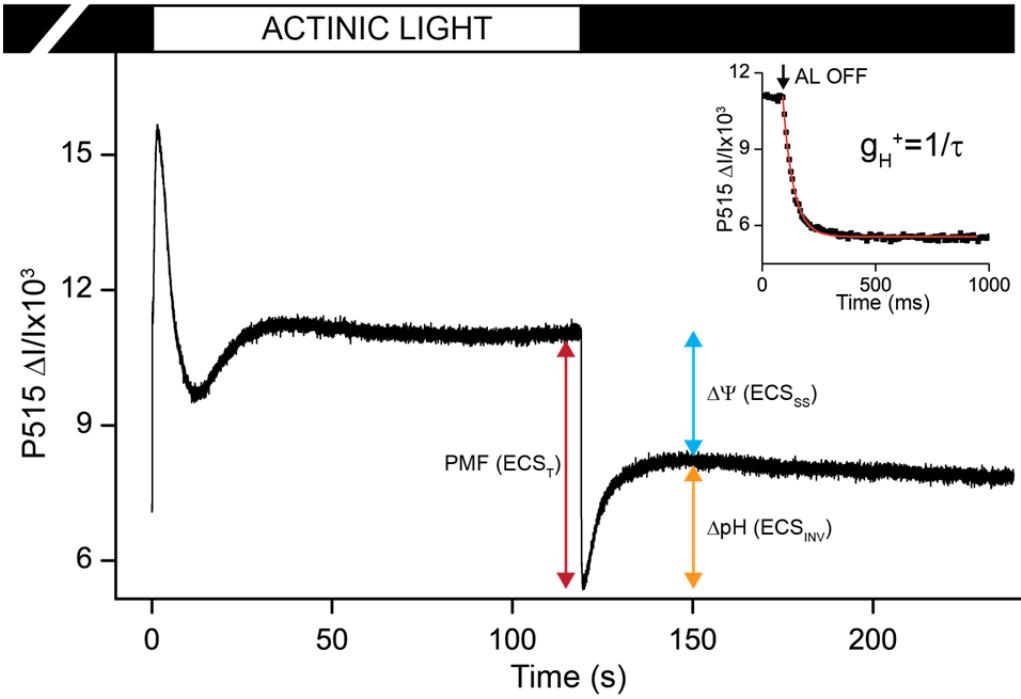

Figure 9. Recording of ECS dark interval relaxation kinetics. Recording of a 30-min dark-adapted leaf of *Arabidopsis thaliana* illuminated with 535 μmol photons m^{-2} s^{-1} actinic red light for 2 min followed by 2 min of darkness. Inset shows the kinetics of the ECS decay at ms time scale when light is turned off; red line indicates a single exponential decay fitting of the curve, and the g_H^+ equation calculated using the decay half time (τ). After illumination is stopped, the ECS signal further relaxes and reaches a new dark baseline within tens of seconds, value that is used to determine the PMF partitioning as shown with blue (ΔΨ) and orange (ΔpH) arrows. Red arrow indicates PMF size.

ROLE OF IONS AND ION TRANSPORTERS IN PHOTOSYNTHESIS

4

4.1 Ion transport mechanisms and regulation of the proton motive force. Ions play a central role in modulation of PMF size, and partitioning into $\Delta\Psi$ and ΔpH. Counterion movement across the thylakoid membrane functions in order to dissipate $\Delta\Psi$ [107-109]. Upon exposure to light, several ion fluxes take place across the thylakoid membrane: (*i*) H^+ are pumped or generated into the lumen (via PQ and the OEC) and hence a significant $\Delta\Psi$ is generated, (*ii*) cations (Mg^{2+}, K^+, etc.) are exported from the lumen while anions are imported in order to quickly dissipate part of the $\Delta\Psi$. By reducing $\Delta\Psi$, it is expected that PMF partitioning is modulated in such a way to allow a fast formation of a significant ΔpH [110] There is an increasing number of mutants defective in thylakoid ion fluxes which are unable to reduce $\Delta\Psi$ and indeed cannot form a significant ΔpH ([33], **Paper IV and V**). Size of total PMF, known to regulate ATP synthase activity [111], can also be modulated by changes in ion homeostasis **(Paper II to V)**. Below, we discuss the specific role of each of the major ions in the chloroplast in regulation of PMF across the thylakoid membrane.

