Hey ho, let's go!

Vesicle transport in chloroplasts

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Abstract

The photosynthetic reactions have been thoroughly studied, but less is known about the biogenesis of the structures harboring the photosynthetic machinery: the thylakoid membranes. Lipids, constituting both the envelopes and thylakoids, are amphipathic molecules with both hydrophobic and hydrophilic ends. Due to this, lipids are not likely to pass the stroma spontaneously, but rather arranged in a way that isolates the hydrophobic parts from the water-based surrounding. As the thylakoid lipids are produced at the envelopes, they have to pass the stroma. Hypotheses about how this is accomplished have been suggested over the years, ranging from invaginations of envelope membranes and direct contact sites between envelope and thylakoid membranes, to lipids being transferred as small spheres, i.e. vesicles. Indeed, vesicles have been identified with electron microscopy, but although repeatedly observed, not much focus has been given to how vesicles in the chloroplast could be regulated.

Vesicle transport is known from the cytosol of both animals and plants. There, vesicles with protein cargo shuttle different compartments and the process is highly regulated by different sets of proteins. In **paper I** we show that vesicles are not only present in the cytosol of plants, but also in chloroplasts and other plastids. These vesicles can be found during different conditions and temperatures, and without chemical inhibitors. This indicates that vesicles are persistent features. How chloroplastic vesicles are regulated is largely unknown, although they are strongly suggested to be of eukaryotic origin and appear to have similarities with cytosolic vesicle systems. In **paper II** and **III**, we used a bioinformatics approach to identify putative components of vesicle transport in the chloroplast. Several homologs to COPII proteins of the cytosol were identified in the chloroplast (**paper II**), but interestingly, homologs related to the cytosolic COPI and CCV systems could not be identified to the same extent (**paper III**). It was therefore suggested that the vesicle system in chloroplasts is most similar to COPII, or even unique. In **paper IV**, one of the homologs was characterized and proposed to have a role in vesicle fusion.

List of publications

I. Lindquist E, Solymosi K, Aronsson H (2016). Vesicles are persistent features of different plastids. Traffic, 17, pp. 1125-1138.

II. Khan NZ, Lindquist E, Aronsson H (2013). New putative chloroplast vesicle transport components and cargo proteins revealed using a bioinformatics approach: An Arabidopsis model. PLoS ONE, 8, e59898.

III. Lindquist E, Alezzawi M, Aronsson H (2014). Bioinformatic indications that COPI- and Clathrin-based transport systems are not present in chloroplasts: An Arabidopsis model. PLoS ONE, 9, e104423.

IV. Karim S, Alezzawi M, Garcia-Petit C, Solymosi K, Khan NZ, Lindquist E, Dahl P, Hohmann S, Aronsson H (2014). A novel chloroplast localized Rab GTPase protein CPRabA5e is involved in stress, development, thylakoid biogenesis and vesicle transport in Arabidopsis. Plant Molecular Biology, 84, pp. 675-692.

Publications not included in this thesis

Lindquist E, Aronsson H (2014). Proteins affecting thylakoid morphology – the key to understanding vesicle transport in chloroplasts? Plant Signaling & Behavior, 9, e977205.

Understanding plastid vesicle transport – could it provide benefit for human medicine? Khan NZ, Lindquist E, Alezzawi M, Aronsson H (2016). Mini-reviews in Medicinal Chemistry, 16, 1-12.

List of abbreviations

ATP	Adenosine triphosphate
CCV	Clathrin coated vesicles
COPI	Coat protein complex I (vesicles)
COPII	Coat protein complex II (vesicles)
CPSAR1	Chloroplast localized Secretion associated Ras related GTPase 1
DGDG	Digalactosyldiacylglycerol
EE	Early endosome
EM	Electron microscopy
ER	Endoplasmic reticulum
ETC	Electron transport chain
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGT	Geranylgeranyl transferase
GTP	Guanosine triphosphate
LE	Late endosome
LHC	Light harvesting complex
LHCI	Light harvesting complex I, of PSI
LHCII	Light harvesting complex II, of PSII
LHCA	Light harvesting chlorophyll a/b binding protein of PSI
LHCB	Light harvesting chlorophyll a/b binding protein of PSII
LPVC	Late prevacuolar compartment
LTP	Lipid transfer protein
MGDG	Monogalactosyldiacylglycerol

MVB	Multivesicular bodies
NADPH	Nicotinamide adenine dinucleotide phosphate
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
POR	NADPH:protochlorophyllide oxidoreductase
PSI	Photosystem I
PSII	Photosystem II
PVC	Prevacuolar compartment
Rab	Ras-related in brain GTPase
REP	Rab escort protein
SAR1	Secretion associated Ras related GTPase 1
SCO2	Snowy cotyledon 2 protein
SQDG	Sulfoquinovosyldiacylglycerol
TEM	Transmission electron microscopy
THF1	Thylakoid formation 1 protein
TIC	Translocon of the inner envelope membrane of chloroplasts
TOC	Translocon of the outer envelope membrane of chloroplasts
VIPP1	Vesicle inducing protein in plastids 1
YFP	Yellow fluorescent protein

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1. Introduction

Without photosynthesis the Earth would look nothing like we know it today and the importance of chloroplasts as photosynthetic organelles of plants can therefore not be overestimated. Chloroplasts in plants and other photosynthetic organisms produce the oxygen we breathe and either directly or indirectly the food we eat; providing the base of the food web to which all animals depend.

