Milton Karlsson Plastidial Phosphate Transport in Plants

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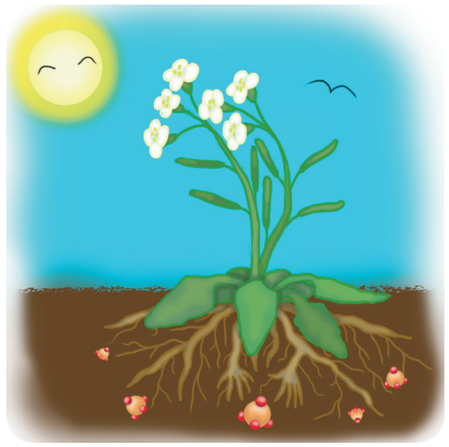

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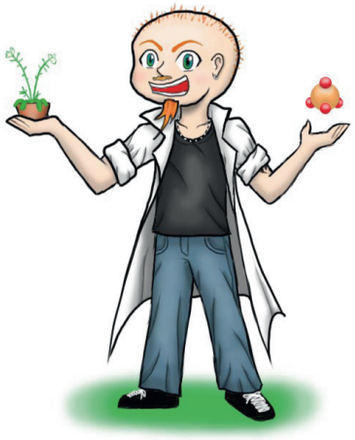

Plastidial Phosphate Transport in Plants

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GÖTEBORGS UNIVERSITET

FACULTY OF SCIENCE
DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

Akademisk avhandling för filosofie doktorsexamen i Naturvetenskap med inriktning Biologi, som med tillstånd från Naturvetenskapliga fakulteten kommer att offentligt försvaras fredagen den 10 oktober 2014 kl. 10.00 i Hörsalen, Institutionen för biologi och miljövetenskap, Carl Skottsbergs gata 22B, Göteborg.

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For me

I know well what I am fleeing from

but not what I am in search of.

There is no wish more natural

than the wish to know.

- Michel de Montaigne -

Plastidial Phosphate Transport in Plants

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ABSTRACT

Phosphorus is an essential element for all living organisms and is central to the genetics and energetics of life. Inorganic phosphate (P_i) is recurrently involved in protein regulation and signal transduction but also in energy transfer as a component of the ATP-molecule. When cells and cell organelles commence a plethora of energy-demanding processes associated with ATP hydrolysis to ADP and P_i, a balancing of the P_i content between compartments is crucial to prevent the ATP hydrolysis to be stalled from accumulation of P_i. The transport of P_i via specialized protein(s) is therefore essential for cellular P_i homeostasis since biological membranes are impermeable to P_i (Paper I, III).

This thesis shows that the plastid-localized P_i transporter PHT4;2 in *Arabidopsis thaliana* is nearly restricted to roots during vegetative growth, where it regulates plastid homeostasis by a Na^+-dependent P_i efflux. The accumulation of P_i in the root plastids of *pht4;2* loss-of function-mutants yields a reduced starch accumulation in roots, which is consistent with the inhibition of starch synthesis by a deficient P_i export. However, the *pht4;2* mutants display a 40% increased rosette area and a twofold larger shoot biomass as compared to wild type (WT) plants, indicating an involvement of PHT4;2 in signaling between roots and leaves. The larger leaf area and biomass accounts from an increased cell proliferation in *pht4;2* mutants compared to the WT plants. Nevertheless, the cell size and the photosynthetic electron transport rate are similar in all genotypes. (Paper I).

Another P_i transporter, PHT4;1, is located in the chloroplast thylakoid membrane of Arabidopsis. By using homology modeling, site directed mutagenesis and functional characterization in *Escherichia coli*, several residues important for P_i transport and its sodium dependency have been identified in PHT4;1 (Paper II). Rosette area and biomass of the *pht4:1* mutants are reduced to 70-80% of the WT plants. Absence of PHT4;1 does not affect the relative electron transport rates, pigment composition, and the expression of photosynthesis-related proteins. However, the ΔpH contribution to the proton-motive force across the thylakoid membrane is significantly higher in the *pht4;1* mutants as compared to the WT plants. Non-photochemical quenching kinetics in *pht4;1* mutants is transiently increased at the initial phase and declines to WT levels during the plateau phase. Moreover, the P_i content is elevated in the *pht4;1* mutants whereas the total Phosphor content is similar to the WT (Paper III).

This thesis shows that, through their activity, plastidial P_i transporters play role in plant growth and behavior under different environmental conditions. This is a subject still in its cradle of being understood. The data acquired in this work not only strengthen the importance for a normal daily life of plants, but also the relevance of P_i transporters as a research field.

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Populärvetenskaplig sammanfattning

Fosfor är ett av de mest nödvändiga näringsämnena i växter och deltar i många av växtens fysiologiska processer. Fosfor är en viktig beståndsdel i bl.a. energimolekyler (exempelvis ATP och GTP), signalmolekyler (proteiner som förändras när de får en fosformolekyl på sig), samt i den genetiska koden (RNA och DNA). I de flesta energikrävande processer används ATP som energikälla där ATP ombildas till ADP och oorganiskt fosfat (P_i). Dessa processer utförs bland annat i membranomslutna plastider (små organeller inuti cellen) där s.k. transportproteiner ser till att återföra P_i till den plats där ATP bildas för att kunna bilda ATP på nytt och upprätthålla P_i-balansen.

Arbetet med denna avhandling har resulterat i karaktäriseringen av två transportproteiner för P_i nämligen PHT4;1 och PHT4;2. Dessa två proteiner transporterar P_i över membran i två olika sorters plastider, nämligen kloroplaster i växters blad samt plastider i rötter hos backtrav (*Arabidopsis thaliana*) (Artikel I, II och III).

Vi har identifierat och karaktäriserat PHT4;2 hos backtrav som endast återfinns i rötternas plastider där fosfat transporteras ut med hjälp av PHT4;2 endast om natrium finns tillgängligt. Fungerar inte denna fosfattransport lyckas inte växten upprätthålla stärkelsenivåerna i rötterna och kompenserar bortfallet med att öka celldelningen i löven vilket i sin tur resulterar i 40 % större blad som har dubbelt så stor biomassa. Intressant nog så påverkas inte de fotosyntetiska processerna av de större bladen. Med ledning av detta samt att PHT4;2 som endast finns i rötterna även påverkar växtens övriga organ (bladen) har vi kunnat visa att fosfatbalansen påverkar signalvägar vi tidigare inte visste fanns (Artikel I).

PHT4;1 är en fosfattransportör som finns i tylakoidmembranet inuti växtcellens kloroplaster. Med hjälp av s.k. jämförande modellering och med kraftfulla datorer har vi tagit fram en proteinstrukturmodell av PHT4;1 där vi lyckats identifiera aminosyror som är viktiga för att känna av närvaron av natrium och som behövs för att kontrollera att inget annat än P_i transporteras av PHT4;1 (Artikel II).

När PHT4;1 inte fungerar blir växterna ca 20-30 % mindre och lättare. Intressant nog påverkas inte fotosyntesens effektivitet utan istället blir den mer beredd på stressande (starkt) ljus som den initialt för över till, och avger som, värme. Växten kompenserar inte för detta genom att tillverka fler eller skyddande pigment utan anpassar sig snabbt till mer normala fysiologiska förhållanden. Vi har kunnat se att om PHT4;1 inte fungerar så ökar andelen P_i i bladen, medan den totala fosforhalten är oförändrad jämfört med om PHT4;1 fungerar (Artikel III).

Som vi alla vet är fosfor en gruvnäring som håller på att ta slut samtidigt som det är en livsnödvändig komponent för växtens överlevnad. Vår forskning, som avhandlats här, är därför ett viktigt bidrag till en framtida ökad förståelse för hur växten använder sig av fosfat och hur vi i framtiden kan minska beroendet av fosfat som näringstillskott för våra grödor.

LIST OF PUBLICATIONS

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

I. Irigoyen S, Karlsson PM, Kuruvilla J, Spetea C, and Versaw WK (2011). The sink-specific plastidic phosphate transporter PHT4;2 influences starch accumulation and leaf size in Arabidopsis. Plant Physiol. 2011 157(4): 1765-1777.

II. Ruiz-Pavon L*, Karlsson PM*, Carlsson J, Samyn D, Persson B, Persson BL, and Spetea C (2010). Functionally important amino acids in the Arabidopsis thylakoid phosphate transporter: homology modeling and site-directed mutagenesis. Biochemistry 49 (30): 6430-6439.

III. Karlsson PM, Herdean A, Beebo A, Irigoyen S, Aronsson H, Versaw WK, Spetea C (2014). On the physiological role of the phosphate transporter PHT4;1 in Arabidopsis with focus on the thylakoid membrane. *Manuscript.*

* Shared first authorship

List of abbreviations

Arabidopsis	*Arabidopsis thaliana*
ANTR	Anion transporter
Chl	Chlorophyll
CP43	Chlorophyll a binding protein of 43 kDa
CP47	Chlorophyll a binding protein of 47 kDa
Cytb_6f	Cytochrome b_6f complex
D1, D2	Reaction-center binding proteins of PSII
E. coli	*Escherichia coli*
ETR	Electron transport rate
Fd	Ferredoxin
FRET	Förster Resonance Energy Transfer
GFP	Green fluorescent protein
GL	Growth light
GlpT	Glycerol 3-phosphate/phosphate antiporter
GUS	β-glucuronidase
HL	High light
LHC	Light harvesting antenna complex
MFS	Major facilitator superfamily
MSA	Multiple sequence alignment
NPQ	Non-photochemical quenching
OEC	Oxygen-evolving complex
PAM	Pulse-Amplitude-Modulation
PC	Plastocyanin
P_i	Inorganic phosphate
Pheo	Pheophytin
PHT	Phosphate transporter
PMF	Proton motive force
PSI	Photosystem I
PSII	Photosystem II
PQ	Plastoquinone
PQH_2	Plastoquinol
RC	Reaction center
ROS	Reactive oxygen species
STN	State transition
TAAC	Thylakoid ATP/ADP carrier
VGLUT	Vesicular glutamate transporters
Q_A	Primary quinone
TM	Transmembrane
VDE	Violaxanthin de-epoxidase
Vio	Violaxanthin
Zea	Zeaxanthin
ZEP	Zeaxanthin epoxidase

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1. Introduction

1.1 Plastids – structure and functions

Plants cells differ in several aspects from animal cells: large water-filled vacuoles for storage of useful and excretion of harmful compounds, cellulose-containing cell walls, plasmodesmata for cell-to-cell communication, and plastids for production and storage of carbohydrates and other compounds.

Plastids are major organelles surrounded by two or more membranes that are found in plant and also alga cells. Plant plastids are divided into different groups depending on their pigment composition, structure and developmental stage. Algae contain only green plastids.