4.1.1 Potassium (K^+) is the second most abundant ion in the chloroplast, second only to H^+. Recent publications [32, 33] and **Paper I** have demonstrated that K^+ plays a crucial role in regulation of the PMF by altering the ΔpH / $\Delta\Psi$ stoichiometry. Two types of transport proteins have been found in the thylakoid membrane of Arabidopsis that mediate K^+ fluxes (Figure 10).

TPK3 is a two-pore K^+ channel, shown to regulate photosynthesis by exporting K^+ from the lumen to allow translocation of more H^+ into the lumen and formation of a significant ΔpH. Knockout mutants displayed a two fold decrease in ΔpH, and consequently a large increase in $\Delta\Psi$ without any changes in PMF size [33]. The physiological effects of this alteration in trans-thylakoid energy storage, when studied in knockout mutants, resulted in reduced plant growth and hypersensitivity to high light due to impaired photoprotective mechanisms (NPQ) dependent on ΔpH.

KEA3 is the second characterized protein ([32], **Paper I**) that regulates K^+ fluxes across the thylakoid membrane. It functions as a K^+/H^+ antiporter, and unlike TPK3, it is proposed to load K^+ into the lumen by using the ΔpH formed during illumination. KEA3 has a KTN domain that may be regulated by the redox state of the chloroplast stroma, and hence may function as part of a PSI feedback mechanisms [32]. *Arabidopsis thaliana* knockout mutants of KEA3 do not have a growth phenotype but the ΔpH / $\Delta\Psi$ stoichiometry is altered in favour of ΔpH, with no changes in PMF size. Its major role has been shown to be under changing light conditions, when transitions from high light to low light occur, as well as transitions from dark to low light.

Together TPK3 and KEA3 form an elegant mechanism for regulating K^+ fluxes across the thylakoid membrane, and indirectly adjusting photosynthesis to the light environment by altering the PMF composition.

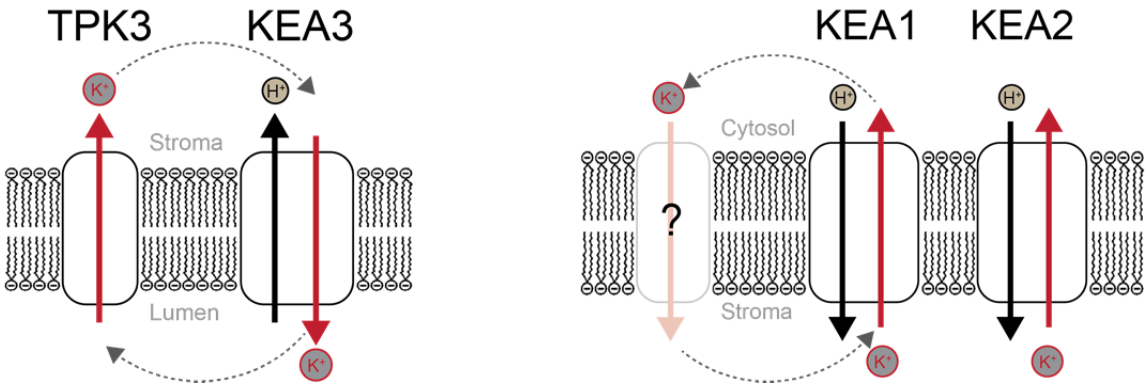

Figure 10. Dual protein mechanism for K^+ cycling in the chloroplast. Schematic representation of TPK3 and KEA3 regulated K^+ fluxes across the thylakoid membrane (left image). A similar mechanism functions across the chloroplast inner envelope where KEA1& KEA2 export K^+ to the cytosol (right image). A chloroplast K^+-loading mechanism is still missing.

KEA1 and KEA2, additional members of the KEA family, have been localized to the chloroplast inner envelope **(Paper I)** and function in a H^+-driven K^+ export system from chloroplast stroma to the cytosol. While these proteins directly regulate the electrochemical gradient across the envelope membrane they do have a downstream effect on the thylakoid membrane. Knockout of either KEA1 or KEA2 did not have a significant impact on plant growth or photosynthesis, however, the knockout of both proteins resulted in a severely stunted growth, disrupted chloroplasts, altered PMF size and partitioning **(Paper I)**.

4.1.2 Calcium (Ca^{2+}) is found in the chloroplast at ten-fold lower concentrations than K^+ [112], however, it may still play an important role in size and composition of PMF. In **Paper II** we show that the Arabidopsis *photosynthesis-affected mutant71* (*pam71*) has an altered Ca^{2+} homeostasis in the chloroplast. The *pam71* mutant accumulates Ca^{2+} inside the thylakoid lumen, which results in a two fold increase in PMF size, altered $\Delta\Psi$ / ΔpH stoichiometry due to significantly increased $\Delta\Psi$ as well as reduced ATP synthase conductivity. TPK3 is Ca^{2+} regulated [33], and our data cannot completely exclude the possibility that the observed phenotype in PMF is due to inhibition of TPK3. The altered Ca^{2+} distribution within the chloroplast of *pam71* may be caused by a defective Ca^{2+} efflux activity in the thylakoid membrane, for which the protein responsible remains to be identified.

4.1.3 Phosphate (P_i) has been proposed to be present in the thylakoid lumen as a component of the lumen-located nucleotide metabolism [113, 114], hence its presence in the thylakoid lumen may contribute to PMF modulation. The current model for import and export of phosphate does not suggest that it is a fast acting mechanism evolved specifically for charge compensation, but rather for the nucleotide-dependent mechanisms. Two proteins are known to be localized to the thylakoid membrane that are part of the phosphate transport mechanism: the thylakoid ATP/ADP carrier (TAAC) [43, 115] and the thylakoid P_i transporter PHT4;1

[46, 47] (Fig. 11). PHT4;1 has been found by other studies in the chloroplast inner envelope [48, 49]. If this is the case, then a lumenal phosphate export mechanism still remains elusive (Fig. 11).

In **Paper III**, we show that PHT4;1 knockout mutants have a transient increase in ΔpH, and consequently a significant decrease in $\Delta\Psi$, whereas the total PMF is not affected. Furthermore, thylakoid membrane conductivity (g_{H^+}) decreases up to 50% in these mutants as compared to WT, a phenomenon that was previously reported under conditions of P_i depletion in the stroma [105]. The observation in **Paper III** that addition of P_i during growth of the PHT4;1 mutants restores the WT g_{H^+} supports the idea of P_i limitation for ATP synthase in the stroma of the mutants. In addition, the reduced growth phenotype of the mutants was suppressed by high phosphate. Another previously reported effect of stromal P_i depletion is a significant increase in PMF size without changing the partitioning [105] which is not observed in PHT4;1 mutants that instead have a transiently increased ΔpH contribution to PMF **(Paper III)**. This difference can be explained by an accumulation of P_i in the lumen of the mutants (due to defective P_i export), that dissipates part of $\Delta\Psi$, and hence keeps PMF size at levels similar to wild-type. The transient nature of this effect is probably due to the fact that the lumenal P_i concentration is significant for membrane depolarization only before the import of other major anions is initiated (*e.g.*, Cl^-), after which lumenal P_i concentration becomes small relative to that of other anions. Taken together, the phenotype of PHT4;1 knockout mutants support the location of PHT4;1 in the thylakoid membrane, where it acts as a local supplier of P_i from the thylakoid lumen to the ATP synthase.