According to the endosymbiont theory, a photosynthetic bacterium was engulfed by a eukaryotic cell which yielded the primary endosymbiosis when most of the genetic material of the retained bacterium was transferred to the nucleus of the host. There are three evolutionary lines of organisms containing primary plastids (1):

- The glaucophytes
  Algae often used to study the evolution of chloroplasts (2). Contain a primitive walled chloroplast called *muroplast.*
- The red lineage
  Often called red algae or *Rhodophyta.* Contain a chloroplast called *rhodoplast* that only contains chlorophyll *a.*
- The green lineage
  Gave rise to the plastids of the green algae and members of the Kingdom Plantae. Contains several plastid variants presented below.

The available diversity of plastids in the green lineage has been an evolutionary advantage when generating the tissue complexity in plants:

- Proplastids
  Proplastids are found in meristematic and embryonic tissues and are undifferentiated and generally very small with a poorly defined internal membrane system. They are the ancestors to all other plastid types.

- Etioplasts
  Plastids in shoot tissues that have been grown in darkness are developmentally arrested as etioplasts during the development from proplastids to chloroplasts. Etioplasts do not form in dark-grown root cells and are only found in white stem and leaf tissue that is deprived of light. Chloroplasts convert into etioplasts when shoots are kept out of light for several days.
- Leucoplast
  Non-pigmented plastids ("leukos" meaning white) are acting as storage compartments and are subdivided in three groups:
  - Amyloplasts
    Starch-synthesizing and starch-storing organelles are typically found in root tissues **(Paper I),** and are involved in gravity sensing.
  - Elaioplasts
    Oil- and lipid-storing leucoplasts are usually small and round ("elaiov" meaning olive), and mainly involved in pollen grain maturation
  - Proteinoplasts
    Sometimes called proteoplast, contains large and visible protein inclusions that can either be crystalline or amorphous.
- Chromoplast
  Brightly colored plastids ("chromo" meaning color) which contain high levels of carotenoids that provide colors to, and acting as attractants or herbivore repellents in flowers, fruits and vegetables.
- Gerontoplast
  A plastid found in senescing green tissues, which is still functioning, but is in a degrading stage of plastids.
- Chloroplast
  Light-exposed proplastids develops into mature and photosynthetically active green organelles, named chloroplasts, containing a plethora of pigments that are vital for the energy conversion in plants and algae **(Paper II & III)**.

1.2 Photosynthetic electron transport

Aerobic organisms on our planet depend on molecular oxygen (O_2) produced by plants, algae, and cyanobacteria through photosynthesis. Oxygen is a waste product produced by these organisms in an effort to convert sunlight into ATP and NADPH. The reaction takes place on thylakoid membranes in cyanobacterial cells and in chloroplasts of algae and plants. ATP and NADPH are then used to fix carbon dioxide (CO_2) into carbohydrates in a series of enzymatic reactions known as the Calvin-Benson cycle.

1.2.1 Linear electron flow

In plants, the photosynthetic process begins in the trimeric light harvesting antenna complexes (LHC) of photosystem II (PSII), which together with the core dimer form a PSII supercomplex. The major PSII core proteins are the reaction center (RC) D1 and D2 proteins, the Chlorophyll (Chl) *a* binding CP43 and CP47 proteins and the lumenal extrinsic PsbO, PsbP and PsbQ proteins (3). In the LHCs of this PSII supercomplex,

photons are captured by chlorophylls and carotenoids. When sufficient amount of excitation energy is obtained in the LHCs for the PSII reaction center chlorophyll P680 to be excited to P680*, one electron is essentially transferred to the primary electron acceptor, pheophytin (Pheo). After this process, commonly known as primary charge separation, the high-energy electron is shuttled through a linear electron flow (LEF), also known as the Z-scheme (4) (Figure 1).

The high-energy electron is transferred from P680* to plastoquinone in several steps. The pigment abbreviation P680 essentially represents a pair of chlorophyll molecules bound to the D1 and D2 subunits in the PSII core where the chlorophyll molecule of D1 (Chl_{D1}) is believed to be the major contributor of the excited P680, denoted as P680*. The high-energy electron in the Chl_{D1} of P680* is transferred via the pheophytin bound to D1 ($Pheo_{D1}$) to the primary electron acceptor Q_A, a quinone molecule bound to D2. The electron is thereafter transferred to Q_B, a secondary quinone electron acceptor molecule, generally called plastoquinone (PQ).

Immediately after two rounds of photon excitation and electrons transferred to PQ, two protons (H^+) originated from the stroma are attached to PQ and become plastoquinol (PQH_2). At this step, PQH_2 is released and laterally migrates from PSII towards the cytochrome b_6f complex (cyt b_6f) in the thylakoid membrane matrix (5). When PQH_2 docks to cyt b_6f, one electron is transferred to an oxidized copper protein plastocyanin (PC), promoting the release of the two protons into the thylakoid lumen. The remaining electron from PQH_2 is recycled by entering the so-called Q-cycle promoting two additional H^+ to be picked up from the stromal side in the second half of the Q-cycle (6) (Figure 1).

The removal of electrons from PSII, and subsequent transfer to cyt b_6f, results in a "vacuum" of electrons in the PSII complex which is refilled by obtaining electrons from water via tyrosine Z and the oxygen-evolving complex (OEC), also known as water splitting complex or Mn_4CaO_5-cluster of PSII. Three manganese, one calcium, and four oxygen atoms form an asymmetrical cubane-like structure, which together with the fourth manganese and the fifth oxygen form a tilted and crooked chair (3, 7, 8).

The absorption of four photons is necessary to complete an oxidation (splitting) of two water molecules into dioxygen (O_2), four H^+, four electrons and subsequently the reduction of two PQ molecules (4). While the oxygen diffuses through the thylakoid membrane the H^+ produced from water splitting and from redox-coupled H^+ transfer by cyt b_6f are trapped and accumulated inside the thylakoid lumen, thus creating a H^+ gradient across the thylakoid membrane between the thylakoid lumen and the stroma. The electrochemical H^+ gradient, termed the proton-motive-force (PMF), is mainly utilized by the ATP synthase, located in the thylakoid membrane, to produce ATP while releasing H^+ into the stroma (Figure 1).

Meanwhile, the electron acquired by cyt b_6f is transferred to PC, which migrates from the cyt b_6f to photosystem I (PSI) in the thylakoid lumen. PSI shares similarities with PSII, however, with some distinct discrepancies. The RC of PSI comprises of a PsaA and PsaB dimer with a P700 chlorophyll molecule pair. The electron received from PC is transferred to P700 and is excited by a photon to P700* where the high-energy electron is transferred through a bound quinone to a set of 4Fe-4S clusters. Ferredoxin (Fd) located in the stroma, transfers the electron to ferredoxin-$NADP^+$-oxidoreductase (FNR). The conversion of $NADP^+$ to NADPH is conducted via the FAD, which acts as an intermediate when assembling $NADP^+$, 2 electrons and H^+ to NADPH (9) (Figure 1).

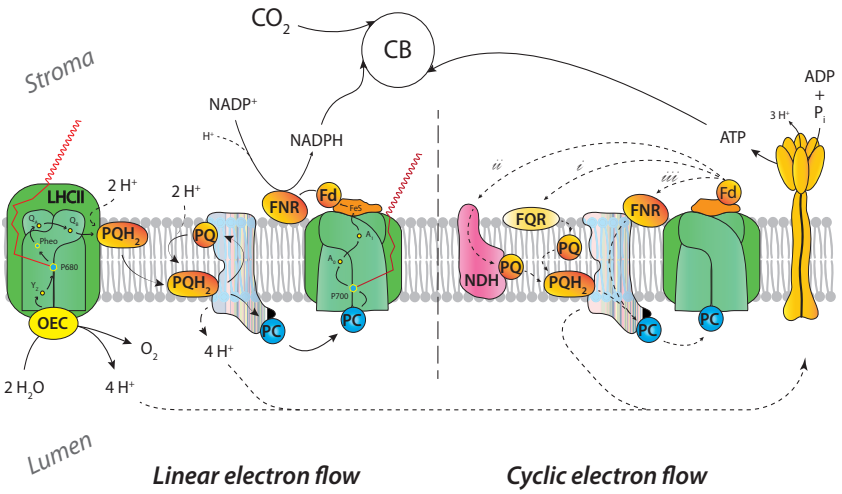

Figure 1. Schematic representation of proteins and cofactors involved in linear electron flow, cyclic electron flow, and H^+ transport in the plant thylakoid membrane. The first complex involved in linear electron flow is photosystem II (PSII) shown in green. The second complex involved in linear electron flow is cytochrome b_6f shown in light blue, is also involved in cyclic electron flow. PSI complex shown in green also participates in both linear and cyclic electron flow. The H^+-translocating ATP synthase is shown in yellow. OEC, Oxygen evolving complex bound to PSII; LHCII, Light harvesting complex bound to PSII; Y_Z, tyrosine-161 on the D1 protein; P680, Reaction center chlorophyll *a* of PSII; Pheo, Pheophytin; Q_A, a tightly bound plastoquinone; Q_B, a plastoquinone that binds and unbinds to PSII; PQ, a pool of mobile plastoquinone molecules; PQH_2, Protonated plastoquinone molecule; PC, Plastocyanin; P700, Reaction center chlorophyll *a* of PSI; A_0, a special chlorophyll *a* molecule; A_1, vitamin K; FeS, Rieske Fe-S protein; Fd, Ferredoxin; FNR, Ferredoxin-$NADP^+$ reductase; $NADP^+$, Nicotinamide-adenine dinucleotide phosphate and NADPH, protonated $NADP^+$; FQR, Ferredoxin-PQ-oxidoreductase and NDH, NADPH-PQ-oxidoreductase. CB denotes the Calvin-Benson cycle. The three carrier pathways proposed to be involved in cyclic electron flow are denoted *i, ii and iii* next to the dashed lines representing the electron flow

To summarize, LEF essentially involves three photosynthetic complexes, namely PSII, cyt b_6f and PSI. Electrons extracted from water by the OEC are transported through PSII reducing sequentially PQ to PQH_2. Oxidation of PQH2 occurs at the cyt b_6f where half of the electrons are linearly transferred via PC and PSI to the $NADP^+$. The other half of the electrons returns to the PQH_2 pool.

1.2.2 Cyclic electron flow

LEF can be bypassed by involving only PSI and cyt b_6f for generating H^+ resulting in an increased lumenal H^+ gradient, which can drive ATP synthase for ATP production via the cyclic electron flow (CEF). Thus, CEF does not generate O_2 or NADPH. The light that excites PSI reduces the FeS centers resulting in oxidation of P700. Similar to LEF, the oxidized $P700^+$ is reduced by an electron from the PQ pool via the cyt b_6f and PC. Three carrier pathways have been proposed for the cycling of electrons from PSI via Fd back to the PQ pool which then reduces the $P700^+$ to complete the cycle: (i) PGR5 pathway, also known as FQR pathway, includes the putative Ferredoxin-PQ-oxidoreductase (FQR) acting as an intermediate between Fd and PQ (10). (ii) NADPH-PQ oxidoreductase (NDH) pathway requires a large multisubunit supercomplex for electron transport back to PQ (11). (iii) A putative ferredoxin:$NADP^+$ oxido-reductase (FNR/b_6f) super complex oxidizes Fd and transports electrons back to cyt b_6f (12).