In contrast to PHT4;1, knockout mutants of envelope located PHT2;1 [51, 52, 116], PHT4;4 [53, 54], PHT4;5 [53] or TPT [53, 117] do not have an apparent growth phenotype or altered photosynthetic performance. It is conceivable that envelope-localized P_i transporters may also alter PMF size and partitioning through a downstream effect by limiting the P_i supply to ATP synthase, however no experimental data is available to confirm this. Interestingly, PHT4;4 has been shown using in vitro assays to be able to transport ascorbate, in addition to P_i into the chloroplast [54]. Authors of the same paper predicted a similar function for PHT4;1 in the thylakoid membrane. However, this possibility is excluded as based on our finding that the PHT4;1 knockout mutants do not display altered ascorbate content in the thylakoid lumen (**Paper III**).

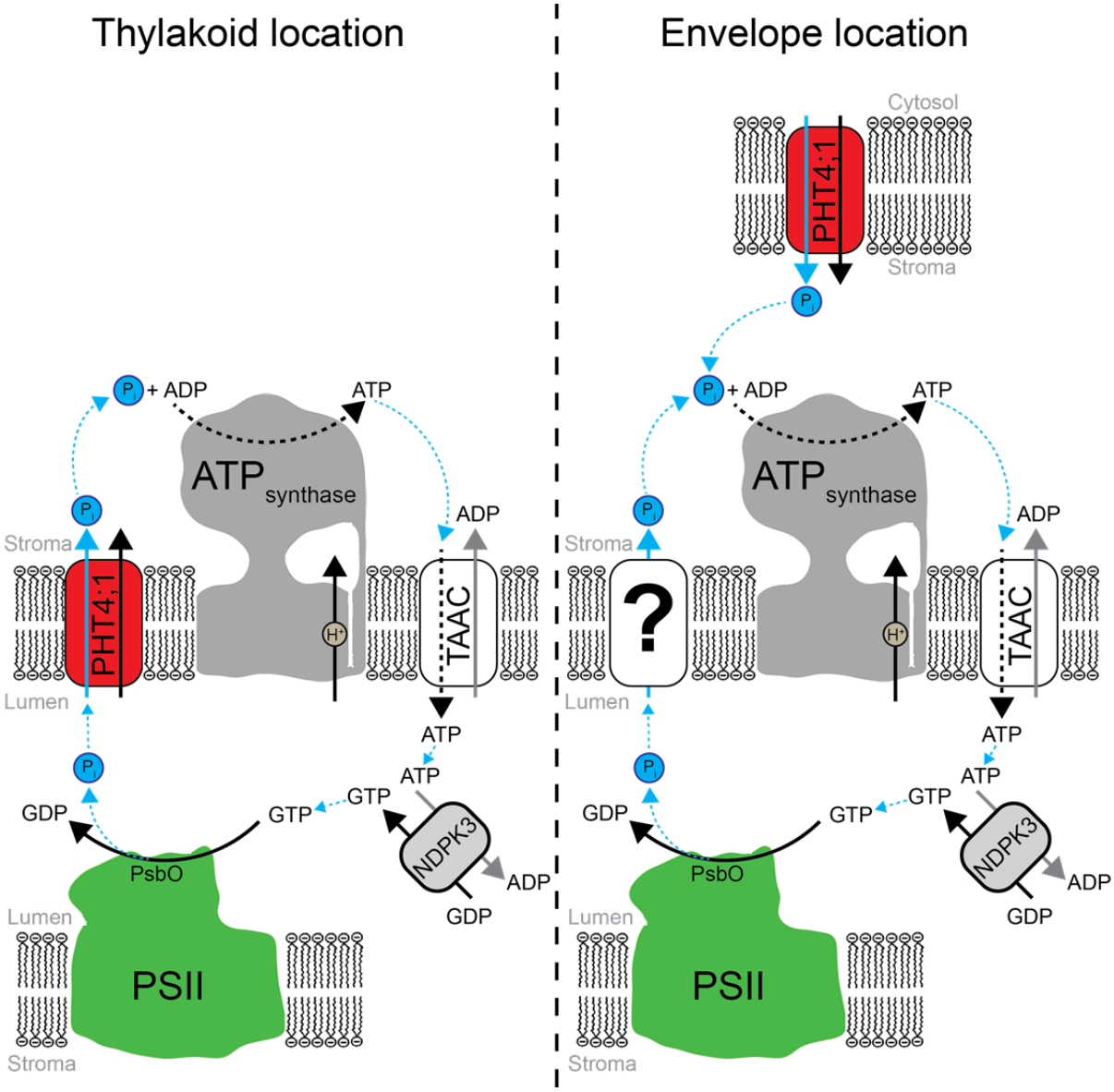

Figure 11. Phosphate cycling across the thylakoid membrane. Blue arrows indicate P_i pathway in the form of nucleoside triphosphates (ATP and GTP) and in free form after GTP hydrolysis by the PsbO extrinsic subunit of PSII. Following import into the lumen via TAAC, ATP is converted to GTP by nucleoside diphosphate kinase 3 (NDPK3). GTP is bound and hydrolysed by PsbO in a process associated with D1 turnover [114, 118], releasing free P_i into the lumen. In the thylakoid-location scenario (left image), PHT4;1 transports P_i back to the chloroplast stroma, where it is used as substrate by the ATP synthase. If however PHT4;1 is located to the chloroplast inner envelope (right image), it will simply act as a P_i chloroplast import system together with other envelope transporters (PHT2;1, PHT4;4 and triose-Pi/Pi transporter). This second possibility however still requires a protein to export P_i from the lumen to the stroma.

4.1.4 Chloride (Cl^-) is the most abundant anion present in the chloroplast and is thought to be important for membrane depolarization. A thylakoid-located Cl^- channel activity has been first reported using a patch-clamp approach on isolated thylakoids from *Peperomia metallica* [109]. A member of the CLC chloride family, CLCe, has been localized to the thylakoid membrane [35, 36], and has been long considered to directly regulate Cl^- or NO_3^- ion fluxes across thylakoids. In **Paper IV**, we identify a second thylakoid-located Cl^- channel in Arabidopsis, VCCN1. ECS measurements of CLCe knockout mutants **(Paper V)** show small but statistically significant changes in PMF size, $\Delta\Psi$ / ΔpH stoichiometry, as well as ATP synthase activity. A similar but greater PMF phenotype was observed in knockout mutants of VCCN1 **(Paper IV)**. The results from ECS measurements confirm that both CLCe and VCCN1 play role in depolarization of the thylakoid membrane.

Despite the identification of two distinct Cl^--translocating proteins in thylakoids, VCCN1 and CLCe (the transport function of the latter remains to be confirmed by heterologous expression), the complete mechanism for Cl^- fluxes has not yet been solved. It is however established that a Cl^- influx to the lumen takes place in the light, and an efflux in darkness [119]. Four hypothetical models are presented in Figure 12 for how these fluxes may take place in the context of VCCN1 and CLCe. Several other possibilities may exist and could also be considered. In order to elucidate this mechanism, more experimental data are required about the type of transport process that CLCe performs (channel *versus* transporter) as well as the substrate specificity, and a more accurate determination of the lumenal and stromal Cl^- concentrations in the light and in the dark.