1.3 Light harvesting

Photons are absorbed by the antenna system which funnels the captured energy from photons to a reaction center. In plants, this antenna system is mainly comprised by LHC trimers aided by more than 200 Chl molecules and more than 60 carotenoid molecules. When light is absorbed by an antenna molecule, an electron is transferred from its electronic ground state to an excited state. Due to the nature and the proximity of other antenna molecules, the energy can be transferred to neighboring antenna molecules by a process known as Förster Resonance Energy Transfer (FRET) (sometimes called resonance). In this way, the energy from the excited electron is "jumping" around between the adjacent antenna molecules until the energy is transferred to an open RC, which performs the charge separation. This charge separation is fully used when Q_A in the RC is oxidized, or "open", promoting a low yield of fluorescence from the supercomplex (i.e. a minor fraction of the excited energy is lost). More than 90% of the absorbed photons can be trapped by a RC and promote charge separation under optimal conditions. However, if Q_A is reduced, "closed", the charge separation is mainly lost to fluorescence (3). Excitation energy that escapes the antenna system as fluorescence comes almost exclusively from Chl *a*, and can be utilized to elucidate the fitness and photosynthetic performance of the plant via Chl fluorescence measurements **(Paper I & III)**.

1.4 High light stress

1.4.1 PSII photoprotection

Under controlled growth light conditions, the efficiency of the plant photosynthetic machinery is nearly optimal after the plant has acclimatized to a given light intensity. However, in their natural environment, plants are continuously exposed to variations in light irradiance, humidity, and temperature that directly, or indirectly, affects photosynthetic activity.

An increased light intensity yields a higher amount of photons, which excite antenna and RC Chl and in turn forces the photosystems, in particular PSII, to work harder. If Q_A is

reduced, P680* will not be able to transfer and release the excited energy into LEF. P680* will then relax back to P680 by transferring the energy to either fluorescence (0.6%-3%), or quenching the corresponding energy to heat, a process known as non-photochemical quenching (NPQ). The overexcitation energy can lead to the production of reactive oxygen species (ROS) via the decay to the triplet state (^{3}Chl*) (13).

NPQ consists of three well-established components, named qE, qI and qT, and two additional components recently proposed as qZ and qM. Upon illumination of the leaf an instant rise of ΔpH forms, which gives rise to the important qE (energy quenching) component of NPQ, and a conversion of violaxanthin (Vio) to zeaxanthin (Zea). A lower lumenal pH activates the PsbS subunit of PSII which together with Zea induce a conformational change of the PSII supercomplex favoring NPQ (14). The qE component is activated within 10-200 s and relaxes within one minute (15). The photoinhibitory component, qI, is activated by NPQ in very high light and is dependent on the accumulation of Zea. The relaxation of qI, with a halftime of approximately 30 min, is proposed to be dependent on D1 re-synthesis (16). The state-transition component, qT, is considered to be less important in high light and is generally attributed to condition in low light intensities (17). The qT component relaxes within minutes and is highly significant in algae, but (probably) not significant in higher plants (15). The newly proposed Zea-dependent component has a slow rise (10-30 min) and a slow relaxation (10-60 min) kinetics, and develops already at medium light intensities. The qZ component is both ΔpH and Zea dependent, however, once activated the qZ component is independent of ΔpH and remains activated while Zea still is available (15). Recently, a fifth NPQ component has been proposed as a chloroplast-moving (qM) component where the plant cell undergoes a photoprotective event by moving the chloroplast closer to the cell wall and thereby avoiding the photons (18).

1.4.2 PSII photoinhibition: damage and repair

Photoinhibition is the process when reduction in the photosynthetic activity is caused by light-induced damage to PSII and occurs continuously during photosynthesis and is elevated and proportional with increased illumination. The repair mechanism, known as PSII repair cycle, is a series of events where the damaged D1 is regenerated (19). If there is an imbalance between the rate of photodamage and the rate of repair the photosynthetic activity will decrease. Lincomycin is a chloroplast protein synthesis inhibitor and can be used to monitor D1 protein degradation kinetics in mutants compared to wild type plants during PSII repair **(Paper III).**

There are essentially two models described for PSII photodamage: (i) the classical one-step scheme and the alternative (ii) two-step scheme. In the classical one-step scheme photosynthetically active light produces ROS which directly attacks the RC of PSII either by charge recombination between the acceptor side and the donor side of PSII, or by excessive reduction of Q_A.

Experiments on initial photodamage of PSII showing a direct proportionality to light intensities but not to ROS levels nourished subsequent investigations towards an alternative model. This model includes photodamage via a two-step process: 1st step, light-dependent destruction of the Mn-cluster of the OEC, which is a slow and rate

limiting step. 2nd step, inactivation of PSII RC by light that has been absorbed by chlorophyll (fast). ROS is believed to increase the extent of photoinhibition by inhibiting the repair of PSII (20).

However, the general mechanism of PSII repair is believed to be feasibly applied on both models of photodamage and is described below.

Upon illumination, the D1 subunits of the PSII dimers are damaged from the strong oxidants, such as ROS. D1 together with PSII core proteins, D2, CP43 and PsbH, are phosphorylated by the STN8 kinase. The phosphorylation of these proteins contributes to the rearrangement, i.e. unstacking of the grana regions of the thylakoid membrane, which provides an easier lateral movement of the damaged PSII complexes. The phosphorylation also contributes to monomerization of the PSII dimer, which is mobilized to the thylakoid stroma lamellae where they become dephosphorylated. The dephosphorylated D1 in stroma lamellae becomes substrate for coordinated degradation by FtsH and Deg proteases. Following D1 degradation, a new D1 protein is *de novo* synthesized and incorporated in the PSII monomer. The newly repaired PSII monomer migrates back to the grana and is reassembled with the structural and peripheral proteins and antenna complexes in the thylakoid grana (Figure 2) (19).

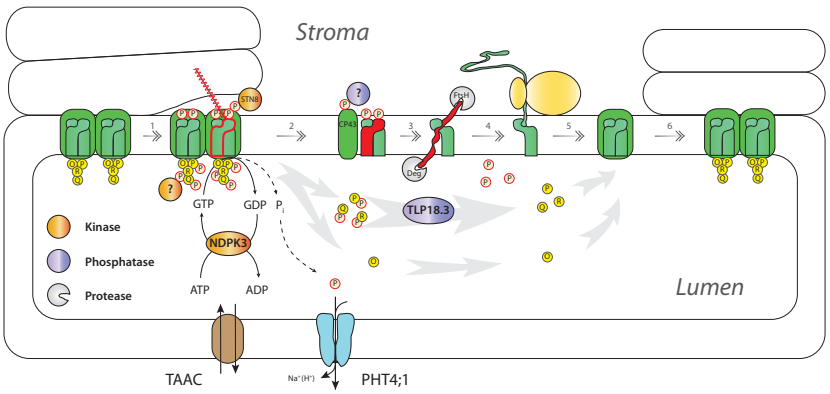

Figure 2. Schematic presentation of PSII repair cycle during HL illumination. PSII complexes are organized as dimers in the thylakoid grana in normal light conditions. The thylakoid ATP/ADP carrier (TAAC) provides ATP to the lumen, in exchange for ADP. The lumenal nucleoside diphosphate kinase 3(NDPK3) kinase transfers P_i from ATP to GTP to be used by PsbO. (1) Upon illumination with high light PSII core proteins are phosphorylated by STN8 kinase. PSII dimers monomerize as a result of PsbO GTPase activity and phosphorylation-induced release of PsbP, PsbQ and PsbR. (2) The monomerized PSII migrates laterally to the thylakoid stroma lamellae, where dephosphorylation of PSII core proteins takes place. (3) FtsH and Deg proteases degrade the damaged D1 protein. TLP18.3 dephosphorylates luminal phosphoproteins. (4) A new D1 polypeptide is synthesized and inserted into the PSII monomer. (5) The PSII and LHCII complexes are assembled. (6) PSII monomers dimerize, and PsbO, PsbP, PsbQ, and PsbR are assembled to the dimers, regenerating a fully functional PSII supercomplex in the grana regions. The resulting P_i in the lumen is recycled to the stroma by the P_i transporter PHT4;1.

Multiple experimental evidence in the recent years indicate a role of nucleotides (ATP and GTP) in PSII repair at several steps, The monomerization of PSII dimer involves the GTPase activity of PsbO and phosphorylation of PsbP, PsbO and PsbR, which facilitates dissociation of the CP43 subunit from the PSII core monomer. GTP is produced by the lumenal nucleoside diphosphate kinase 3 (NDPK3), which catalyzes the interconversion of ATP to GTP by transferring P_i from ATP to GDP. In turn, ATP is translocated into the thylakoid lumen by the thylakoid ATP/ADP carrier (TAAC), in exchange for ADP (21). Lumenal phosphor-PsbP, PsbQ, and PsbR are dephosphorylated by a thylakoid lumenal acid phosphatase named TLP18.3 yielding an excess of lumenal phosphate. Hence, PHT4;1 is proposed to be involved in balancing the P_i homeostasis in the current model of PSII repair cycle by exporting lumenal P_i to the stroma (Figure 2) (21).

1.5 Ion transport and photosynthesis

There are several thylakoid localized ion transporters that are directly or indirectly involved in photosynthesis. The ATP synthase, an F-type ATPase, is the enzymatic machinery that produces ATP using the transthylakoid H^+-gradient. Several other thylakoid transport proteins were identified and characterized previously: PAA2, a Cu^{2+}-transporting ATPase facilitating scavenging of ROS molecules and PC function; CLCe, a chloride channel proposed to balance abiotic and biotic stress related signaling pathways, and to maintain the electrochemical gradient across the thylakoid membrane; and the P_i transporter PHT4;1 studied in this work (Figure 3) (Paper II, III), (22, 23).

Potassium (K^+) is an important balancing agent for photosynthesis and its tandem pore channel, TPK3, has recently been localized to the thylakoid membrane where it is proposed to modulate the partitioning between PMF components (24). Moreover, an additional K^+ transport protein, KEA3, have been identified and localized to the thylakoid membrane, however, its true relevance for photosynthesis remains to be elucidated (Figure 3) (25).

The chlorophyll magnesium, together with calcium and manganese ions involved in OEC, embrace its obvious importance in photosynthesis. However, proteins responsible for their translocation across the thylakoid have not yet been found. Ascorbate has several important roles in the lumen, e.g. scavenging stromal ROS and acting as a cofactor for violoxanthin-de-epoxidase (VDE) facilitating the conversion of violoxanthin (Vio) to zea which is important for NPQ. Its corresponding transporter in thylakoid remains to be elucidated (Figure 3) (26).

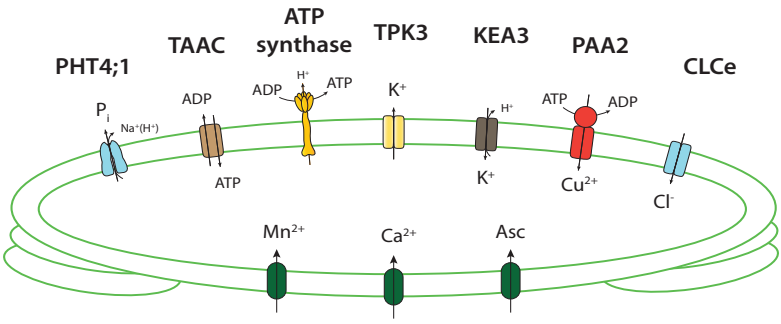

Figure 3. Schematic overview of identified and putative transporters in the thylakoid membrane. Upper panel illustrates a collection of identified transporters: P_i transporter, PHT4;1 (Paper II & III); Thylakoid ATP/ADP carrier, TAAC; ATP synthase; Tandem pore K^+ channel, TPK3; K^+ efflux antiporter, KEA3, Cu^{2+}-transporting ATPase, PAA2 and Chloride channel protein, CLCe. Lower panel illustrates important transporters awaiting identification.

2. Useful methods for studying ion transporters

2.1 Fluorescence- and absorption techniques in photosynthesis

Chlorophyll *a* fluorescence is a powerful, mostly non-invasive, tool to examine photosynthetic performance and stress responses in plants. The user friendly setup and the ease with which one can get reproducible, reliable and detailed information of, primarily, PSII activity are strong factors that explain its immense popularity among researchers. The relative low prices of handheld units, able to monitor a few of the most important photosynthetic parameters, also bring fuel to its popularity in the research field.

Pulse-Amplitude-Modulation (PAM) Chl fluorometers, such as the Dual-PAM-100 from WALZ, have been developed to measure the relative Chl fluorescence quantum yield on plants. The technique can be applied on both crude extracts, such as isolated thylakoids, or directly on leaves in a non-destructive fashion providing information of photosynthesis-related activities *in vivo.* The application of the saturation pulse method on PAM-fluorometers has allowed the assessment of photosynthetic energy conversion to be entailed, not only in laboratory or greenhouse environment, but also outside in the field.

Light energy absorbed by Chl molecules and generating excited states in PSII can undergo one of the following three fates:

1. Drive photosynthesis. Generally named photochemistry.
2. Be re-emitted as heat. Generally named thermal dissipation.
3. Be re-emitted as light. Generally named fluorescence.

The sum of these processes is always equal to 1, hence they do not work or exist as isolated processes but are in competition of each other. Therefore, the yield of fluorescence gives an indirect measure of the other two components: quantum efficiency of photochemistry and heat dissipation (27). However, since large changes in the rate constant for heat loss from PSII can occur, it is crucial to determine the fluorescence quenching that results from both photochemical and non-photochemical process, which normally is done combining a weak modulated measuring beam together with a strong modulated pulse of approximately 1 s to saturate, or “close”, all PSII RCs, i.e. achieving maximal reduction of the Q_A pool in the sample. Thus, by applying a weak non-actinic[1] modulated measuring beam (approx. 0.1 µmol m^{-2} s^{-1}) to a dark-adapted leaf with fully oxidized Q_A, it is possible to determine the fluorescence from the dark-adapted state of the Q_A pool when the PSII RCs are “open”, called F_0. To determine the fluorescence of the fully reduced Q_A pool, i.e. when the PSII RCs are “closed”, a saturating pulse of acting light of several thousands of µmol m^{-2} s^{-1} is applied for approx. 1 s and the fluorescence is measured. This maximum fluorescence, when the Q_A pool is

[1] Actinic light – Light that can drive photosynthesis ranging from UV light to light in the visible spectra. Non-actinic light is either light ranging outside this spectra or light that is too weak to drive photosynthesis.

fully reduced, is called F_m. The difference between F_0 and F_m is defined as the variable fluorescence, F_v, and is used in maximum PSII quantum yield measurements (F_v/F_m, see below).

Actinic light applied subsequent of the saturating pulse reduces the Q_A pool and gives rise to fluorescence. When the exposed leaf has reached a steady-state photochemistry, under the continuous actinic light, the leaf has a fluorescence level termed *F'* (sometimes also called F_t). A prime notation (') used after a fluorescence parameter indicates that the sample has been exposed to actinic light. Applying a short saturation pulse at this stage yields a maximally reduced Q_A pool which in turn gives rise to the fluorescence maximum, F_m'. The difference between F_m' and F' is denoted F_q'. To determine the proportional quantum yield of photochemistry, sometimes denoted Φ_{PSII}, the F_q'/F_m' ratio is used which is also implemented when measuring the relative electron transport rate (ETR, see below).

2.1.1 F_v/F_m

In order to estimate the maximum quantum yield of Q_A reduction, i.e. PSII photochemistry, F_v/F_m can be used (28). F_v/F_m is highly consistent for non-stressed leaves with values of approx. 0.8. Lower values indicate stressed plants as can be seen in e.g. high light experiments **(Paper III).** The protocol is very fast and takes only a few seconds to conduct on a dark-adapted leaf. F_v/F_m is denoted without units. (17, 28)

2.1.2 ETR

There is a linear relationship between the operating efficiency of PSII and LEF in photosynthesis, generally named relative electron transfer rate (ETR). ETR is generally measured on light-adapted leaves, i.e. leaves that have reached steady-state photosynthesis. By applying sequentially increasing actinic light intensities while measuring the fluorescence quantum yield, F_q'/F_m', it is possible to estimate ETR through PSII via the equation (17):

$$ETR = I * A_{leaf} * fraction_{PSII} * \left(F_q'/F_m'\right)$$

Where *I* is the photosynthetically active radiation (PAR, µmol m^{-2} s^{-1}) incident on the leaf, A_{leaf} is the proportion of incident PAR that is absorbed by the leaf and, $fraction_{PSII}$ is the fraction of absorbed PAR that is received by PSII (17).

When measuring ETR, the instrument is often by default set to assume that the ratio of PSII and PSI in the leaf is equally distributed, i.e. the fraction of PSII is 0.5 (50%) of the total number of photosystem complexes. Moreover, the proportion of the absorbed light by the leaf is assumed to be 84%, i.e. 84% of the incident light is absorbed and available to drive photosynthesis. Hence, in simpler terms and with corresponding values, the equation is sometimes written as (17):

$$ETR = PAR * 0.84 * 0.5 * \left(F_q'/F_m'\right)$$

Since ETR measurements are highly sensitive to small variations in levels of pigment components and the stoichiometry between PSII and PSI, it is very difficult, if not impossible, to compare ETR values between a plant grown in normal conditions with a

plant exposed to stress (17, 28). However, ETR values compared in this work are solely related to the absence or presence of PHT4;1 **(Paper III)** or PHT4;2 **(Paper I)**, which did not alter the pigment composition of the plant.

2.1.3 NPQ

Under condition of excess light, the light-harvesting system of PSII is switched into a state in which unwanted and potentially harmful energy is dissipated as heat. This process is known as non-photochemical quenching (NPQ) which together with photochemical quenching (qP) aims to maintain a low steady-state fluorescence yield, i.e. to keep open as many RCs as possible. By dissipating heat, NPQ prevents any damage from excess excitation energy from the chlorophyll binding complexes, which would form harmful ROS from "over-excited" chlorophyll molecules called ^{3}Chl*.

According to the allosteric conformational change model, NPQ is initially activated by a decrease of pH, i.e. an elevated ΔpH immediately upon the onset of illumination, and after a few minutes by a light-dependent subsequent conversion of Vio to Zea by VDE. While Zea is forming, the system gradually shifts towards a de-epoxidation step where the qE component no longer is directly dependent on ΔpH and further increases as the illumination continues. When the illumination stops, the ΔpH collapses and the qE component relaxes back to its original state (29). If the leaf is re-subjected to illumination, a very rapid accumulation of qE back to its maximum value is recorded due to the residual light activated Zea in the system. Interestingly, dark-adapted *pht4;1* leaves mutants shows a similar behavior as a "light-activated" leaf with a high transient peak in the initial phase of NPQ albeit with a lower secondary phase **(Paper III)**.

2.1.4 ECS – PMF – P515

The movement of H^+ and electrons through the membrane generates a proton motive force (PMF or $\Delta\mu_{H+}$), which comprises of an electric field (ΔΨ) and a H^+ concentration gradient (ΔpH). The energy of the PMF is utilized for ATP synthesis by the activity of the thylakoid ATP-synthase complex.

The absorption of photons by photosynthetic pigments yields a modification on the spectrum owed to the phenomenon called Stark effect, also known as electrochromism, which brings various modifications to chromophores in an electric field, as in the case of pigments embedded in a lipid membrane. In analogy, photon absorption by the pigment results in an energetic transition of the pigment from the ground state to an excited state. When these two stages are represented by different dipole moments, the energy difference between them can be changed by an electric field. Hence, if an electric field such as a membrane potential is applied, the spectral frequency of the pigment will be changed. This absorbance change is generally known as electrochromic shift (ECS), signal which in most photosynthetic systems shows a linear response to PMF and can be used as an intrinsic membrane voltmeter (30).

In plants and algae, the maximal spectral change related to ECS is around 515 nm and results from either a formation or decay from the carotenoid pool including e.g. Zea. Hence the term P515 arises from the photon absorption by a broad peak at 515 nm (515-525 nm) by photosynthetic pigments that are closely related to PMF (31). As for

the equipment used in this work, a 520 nm beam is measured against a 550 nm reference. By utilizing the ECS of P515 and apply the dark interval relaxation kinetic (DIRK) technique, it is possible to extract H^+-gradient (ΔpH) and electric field (ΔΨ) components of the thylakoid membrane from PMF absorbance data (32) **(Paper III)**.