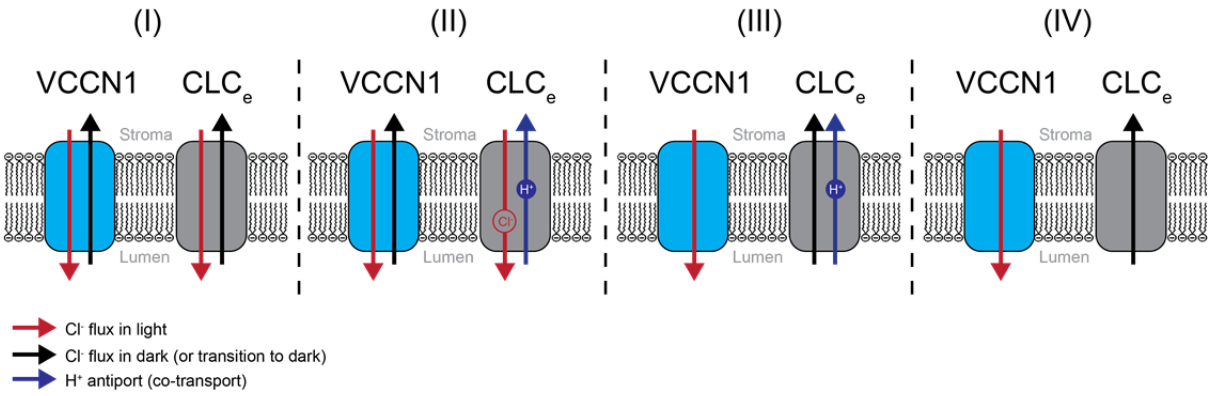

Figure 12. Hypothetical models of Cl^- transport across the thylakoid membrane. (I) VCCN1 and CLCe are driven by $\Delta\Psi$ and both transport Cl^- in and out of the lumen, but are differently regulated or have different levels of specificity. Export can take place immediately after light is switched off due to change in electrical polarity across the membrane. (II) VCCN1 is driven in the same way by $\Delta\Psi$ for import and export, whereas CLCe uses ΔpH to drive import of Cl^- against its concentration gradient. (III) VCCN1 imports Cl^- in light but does not perform export (the inverted polarity in transition to darkness may not be energy sufficient), whereas CLCe uses ΔpH upon transition to darkness for Cl^- efflux. (IV) Both proteins use $\Delta\Psi$

but are differently gated and VCCN1 provides the influx, whereas CLCe the efflux of Cl^-.

4.1.5 Other ions like Mg^{2+}, which is present in mM concentrations in the chloroplast, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, NO_3^- or NO_2^- have been less studied in the context of their role in regulation of the PMF. Nevertheless, they may contribute directly or indirectly to the overall electrochemical gradient across the thylakoid membrane. The proteins responsible for mediating these ion fluxes remain largely elusive.

4.2 Regulation of electron transfer.

Arguably the most important component of the ETC, that is dependent on a specific configuration of ions, is the OEC. The assembly of the Mn_4CaO_5 inorganic cluster at the donor side of PSII is not fully understood, but it is obvious that during the assembly process Ca^{2+} and Mn^{2+} are strictly required in the thylakoid lumen. Cl^- ions have also been found in the vicinity of the Mn_4CaO_5 cluster, and may have a role in H^+ channelling from the core of the OEC after oxidation of water [11, 120, 121].

The Arabidopsis *pam71* mutant has a significant loss of PSII activity due to impaired and unstable OEC, and resembles a double *psbo1×psbo2* mutant **(Paper II)**. Since *pam71* accumulates Ca^{2+} inside the thylakoid lumen, it is plausible that this causes the observed defect at OEC site. This possibility is supported by the notion that PsbO, known to stabilize the OEC, is washed off the PSII complex in the presence of excess Ca^{2+} [122]. This finding emphasizes the importance of Ca^{2+} homeostasis for the optimal function of OEC and entire ETC.

Although chloride is known to have a role in oxygen evolution, knockout mutants of VCCN1 or CLCe do not seem to have a significant impact on the OEC **(Paper IV and V)**.