3. Strategies to characterize biochemical function of new transporters

Chlorophyll fluorescence and absorption spectrometry are powerful tools for studying role of transporters in plant photosynthesis providing secondary information of its presence (or when characterizing mutants, its absence). However, to identify and fully characterize a putative transporter, additional methods need to be applied to assess its biochemical function. By utilizing bioinformatics, it is possible to predict location and function of new transporters in model organisms with sequenced genome, such as *Arabidopsis thaliana* (22). Localization studies using green fluorescent protein (GFP), gene expression studies using GUS **(Paper I)**, and immunological techniques **(Paper I, II and III)** serve as experimental validation of predicted location, often in parallel or together with utilizing loss-of-function mutants, such as transfer DNA (T-DNA) **(Paper I)** or transposon (Tn) mutants **(Paper III)**. Functional studies include the use of homology modelling **(Paper II)**, heterologous expression and activity assays **(Paper II)**, and phenotypic analysis of knockout mutants **(Paper I and III).**

3.1 Homology modelling

When a three-dimensional (3D) structure of a protein such as the P_i transporter PHT4;1 is not available, a target-template approach known as homology modelling or comparative modelling can be utilized to elucidate its 3D properties and amino acids important for its function **(Paper II)**. By using a crystal structure of a related protein (template) from the same family, it is often possible to construct a highly reliable model (target) that can be utilized in subsequent experimental validation and functional characterization **(Paper II)**, (33).

Due to the hydrophobic nature of membrane embedded proteins, transporters and ion channels are difficult to express and purify in amounts required for crystallization and nuclear-magnetic resonance (NMR) spectroscopy (34). Therefore, a relatively low number of transporters have been crystallized or determined by its tertiary structure from different species. However, a few of them are worth mentioning; Glycerol-3-phosphate transporter, GlpT, from *Escherichia coli* (*E. coli*) *(35)*; Oxalate transporter, OxlT, of *O. formigenes (36),* Lactose permease, LacY, from *E. coli* (37), and Multidrug transporter, EmrD, from *E. coli* (38).

The power of assessing a 3D structure relies in the decoding of the information on the primary structure of the protein since the information of how the protein should fold to function is contained in the amino acid sequence. The idea of elucidating the unknown 3D structure generally relies on comparison of hydrophobic and hydrophilic regions of the target protein and thereby establishing a reliable foundation for the subsequent comparison, known as multiple sequence alignment (MSA). During the MSA, each individual amino acid in the target is compared to its template counterpart revealing identical and conserved amino acids. This information is sometimes used to establish the important amino acid residues that might be involved in certain tasks, but can also reveal differences in between conserved regions, and thereby indicate amino acids that

are important for a certain function or motif. However, the initial analysis of the MSA generally gives information on the reliability of the proposed target-template combination, and also emphasizes the importance of conducting parallel analysis of hydrophobic regions using several other prediction tools for both target and template.

The next step, when the MSA is finalized, is to make a homology model using e.g. ICM 3.5 from Molsoft (39). There are several other programs available and most of them are freeware. By endorsing the information given from the Ramachandran plots, the target model with the lowest constrains or energy level is selected. An additional analysis of the selected model verifies the structure and validates the geometrical and restraint violations (40). The validated model can thereafter be used for substrate docking analysis etc.

We continuously acquire computers with advancing calculation power that can be applied on a comparative target-template approach known as homology modelling. Hence, it is undoubtedly realistic to believe that computers will aid and assist us even more in the future in elucidating the 3D structure of numerous putative transport proteins lacking a tertiary structure.

3.2 Heterologous expression using E. coli

Escherichia coli (*E. coli*) is a gram-negative bacterial strain normally found in the lower intestine of warm-blooded organisms and encompass a 4.3 Mbp genome coding for almost 4,300 genes. The common *E. coli* strain used in laboratories (*E.* coliK-12) was fully sequenced in 1997 (41).and is, together with many other strains, one of the most widely used organism for the production of recombinant proteins in scientific and industrial fields. Heterologous expression of recombinant proteins provides great advances in research when characterizing low abundant proteins **(Paper II),** provides cheaper medicines such as insulin (42-44), and has the potential to contribute to a large scale biofuel production (45). There are several advantages of using *E. coli* as a single cell factory. Its fast growth kinetics with a doubling time of 20 min makes it easy to achieve a high-density cell culture even when using rich complex media and the potential use of exogenous DNA and its relative ease of transforming them into *E. coli* have by far nourished *E. coli's* popularity as a choice of work-horse in the laboratory.

3.2.1 An alternative to E. coli – Brewer's yeast, Saccharomyces cerevisiae

Since ancient times, *Saccharomyces cerevisiae* (*S. cerevisiae*) has been used in wine making, brewery, and baking and is extensively used as a eukaryotic model organism in laboratories for studying DNA damage and repair mechanisms. *S. cerevisiae* is also commonly used for characterizing heterologous proteins and when studying protein interactions using yeast-two-hybrid systems. The 12 Mbp genome coding for approximately 6,000 genes was fully sequenced in 1996 (46). The genome of *S. cerevisiae* is smaller and more compact compared to the human genome (12 million base pairs and ~6,000 genes, compared with 3 billion base pairs and ~20-25,000 protein-coding genes, respectively). Yet, comparisons of the two genomes indicate that ~31% of yeast genes are very similar to the human ones and 20% of human genes associated

with genetic disorders have counterparts in yeast. One of the early characterization studies on PHT4;1 was conducted using *S. cerevisiae* and found PHT4;1 to be a H^+-dependent P_i transporter (47).

3.2.2 Applications and comparisons

Different expression systems might result in different outcomes of the assays when trying to characterize the biochemical function of a heterologously expressed protein. One example is the former work of the P_i transporter PHT4;1 where expression in yeast showed a H^+ dependent transport of P_i requiring a pH 5, but was completely abolished at pH7 (47). However, when was expressed in *E. coli*, PHT4;1 displayed a Na^+-dependent P_i transport activity peaking around pH7 and with a lower transporting efficiency at pH5 (48). The findings of characterizing PHT4;1 in two different model organisms show that it is still not clear whether PHT4;1 in fact is a Na^+- or H^+-dependent P_i transporter, as it is investigated *in situ*. To further elucidate the regulation of its function one must find alternate experimental pathways in the quest of revealing its mode-of-function *in vivo* **(Paper III)**.

Moreover, application of *E. coli* or *S. cerevisiae* for production of recombinant protein is theoretically pretty straightforward: extract the gene of interest without any introns (i.e. complementary DNA, cDNA); insert the cDNA into an appropriate vector plasmid; perform the cloning by transforming the vector plasmid construct, and finally induce the expression. The expressed protein of interest is ready for characterization assays **(Paper II)**. However, the reality is a little bit more complex than that and dozens of things might go wrong; poor or no protein expression, inactive protein, and poor growth of selected host to name a few (49). Being a powerful tool in the laboratory, heterologous expression still cannot fully validate the function of the protein of interest, such as the PHT4;1, since it is raised in a non-natural habitat. To elucidate its true biochemical function and physiological role in Arabidopsis, one needs to design experiments and use methods for characterization in its native membrane (**Paper III**).

3.3 Arabidopsis as model plant in phenotypic analysis of knockout mutants

Arabidopsis thaliana (Arabidopsis) is a small flowering plant and a member of the mustard (Brassicaceae) family that is more commonly known as thale cress, mouse-ear cress, or simply Arabidopsis. Over 750 accessions have been collected throughout the world and it is one of the most widely used model organism in plant research. Due to its relatively short life cycle (generation time – from germination to mature seeds) of six weeks when grown in a 16 h light period per day together with the prolific seed production and abilities of cultivation in limited spaces, Arabidopsis has further gained its popularity as a model plant in the laboratory research. Moreover, if grown in conditions with shorter daylight cycles, Arabidopsis generally gains shoot biomass and retrieves a prolonged life cycle to at least nine weeks. Growing Arabidopsis in shorter day light cycles might be beneficial when utilizing protocols that require a substantial amount of raw material **(Paper I & III)**.

Another major advantage of Arabidopsis is the relatively small genome of 157 Mbp (50), coding for approximately 25,500 genes, which was fully sequenced in 2000 (51) giving the opportunity to retrieve mutant lines and other genomic resources for virtually every gene (http://arabidopsis.org) (52). Public collections have been setup and have significantly reduced the laborious work and time of preparing mutant lines for a particular gene of interest. One of the most common resources is the Arabidopsis Biological Resource Centre (ABRC) (http://abrc.osu.edu/), a collection of nearly 1,000,000 stocks including T-DNA insertion lines, transposon (Tn) insertion lines, and the TILLING population. Arabidopsis is not of major agronomic significance, but it offers important advantages for basic research in genetics and molecular biology.

4. Transporters and phosphate

4.1 Families of transporters

Membrane transport proteins are classified according to the Transporter Classification (TC) system in three categories: channels/porins, secondary transporters, and primary transporters/pumps (53).

4.1.1 TC#1: Channels/porins

The non-energy-consuming channels/porins transport the substrate with high velocity (10^7-10^8 molecules s^{-1}) down the concentration gradient and are mainly represented by voltage-gated channels, aquaporins and porins.

4.1.2 TC#2: Secondary transporters

The category of secondary transporters includes three subgroups of transport proteins: uniporters, antiporters and symporters, which work at an intermediate rate (10^2-10^4 molecules s^{-1}). Uniporters utilize facilitated diffusion of a single molecule or ion for passive transport across the membrane. Antiporters, sometimes also called exchangers, transport two chemical species in opposite directions. The energy for transport originates from the chemiosmotic gradient of the co-transported ion or molecule. Symporters transports two or more ions/molecules in the same direction where at least one of the compounds is transported down the chemiosmotic gradient. The two P_i transporters studied in this work, PHT4;1 and PHT4;2 **(Paper I, II and III),** belong to the secondary transporters family, more specifically to the major facilitator superfamily (MFS), which also includes the glycerol 3-phosphate/phosphate antiporter (GlpT) used as a template in the homology modelling of PHT4;1 **(Paper II).**

4.1.3 TC#3: Primary active transporters/pumps

Primary active transporters/pumps use a primary energy source, such as ATP, to facilitate ion or molecule transport against the chemiosmotic gradient. Hence, they do not use a secondary ion gradient as an energy source. The primary active transporters are slow (1-10^3 molecules s^{-1}) and include four types of ATP-utilizing pumps in biological membranes: ATP-binding cassette (ABC) transporters, H^+-translocating F-type ATPases, vacuolar V-type ATPases, and metal ion P-type ATPases.

4.2 Phosphate and its role in the cell

P_i is an inorganic salt of phosphoric acid comprised of a phosphorous atom surrounded by four oxygen atoms where one of them is double bound to the phosphorous atom yielding a negative three formal charge in a tetrahedral arrangement. P_i in its aqueous form exists in four forms ranging from PO_4^{3-} in a strongly basic environment to H_3PO_4 in a very acidic environment. In neutral pH solution the two by far most common forms are HPO_4^{2-} and $H_2PO_4^-$. Nevertheless, P_i can take a different form than the expected one in a particular pH if the ionic strength in the solution, or in the microenvironment (the closest proximity of the molecule), is altered.

Plants require more than 14 essential nutrients for its survival and growth, phosphorous being one of "macronutrients" that is required in larger amounts. However, phosphorous does not occur in its elemental form in the soil and must therefore be acquired by the plants in its P_i form which is very low abundant (<10% of total P content in soil). Moreover, phosphorous is vital for plant growth and is involved in several key plant functions including nutrient movement and energy transfer (phosphoenol-pyruvate, ATP), photosynthesis and cell signaling (phosphorylation of proteins, membrane phospholipids), transformation of sugars and starch (glucose-6-phosphate), and transfer of genetic characteristics from one generation to the next (RNA, DNA).

4.3 Phosphate starvation effects

The sensation of P_i starvation induces both local- and systemic responses in complex crosstalks of developmental and metabolic adaptations. The local response affects the root system architecture by inhibiting the primary root growth whilst inducing a lateral root formation and growth including enhanced root hair formation.

The systemic response to low P_i induce the expression of high-affinity transporters, an intense recovery of P_i via secretion of phosphatases, and P_i recycling via catabolism of phospholipids (54), mainly from mature leaves to young leaves, but also to roots, in order to sustain root meristem activity (55). P_i deficiency is believed to affect sugar levels which induce expression of several P_i transporters, such as *PHT1;4 and PHT3;1* in roots acting upstream of the hexokinase sugar sensing pathway (56).

Moreover, under normal conditions P_i acts as a suppressor for the expression of miR399, one of the regulatory modules controlling P_i homeostasis. Expression of miR399 is strongly stimulated upon P_i starvation in shoot and is transported to the root system via the phloem, where it silences *PHO2* expression. The inactivation of *PHO2* leads to increased expression of *PHT1;8* and *PHT1;9* which facilitate the P_i uptake in roots and transport of P_i back to the shoot. Interestingly, the silencing of miR399 is rebalanced by the increased P_i levels in the shoot, but is inhibited via the expression of *IPS/At4,* which is slowly induced by the systemic and general progression responses of P_i starvation response (55).

An alternative route of response to P_i limitation acting on P_i transporters in roots is via the *SIZ1* gene which is involved in regulating responses to many types of abiotic stress factors and not specifically involved in P_i signaling. *SIZ1* has been shown to modify the putative transcription factor *Ph*osphate starvation *R*esponse 1, *PHR1* (55) which in turn induce expression of *PHT1;4* and *PHT1;5* via SPX3 domains found in numerous proteins responsible for the fine tuning of P_i homeostasis (57).

In chloroplasts, the ATP synthase activity is impaired if the stromal P_i levels reach levels below its K_m (~1mM). The impairment results in lowered H^+ flux, which leads to an elevated PMF and a subsequent down-regulation of photosynthetic light capture (58).

Moreover, it has been reported that phosphate starvation in oat affects the lipid composition in plasma membrane and tonoplast where phosphoglycerolipids are being

replaced to a large extent by digalactosyldiacylglycerol. Interestingly, the replacement did not occur to any greater extent in endoplasmic reticulum, Golgi apparatus or mitochondrial inner membrane (59, 60).

4.4 Phosphate uptake and transport

As mentioned earlier, the P_i concentration in soil is very low, (0.1-1 μM), whereas the required P_i concentration in roots is closer to the millimolar range. Hence, the plants acquire P_i against a steep concentration gradient across the plasma membrane. Moreover, the pH in soil varies extensively depending on the geographic location and chemical composition. The "ideal" pH range for the plant to acquire essential nutrients more efficient is ranging from pH 6 to 7.5. In that range, P_i is negatively charged by at least one negative form and thus cannot diffuse from soil into the root across the membranes. The inability to diffuse P_i into roots together with steep concentration gradient makes the plant solely dependent on active transporters. In this energy demanding process P_i is generally transported using the H^+ gradient commonly generated by a plasma membrane H^+-ATPase. Hence, most of the P_i transporters characterized in roots are H^+- P_i symporters.

Many P_i transporters have been characterized and classified into five phosphate transporter families: PHT1, PHT2, PHT3, pPT and PHT4. All four PHT families belong to the MFS superfamily. MFS members are single-polypeptide secondary transporters capable of transporting small solutes in response to chemiosmotic ion gradients. The pPT family includes only plastid P_i transporters.

PHT1 family consists of nine members ubiquitously expressed in *Arabidopsis.* Most members except *PHT1;6* are expressed in roots and root epidermis, however, some of them can be found in leaves (PHT1;1 and PHT1;3, PHT1;4 and PHT1;5), flowers (PHT1;3 to PHT1;7), and senescing leaves (PHT1;4 and PHT1;5) (61). The only PHT1 members that have been functionally characterized are PHT1;1 and PHT1;4, involved in P_i acquisition in soil (62), and PHT1;5, predominantly expressed in senescent leaves and important for the source-to-sink organ P_i remobilization via phloem (63). Subcellular localization studies indicate the presence of PHT1;1 PHT1;4, PHT1;9 in the plasma membrane (64-66).

PHT2;1 is currently the only member of the PHT2 family characterized in Arabidopsis so far and is mainly expressed in the chloroplast inner envelope membrane (67). PHT2;1 is structurally similar to the members of the PHT1 family, but deviates in having a large hydrophilic loop between TM8 and TM9 and has similarities to a Na^+-coupled P_i transporter that can be found in *S. cerevisiae* (68). However, PHT2;1 shows a H^+-dependent low-affinity P_i transport when expressed in yeast. *PHT2;1* gene knockout mutant indicated an altered P_i distribution pattern in response to different P_i availability (67).

The P_i transporter PHT3 family consists of at least three different genes in *Arabidopsis*. Genes from the PHT3 family are highly conserved within the mitochondrial carrier family

(69), and are sometimes named AT3 or MPT3 (70). PHT3;1 is the only member that has been localized and is found in the mitochondrial inner membrane (71). The expression of *MPT3/PHT3* genes is up-regulated during salt stress, and it has been suggested that MPT3s are involved in mitochondrial ATP content with a possible link to gibberellin metabolism in response to high salinity stress (70). However, the true physiological role of PHT3 remains to be elucidated.

The pPT family includes the triose phosphate/phosphate translocator (TPT), Phosphoenolpyruvate (PEP) translocator (PPT), and the glucose 6-phosphate (Glc-6-P)/ P_i translocator (GPT). TPT is an anti-porter located in the chloroplast inner envelope membrane, where it exports the fixed carbon in the form of triose phosphate to the cytosol in exchange for P_i. The *TPT* gene is expressed in shoots, leaves and flowers, and follows a circadian expression (72)

The Arabidopsis PPT family comprises of *PPT1* and *PPT2,* where *PPT1* mainly is expressed in root plastids, in particular in xylem parenchyma cells, and the vascular region of leaves, whereas *PPT2* is found uniformly expressed in leaves. Both PPT1 and PPT2 anti-porters are located in the chloroplast inner envelope membrane and facilitate the translocation of PEP in exchange for P_i export and function as dimers.

The GPT family comprises of *GPT1* and *GPT2,* where *GPT1* is ubiquitously expressed whereas *GPT2* mainly is expressed senescent leaves, sepals and seeds (73). The major role of GPT is believed to be the import of Glc-6-P into root plastids in exchange for either triose-phosphate or P_i. Hence, TPT, PPT and GPT contribute to P_i homeostasis in plastids of leaves and roots as a complement to P_i transporters from the PHT families (68, 74).

5. Phosphate transporter family 4 – PHT4

PHT4 is the fourth P_i transporter family in Arabidopsis, and consists of six members. They all share similarities with the SLC17/type I P_i transporters family including mammalian vesicular glutamate transporters (VGLUT). Several members of the PHT4 family have a history from the *an*ion *tr*ansporter (ANTR) family nomenclature (75), but are now designated as members of the PHT4 family (47, 76). Several expression and localization studies suggest that members of the PHT4 family can be found in chloroplasts, non-photosynthetic plastids and the Golgi apparatus and will be discussed herein in reverse chronological order, starting with PHT4;6 and closing with PHT4;1.

5.1 PHT4;6 – Ubiquitously expressed

PHT4;6 is the only gene in the PHT4 family that is composed of a single exon while the genes for all other members are composed of at least eight exons. Early bioinformatics studies suggested that PHT4;6 appeared to be structurally different from the other members of the PHT4 family in that it lacked an extensive N-terminal hydrophobic domain (75).

PHT4;6 was later identified as a Golgi-located P_i transporter (47), and has been targeted to the *trans*-Golgi compartment by localization studies using GFP (77), where it is involved in release of P_i (78). Loss of function of PHT4;6 has a severe impact on growth and development exhibiting a dwarf phenotype and influences important Golgi-related characteristics like alteration of N-glycosylated proteins and altered composition of cell-wall hemicellulose (77). Interestingly, the *pht4;6* mutant had similar P_i content compared to the wild type, even though the *pht4;6* mutant was smaller. Adding P_i to the daily supplied water stimulated growth of the *pht4;6* mutant but not of the wild type. However, the vacuolar P_i content was 40% higher in *pht4;6* mutants compared to wild type. Moreover, leaves from *pht4;6* plants showed spontaneous necrotic lesions, increased salicylic acid and H_2O_2 levels. ROS probably triggered the hypersensitive response in terms of programmed cell death and accumulation of callose, which is a response to wounding and infections by pathogens. The *pht4;6* mutants also showed an elevated expression of several pathogen-related genes, which probably are involved as a response to the elevated H_2O_2. Hence, the *pht4;6* mutants showed morphological, physiological and molecular symptoms of infection without direct contact with pathogens which is sometimes also known as 'mimic disease' (77).

The *pht4;6* mutants also displayed an increased sensitivity against Na^+ ion stress, as revealed by altered root morphology, which is dependent on elevated NaCl concentrations (78). That observation is in line with a critical function of the Golgi apparatus in cell-wall synthesis (77). The expression of *PHT4;6* gene is not affected by light (79).

5.2 PHT4;5 – Found in flowers and phloem of leaves

When using GUS-expression driven by the *PHT4;5* promoter, the expression was restricted to the sepals in flowers and the phloem portion of the vascular bundle in leaves and cotyledons. The expression of *PHT4;5* gene is not affected by light (79). PHT4;5 was formerly named ANTR6.

5.3 PHT4;4 – Localized to the inner envelope membrane of chloroplast

PHT4;4 is mainly expressed in leaves, stems, and developing siliques (79). PHT4;4 is a P_i transporter located in the inner envelope of chloroplasts (48, 75). The expression of *PHT4;4* gene is affected by light and increases 20-fold 3h after initiation of illumination. PHT4;4 was formerly named ANTR2.

5.4 PHT4;3 – Shares similarities to PHT4;5

PHT4;3 promoter-GUS fusion displays similar expression pattern as *PHT4;5* in leaves and is restricted to veins, detected at low levels in root caps, but not detectable in flowers. The expression of *PHT4;3* gene is not affected by light (79). PHT4;3 was formerly named ANTR4.