PSII, Cyt b_6f and PSI have iron clusters that directly participate in transfer of e^-. Approximately 80% of cellular Fe^{2+} is stored in the chloroplast [38], and under Fe^{2+} starvation plants undergo chlorosis. Fe^{2+} transport in to the chloroplast takes places via several transporter proteins: MAR1 [57], NAP14 [58], YLS4 [59, 60], YLS6 [59, 60], PIC1 [61, 62], and NiCo [61]. No Fe^{2+} transport mechanism or requirement has been proposed across the thylakoid membrane. Cu^{2+} is a redox cofactor for PC [123], hence is directly required for electron transfer between Cyt b_6f and PSI. Cu^{2+} import to the chloroplast is proposed to take place via HMA6 [66, 68] and HMA1, whereas import to the thylakoid lumen via HMA8 [48, 66].

4.3 Regulation of thylakoid architecture.

Regulation of thylakoid stacking and unstacking is an important process for PSII repair [3], state transition [124], NPQ [125] and spill-over of excitation energy [126]. Two distinct but interconnected phenomena govern the thylakoid stacking and

unstacking: electrostatic screening of the negative charges from membrane bound proteins at the surface of the membrane [127, 128], and increased osmotic pressure in the lumen [3]. $\Delta\Psi$-driven influx of Cl^- in the lumen increases the osmotic potential, hence induces lumen swelling [119]. Knockout mutants of the thylakoid chloride channel VCCN1 have altered thylakoid structures, confirming the role of Cl^- homeostasis in regulation of thylakoid ultrastructure **(Paper IV)**. Mg^{2+} is well known to be a catalyst for grana stacking [129, 130], whereas Ca^{2+}, Mn^{2+} and K^+ are known to be less effective in this process [131]. Nevertheless, knockout mutants of the thylakoid potassium channel TPK3 have an altered ultrastructure under certain light intensities [33].

4.4 Signalling and enzymatic activation.

Calcium is predominantly bound to the negatively charged thylakoid membrane or to calcium-binding proteins, and only a small fraction is free resting. Light-dependent Ca^{2+} influx into the lumen has been associated with Mg^{2+} efflux to the stroma [132, 133]. Ca^{2+} is involved in a wide range of regulatory processes in the chloroplast, by either direct binding[1] of Ca^{2+}, Ca^{2+} dependent phosphorylation[2], or calmodulin (CaM) binding[3]:

- linear electron flow (PsbO[1])
- cyclic electron flow (NDH-F[2])
- state transition (PsaH[2])
- PSII repair (FtsH2[2] and 5[2])
- carbon fixation (FBPase, SBPase, TKL[2])
- transporters and channels gating (TPK3[2], TIC110[2], TIC32[3])
- regulation of stomata function and photoaclimation (CAM[1-3]) [112].

Copper is required in the thylakoid lumen for PC maturation [123], and additionally it regulates its thylakoid loading mechanism by binding to HMA8 (ATP-dependent Cu^{2+} transporter) to initiate its degradation by Clp proteases [134]. Cu^{2+}, Zn^{2+} and Fe^{2+} are required for the activity of superoxide dismutase, a scavenger of reactive oxygen species [135].

Zinc has been shown to stimulate the proteolytic activity of FtsH2, and to promote specific degradation of Rieske FeS protein of the Cyt b_6f [136, 137].

Magnesium catalyses enzymatic reactions of acetohydroxy acid isomeroreductase that is involved in biosynthesis of the amino acids isoleucine, valine, and leucine [138]. The ATP synthase CF1 subunit also binds Mg^{2+} and becomes reversibly inactivated [139].

4.5 Regulation of photoprotective mechanisms.

In natural environments plants absorb more energy from light that can be used for photochemistry, and in order to avoid photodamage the excess energy is dissipated as heat in a process called non-photochemical quenching (NPQ). NPQ incorporates several mechanisms that are directly or indirectly dependent on ∆pH (See section 3.3.2).