5.5 PHT4;2 – A phosphate transporter in root plastids

The expression of *PHT4;2* is mainly restricted to roots and is very low, less than 2% of the lowest value of the other genes in the PHT4 family. Similarly to *PHT4;1 and PHT4;4,* the expression level of *PHT4;2* is affected by light, however, in contrast to *PHT4;4* and *PHT4;1* the expression drops almost 80% 3h after light induction. Albeit PHT4;2 is affected by light it doesn't follow a diurnal expression pattern (79).

GUS expression confirmed that *PHT4;2* is expressed in roots, but not in leaves, yet *PHT4;2* is also found in stamens, sepals and carpels as well as siliques, but not in mature seeds. The expression is greatest in roots, followed by intermediate expression levels in flowers and siliques and barely detectable in rosette and cauline leaves **(Paper I)**.

PHT4;2 encodes a 512-amino acid protein which catalyzes a Na^+-dependent P_i export in Arabidopsis root plastids and reaches its kinetic maximum at pH 7.5 . It is predicted as a monomeric 50.5 kDa protein when lacking its 44 amino acid N-terminal transit peptide, but migrates as a 35 kDa band in SDS-PAGE-gels **(Paper I)**. The migration discrepancy can be explained by an over-proportional binding of SDS, which is common for hydrophobic integral membrane proteins (80). Immunodetection using western blot could detect a small amount of PHT4;2 in roots, but not in other organs. **(Paper I).**

5.5.1 Pht4;2 mutants display an increased growth phenotype in leaves

When grown using a 14 h photoperiod, *pht4;2* mutants shows no phenotype compared to wild type plants. However, an 8 h photoperiod reveals increased leaf area and shoot biomass, detected after five weeks of growth **(Paper I)**. The increased rosette size of *pht4;*2 originates from an increased number of e P_i dermal cells. However, the increased

cell number occurs predominantly at early stages in leaf development. Hence the lack of apparent phenotype is compensated by reduced cell area during the first five weeks, but becomes visible during the subsequent developmental and mature stage when the cell area is the same as for the wild type. Cell proliferation is restricted to leaves, hence root size is not affected in *pht4;*2 mutants **(Paper I)**.

5.5.2 Lack of PHT4;2 does not affect photosynthesis

The increased rosette area together with the reduced starch levels in illuminated leaves suggest that PHT4;2 might play a role in photosynthesis. However, there are no differences in either F_v/F_m or ETR between *pht4;2* mutants and wild type plants. Thus the lack of PHT4;2 does not modify the photosynthetic electron transport in leaves. Moreover, there is no significant difference between *pht4;2* and wild type in terms of chlorophyll content **(Paper I)**.

5.5.3 Starch levels and several starch related genes are altered

Interestingly, *pht4;2* mutants display altered starch accumulation throughout the plant. PHT4;2 is important for P_i export in Arabidopsis root plastids and is mediated in a Na^+-dependent fashion resulting in a P_i accumulation in plastids **(Paper I)**, which in turn inhibits starch biosynthesis (Figure 4) via allosteric inhibition of AGPase (81). Even though *PHT4;2* is not expressed in leaves, there is a reduction of starch content in rosette leaves of *pht4;2* mutants compared to the wild type. The difference in starch content in leaves is significant during the illumination period, and is abolished during the dark period. However, starch levels are lower in roots of *pht4;2* both during the light- and the dark period of the diurnal cycle. The finding of reduced starch levels is consistent with P_i inhibition of starch synthesis in roots, while the reduced starch content in leaves indicates a previously unknown signaling pathway between roots and leaves, since the expression of *PHT4;2* is detectable in roots but not in leaves. Surprisingly, even though the starch is depleted in root plastids, sucrose does not compensate for the lack of starch as a carbon source, and is maintained at levels similar to the wild type **(Paper I)**.

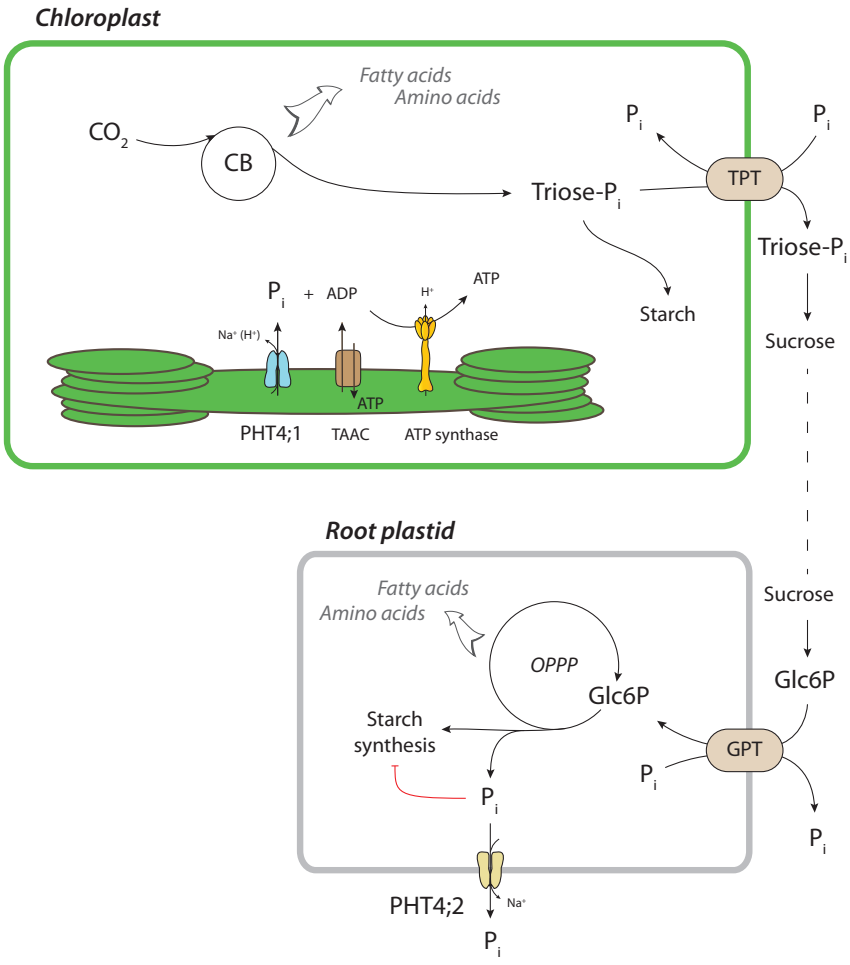

Figure 4. Schematic overview of transporters involved in carbon metabolism and phosphate homeostasis in chloroplasts and root plastids. Triose-phosphate/phosphate translocator (TPT) and glucose-6-phosphate/phosphate translocator (GPT) works in concert to supply root plastids with carbon skeletons. Phosphate transporter PHT4;2 in root plastids exports P_i from the root plastid, preventing accumulation of P_i and thereby inhibiting starch synthesis in root plastids. Similarly, PHT4;1 is believed to export accumulated P_i as a result of the ATP transport by the thylakoid ATP/ADP carrier (TAAC) and nucleotide metabolism in the thylakoid lumen.

At the transcriptional level, *PHT4;2* mutants show an altered expression of starch synthesis related gene (*APS1*), GPT, and ATP/ADP nucleotide translocator genes in root plastids, but not in leaves.

In contrast, a subset of genes responsible for the metabolism of starch and sucrose is affected in leaves (APL1 & APL3) but not roots. The inverse correlation of genes in the same family but in different organs, i.e. APL1 and APL3 in leaves and APS1 in roots, suggests an importance of *PHT4;2* for the long distance signaling between roots and

leaves and for P_i homeostasis in root plastids regarding plant growth and physiology (Figure 4). Moreover, cFBF, one of the genes responsible for sucrose synthesis, is elevated in leaves, but not in roots where the starch is limited. Together with the compensatory mechanisms of starch contents and starch related genes in-between roots and leaves, these findings emphasize a novel signaling mechanism between roots and leaves.

5.6 PHT4;1 – Formerly known as ANTR1

5.6.1 Expression pattern and localization of PHT4;1

PHT4;1 displays a nearly identical GUS expression pattern as *PHT4;4*, including the expression throughout the green tissue of seedlings. Interestingly, the transcription levels of *PHT4;1* in roots are lower than the expression levels of *PHT4;4* in roots (47). The GUS-expression driven by *PHT4;1* show a low yet a detectable level while the GUS-expression driven by *PHT4;4* are not detectable at all. The circadian expression of *PHT4;1* gene is affected by light and increases 10-fold subsequent of a 3h illumination (79). Interestingly, expression of *PHT4;1* is not affected by abiotic stress in general, however *PHT4;1* expression is lowered by HL and oxidative stress **(Paper III)**.

PHT4;1 has been localized using GFP-microscopy to the chloroplast (75) and later on using peptide specific antibodies to the thylakoid membrane in Arabidopsis (48). Subfractionation of the thylakoids revealed the stroma regions as the most likely final destination of PHT4;1 *(82).* Proteomic analysis claim this protein is in the envelope (83). However, the purity of the envelope from stroma lamellae has not been verified in those studies.

5.6.2 Biochemical function

PHT4;1, formerly known as ANTR1, is located in the thylakoid membrane of Arabidopsis and has been characterized as a Na^+-dependent P_i transporter when expressed in *E. coli* (48) and as a H^+-dependent P_i transporter when expressed in yeast (47). The homology model suggests that PHT4;1 indeed is a Na^+-dependent transporter which is verified by heterologous expression and characterization in *E.coli* **(Paper II)**.

5.6.2.1 Important amino acid residues for P_i transport

The homology model of PHT4;1 emerged by using the 3D structure of the crystallized GlpT with 3.3 Å resolution (35) as a template. PHT4;1 is predicted to encompass 12 transmembrane (TM) helices consisting of two six-TM-containing domains connected by a large loop **(Paper II)**, which corresponds well to the 12-TM topology of several crystallized MFS members such as GlpT (35-38).

Multiple sequence alignment with all members of the PHT4 family and eukaryotic homologues revealed 25 highly conserved amino acid residues. However, after mapping onto the GlpT based structure model of PHT4;1, 20 of them were found to be either located inside a small closed cavity, or believed to be solely important for the stability of the protein structure. The model forms a central cavity open toward the cytoplasm with diameters ranging from 9 Å at the bottom, 6 Å in the middle, and 5 Å at the entrance.

Docking predictions for P_i and glutamate show that glutamate is bound to the bottom of the cavity, in the close proximity of Arg-201, whereas P_i is bound 6 Å closer to the middle of the cavity, in the proximity of Arg-120 **(Paper II)**. Interestingly, an arginine residue found to be important for P_i transport in VGLUT1 (84) corresponds to Arg-120 in PHT4;1, which further supports these findings **(Paper II)**.

Heterologous expression experiments combined with site-directed mutagenesis confirmed that three amino acids in the cavity, Arg-120, Ser-124, and Arg-201, are important for binding/translocation of the P_i substrate while two other amino acids, Arg-228 and Asp-382 on the cytoplasmic surface of PHT4;1, instead are important for intermolecular binding in the protein structure.

Arg-120 is found to play an important role in P_i binding and associated conformational changes. The corresponding arginine residue in GlpT has been shown to be important for P_i transport.

Ser-124 is found to be a key residue for the Na^+-dependency of the transport and probably functions as a transient binding site for Na^+ ions, in line with the crystal structure of Na^+-dependent neurotransmitter transporter LeuTA (85). Ser-124 allows opening at the periplasmic side and access of P_i inside the cavity. Interestingly, Ser-124 is not found in the animal homologues of Na^+-dependent glutamate transporters SLC17 and VGLUT, but is fully conserved among the plant homologues, and animal homologues of Na^+-dependent P_i transporters such as NPT3 and NPT4.

Arg-201 is the most critical residue for substrate binding and translocation. It was fully conserved among PHT4, VGLUTs and other proteins in MSAs. It is essential for glutamate transport but not for P_i transport in VGLUT2 (86). The positively charged Arg-201 probably attracts P_i in the cavity before reaching its binding position close to Arg-120.

Histidine has been proposed to be involved in H^+ cotransport in LacY (87). However, three histidine residues found in PHT4;1 is neither conserved among the PHT4 family members nor do they have direct access to the cavity. It would require a substantial amount of energy to change the conformation for any of them to acquire access to the cavity, which further supports a Na^+-dependent co-transporting mechanism for PHT4;1.

5.6.2.2 Proposed mechanism of PHT4;1

The P_i transport mechanism of PHT4;1 follows a rocker switch movement involving conformational change where the cavity alternately expose its residues towards the cytoplasm and periplasmic side. The periplasmic side of PHT4;1, which most likely correspond to the luminal side of the thylakoid membrane, exposes Arg-120, Arg-201 and Ser-124 in its initial transporting cycle where P_i binds to Arg-201 and Na^+ binds to Ser-124, inducing the conformational change. During conformational change and the proposed intermediate step, P_i is translocated from Arg-201 to Arg-120. The subsequent reaction yields a second conformational change closing the cavity against the periplasmic side and opening the cavity against the cytoplasm (stroma) where P_i and Na^+ are released. **(Paper II)**.

5.6.3 Physiological role from phenotypic analyses of loss-of-function mutants

5.6.3.1 Role in response to pathogen attack

By using *Pseudomonas syringae*, a truncated PHT4;1 has been reported to be involved in Arabidopsis defense acting upstream of the salicylic acid pathway as a type II salicylic acid regulator contributing to both *SID2*-dependent and -independent SA accumulation, i.e. PHT4;1 is a negative regulator of basal defense in Arabidopsis (88). However in the same report, two *pht4;1* mutants were found similar to wild type in their immune response to Pseudomonas resistance. Therefore, the wild type PHT4;1 may not play such a role in Arabidopsis.

5.6.3.2 Transcript levels of co-expressed genes

Transcript levels of several genes involved in thylakoid associated processes are reduced in *pht4;1* mutants compared to the wild type. The ATTED-II coexpression database displayed a relatively strong co-expression of zeaxanthin epoxidase (ZEP) (89). Indeed, this gene displays an altered expression in *pht4;1* mutants. In addition, the genes coding for proteins involved in the nucleotide-dependent reactions of PSII repair, such as lumenal phosphatase TLP18.3, the extrinsic PsbO, PsbP, PsbQ and PsbR (Figure 2) show reduced expression levels in the *pht4;1* mutants while the gene for thylakoid ATP/ADP carrier (TAAC) have similar expression levels in mutants compared to wild type. The lowered expression of the investigated genes, with the exception of *TAAC*, indicated a co-regulation with *PHT4;1*. However, western blot analysis revealed no alteration of protein levels of the corresponding genes in *pht4;1* mutants compared to the wild type **(Paper III)**.

5.6.3.3 Susceptibility to photoinhibition

When grown in an 8h diurnal GL photoperiod, *pht4;1* mutant plants reveals a smaller rosette area and biomass after six weeks of growth and throughout the growth period, compared to wild type plants. Interestingly, the *pht4;1 mutants* recover and show a similar rosette area and biomass as wild type plants after 2-3 weeks of subsequent growth in HL conditions. Hence, plants lacking PHT4;1 seem to better cope with HL stress than wild type plants which is confirmed by the recovery of Fv/Fm parameters in *pht4;1* mutants **(Paper III)**

5.6.3.4 Lack of PHT4;1 does not affect photosynthetic pigment and protein composition

The pht4;1 mutants do not display altered composition of photosynthetic pigments i.e. Chlorophyll (*a* and *b*) and carotenoids (β-carotenoids, violaxanthin and lutein). Also the relative amounts of photosynthetic proteins are similar in *pht4;1* mutants and wild type plants.

5.6.3.5 Photosynthetic mechanisms

It was proposed that PHT4;1 exports P_i from the thylakoid lumen and may be involved in photosynthesis. In **Paper III** it is shown that PSII activity and LEF are not affected in the *pht4;1*, mutants. However, the lack of PHT4;1 seems to have a more direct effect on the initial phase of NPQ, since the mutants reach a transient maximum faster and with a higher amplitude than the wild type, indicating an lower pH level in the thylakoid lumen. The second phase of NPQ is slow and displays no significant differences between the mutants and the wild type. PHT4;1 has been shown to transport P_i in a H^+- or a Na^+-

dependent manner, depending on the expression system used. Based on the NPQ readings, it is plausible that PHT4;1 in fact is a H^+-dependent. Alternatively, it may be Na^+-dependent, as suggested by the homology modeling studies and may be coupled to a Na^+/H^+ transport system yet to be identified.

The *pht4;1* mutants harbor significantly lower PMF, with an altered partitioning between ΔΨ and ΔpH, i.e. an elevated ΔpH and a lowered ΔΨ, supporting the finding of a higher transient maximum of the initial phase of NPQ

5.6.3.6 D1 degradation during PSII repair

By using detached leaves that have been incubated overnight in deionized water, as control, or Lincomycin which blocks the translation of chloroplast encoded proteins, it is possible to monitor the PSII inactivation and turnover of its D1 subunit. Wild type and *pht4;1* mutants show a similar inactivation of PSII and turnover of its D1 subunit, indicating that PHT4;1 does not play an essential role in PSII repair cycle. This is confirmed by the Fv/Fm parameter which showed a similar decay in both *pht4;1* mutants and the wild type **(Paper III)**.

5.6.3.7 PHT4;1 alters the phosphorylation patterns in leaves but not in thylakoids

The levels of photosynthetic proteins between the *pht4;*1 mutants and L*er* do not differ and the steady-state phosphorylation of LHCII and PSII core proteins CP43, D2, and D1 in thylakoids is not affected by the absence of PHT4;1. **(Paper III)**. However, the phosphorylation patterns between the *pht4;1* mutants and L*er* differs significantly when using leaf extracts (which includes proteins from all cells and organelle types in the leaf). Interestingly, several (unknown) proteins are phosphorylated to a greater extent in *pht4;1* mutants in darkness and in growth light as compared to wild type plants in the same conditions (Figure 5).

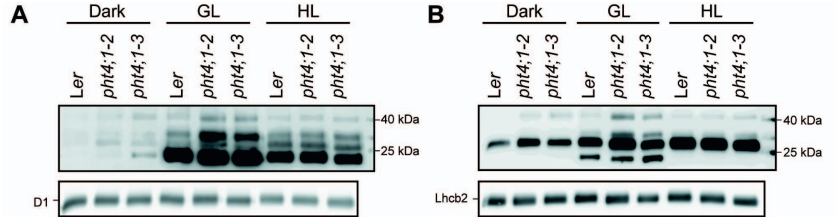

Figure 5. Quantative immunoblotting analysis of phosphorylated proteins in total leaf extracts using anti-phospho-threonine antibodies from (A) Cell signaling and (B) Zymed.

Moreover, *pht4;1* mutants significantly accumulate greater amounts of Pi compared to wild type whereas organic phosphate levels are similar in all genotypes. Since the transport direction of PHT4;1 not yet have been determined, the elevated P_i in leaves might originate from thylakoid lumen or another cell organelle, e.g. the vacuole which frequently is used as a storing compartment.

Taken together, these data indicate an involvement of PHT4;1 in a potential phosphorylation signaling pathway, or perhaps a signaling pathway that is dependent on P_i, in Arabidopsis.

6. Conclusions and future perspectives

Until now, the tertiary structure of PHT4;1 has remained unknown. However, this thesis work has brought new light on the Pi transporting mechanism by revealing amino acids that are important for Pi selectivity and Na^+ dependency. We have also shown that the trans-thylakoid Pi transport of PHT4;1 regulates Pi homeostasis in leaves which, together with the reduced leaf area and biomass of *pht4;1* mutants, further implicates an important role of PHT4;1 for plant growth.

The elevated ΔpH fraction of PMF, together with the response of the H^+-dependent NPQ-mechanism from *pht4;*1 plants, indicate a H^+-dependent transport mechanism of PHT4;1. Nevertheless, it is plausible that a secondary Na^+/H^+-exchanging mechanism plays a confounding role in parallel with PHT4;1. Hence, the question still remains whether the activity of PHT4;1 *in vivo* is Na^+- or H^+-dependent.

This thesis work also shows that PHT4;2 catalyzes a Na^+-dependent Pi efflux in root plastids. The root of *pht4;2* mutants accumulate more Pi, which results in lower starch content than in the wild type roots. Moreover, *pht4;2* mutants display larger leaves with increased biomass, due to increased cell proliferation indicating a potential role of PHT4;2 in a Pi-dependent signaling pathway between roots and shoots.

The physiological roles of PHT4;1 and PHt4;2 in response to phosphate starvation has, to our knowledge, not yet been studied. In oat, phosphate starvation results in replacement of phospholipids with lipids containing less phosphate. A similar investigation would bring light whether a similar approach is used in Arabidopsis. Moreover, a phosphate starvation analysis might also reveal potential phenotypes previously unseen which might give useful hints and indications of secondary- or coordinative processes working together with PHT4;1 or PHT4;2.

Based on the finding that PHT4;1 is involved in pathogen attack resistance, albeit in a truncated form, together with the fact that PHT4;1 is involved in specific abiotic stress, it is plausible that PHT4;2 might be involved in stress responses as well. The indications of PHT4;2 being involved in a signaling pathway responsible for cell division, which are responsible for larger leaves of *pht4;2* mutants might encompass a role in plant response to stress.

Future studies of the role of PHTs under stress conditions mentioned above will certainly help to evaluate their precise participation of PHTs in these processes and bring light into the still undefined role of P_i and transporters.

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