Knockout mutants of chloroplast thylakoid and inner envelope ion transporters have almost always an altered NPQ [32, 33, 54] **(Paper I, III, IV, and V)**. However, this effect cannot be attributed to the transporters themselves, since they do not directly participate in NPQ, but to a secondary effect of the altered ∆pH (in the case of TPK3, KEA1-3, PHT4;1 and VCCN1 in **Papers I, III and IV**), impaired enzymatic activity of the xanthophyll cycle (PHT4;4, [54]) or possible changes in thylakoid architecture (CLCe in **Paper V**). PHT4;1 changes the kinetics of NPQ by altering P_i availability to ATP synthase, which most likely results in a faster acidification of the thylakoid lumen that in turn affects NPQ **(Paper III)**.

In nature, plants experience rapid fluctuations in light intensity that change up to 100 fold within seconds to minutes [140]. In order to avoid photodamage, plants can modulate the response time of NPQ by quickly adjusting the ∆pH. Previously KEA3 has been shown to modulate NPQ upon rapid shifts from high to low light [32]. In **Paper IV** we show that VCCN1 is able to modulate the NPQ response time upon rapid shifts from low to high light.

CONCLUSIONS AND OUTLOOK

5

The work presented in this thesis answers several questions about the role in regulation of photosynthesis of a number of ion channels and transporters from the thylakoid membrane and chloroplast inner envelope of Arabidopsis.

Regulation of K^+ transport in chloroplasts is presented as a complete mechanism governed by KEA3 and TPK3 across the thylakoid membrane and partially by KEA1 and KEA2 across the inner envelope **(Paper I)**. The cytosol to stroma import mechanism still remains to be resolved. Nevertheless, the importance of KEA1, KEA2 and KEA3 for plant growth is studied, and their role in K^+ homeostasis, regulation of PMF and photoprotection is presented in **Paper I**.

Ca^{2+} transport across the thylakoid membrane has been demonstrated since 1999 [133], however the protein(s) responsible for these fluxes have not been identified. In **Paper II**, a mutant affected in Ca^{2+} homeostasis is identified (*pam71*). This alteration is shown to have implications for PSII function and regulation of PMF. A defective activity for Ca^{2+} efflux from the thylakoid lumen may be responsible for the observed phenotype, for which the responsible protein remains to be identified.

In **Paper III**, role of the PHT4;1 in phosphate transport across the thylakoid membrane is addressed. We present a new model for P_i metabolism where PHT4;1 is a localized supplier of P_i to ATP synthase. This discovery has implications for plant growth, $\Delta\Psi$ / ΔpH stoichiometry and photoprotection.

In **Paper IV** a novel voltage-dependent Cl^- channel (VCCN1) is identified in thylakoids. VCCN1 is the first confirmed (by electrophysiology) Cl^- channel from the thylakoid membrane. Cl^- fluxes in the chloroplast have long been thought to regulate membrane depolarization, and to induce changes in thylakoid ultrastructure. These predictions are confirmed in **Paper IV**, and additionally we show that PMF-driven Cl^- fluxes also regulate formation of NPQ upon rapid shifts from low to high light.

Before the discovery of VCCN1 in the thylakoids, another protein from the CLC family (CLCe) has been localized to the same membrane, and was thought to mediate Cl^- fluxes. The Cl^- transport function of CLCe has not yet been confirmed by heterologous expression. However, in **Paper V** its role in photosynthesis is investigated, and similarly to VCCN1, it was found to regulate membrane depolarization but to a smaller extent. Additionally, a specific function in state transition and regulation of the electron transfer was found.

Taken together, the findings presented in this thesis contribute to the increasing picture of ion channels and transporters from the chloroplast and their importance in photosynthesis. Furthermore, studies of the possible interactions between these proteins will help to better describe the network of ion fluxes in the chloroplast.

Due to their role in modulation of PMF and its partitioning, ion transporters could potentially be used for engineering photosynthetic organisms with maximized light use efficiency, and shortened response time of photoprotective mechanisms. VCCN1 together with KEA3 may be used to accelerate or slow down the response time of NPQ upon fast changes in light intensity. CLCe on the other hand may be used to modulate the response time for changes in light quality. Several other features of transporters and channels may be further employed for optimization or improvement of photosynthesis.

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6

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