Earth is considered to be 4.5 billion years old, but more than 3 billion years passed before chloroplasts were formed. The first photosynthetic eukaryotes developed as a eukaryotic host engulfed a cyanobacterium, a process known as endosymbiosis that occurred 1.2-1.5 billion years ago [1-3]. The hypothesis of endosymbiosis was formed in the late 1800s – early 1900s as Schimper and Mereschowsky discussed similarities of cyanobacteria with plastids. About a hundred years later the hypothesis of endosymbiosis was considered a theory to which "it seems pointless to consider seriously alternative explanations" (Michael W. Gray, 1991 in [4]). During primary endosymbiosis, a cyanobacterium was engulfed by a eukaryotic cell, forming primary plastids: chloroplasts in green algae and plants, rhodoplasts in red algae and cyanelles in glaucophytes [2]. That primary plastids in all members of the kingdom Plantae (green plants, red - and glaucophyte algae) derive from one endosymbiotic event and a common ancestor is now considered to be consensus [3, 5]. During secondary and tertiary endosymbiosis, the primary plastids were engulfed again, resulting in diversification of the kingdom [3-5].

Over time, a large proportion (~95%) of the chloroplast genome has been transferred to the nucleus [6, 7]. One reason for this gene transfer could be the mutagenesis rate, which is high in the chloroplast due to abundance of reactive oxygen species [8]. Another hypothesis is that once the cyanobacteria entered its eukaryotic host, the chloroplast was isolated and probably became clonal (asexual). Transfer of genes to the nucleus would mean a transition from asexual to sexual genome, thereby increasing the possibilities to recombine out deleterious mutations [9]. The numbers of protein coding genes residing in the chloroplast of land plants differ and recent investigations seem to be lacking, though most estimations concern less than 100 to 200 [2, 9-11]. In cyanobacteria (*Synechocystis* sp. strain PCC6803, hereafter *Synechocystis*) the total number of protein-coding genes is >3000 [12, 13]. In *Arabidopsi*s sp. (hereafter Arabidopsis) only 87 proteins are considered to be chloroplast encoded, but approximately 1500 proteins are found in the chloroplast in total [2, 7].

Chloroplasts have double bilayer membranes (the outer and inner envelope), limiting the organelle from its surrounding and enclosing the stroma. Stroma is a semi-liquid water-based solution containing proteins and ribosomes, in addition to thylakoid bilayer membranes arranged in grana and stroma lamellae. Grana and stroma lamellae show differences in protein composition (lateral heterogeneity): the photosystem II (PSII) and its

light harvesting complex (LHCII) are concentrated to grana, and photosystem I (PSI), its light harvesting complex (LHCI) and ATP synthase are localized in unstacked regions named stroma lamellae [2]. The chloroplast is the site of all photosynthetic reactions, which start when sunlight reaches the thylakoids and provides energy to the electron transport chain (ETC). The ETC reactions, or light reactions, result in oxygen, NADPH and ATP. NADPH and ATP are subsequently used in the Calvin Benson cycle. There, carbon dioxide is fixed and converted into the three-carbon sugar glyceraldehyde-3-phosphate, which in turn can form other carbohydrates.

The first part of photosynthetic reactions is driven by the ETC, as sunlight reaches the thylakoid membranes. The energy of the photons is captured by antenna complexes, consisting of light harvesting complex proteins, chlorophylls and other pigments. In the light harvesting complex, proteins binding chlorophyll are found (light harvesting chlorophyll a/b binding proteins), designated LHCBs if associated to LHCII and LHCAs if associated with LHCI [14, 15]. The energy is transferred within the antenna complex until it reaches a special pair of chlorophyll *a* molecules in the reaction center. The pair is named by its absorption maximum, which in PSII is 680 nm. The energy from the sunlight excites the special pair (P680), causing it to release an electron that is transferred to a primary acceptor of the ETC. Simultaneously, the water-splitting complex splits water into protons, oxygen and electrons; electrons that can reduce P680. As the electron is transferred from one acceptor to another it loses energy and is subsequently re-energized when reaching the second photosystem. This photosystem (PSI) functions much in the same way as PSII, but the special pair of chlorophyll *a* molecules are there named P700, as the absorption maximum is 700 nm. Once excited, P700 releases an electron. P700 can then be reduced by the electron arriving from the water splitting process and PSII. This creates a flow of electrons transported through a series of protein complexes, which is reflected in the name of the process: the electron transport chain. At the end of the ETC, the electron is accepted and involved in reducing $NADP^+$ to NADPH. As electrons are transported in the ETC, protons are transferred from the stroma to the inside of thylakoid membranes, the lumen. These add to the proton concentration from the water splitting process and results in a surplus of protons in the lumen that drives the ATP synthase. The ATP synthase transfers the protons across the thylakoid membrane, to the stroma, a process resulting in the production of ATP (figure 1).

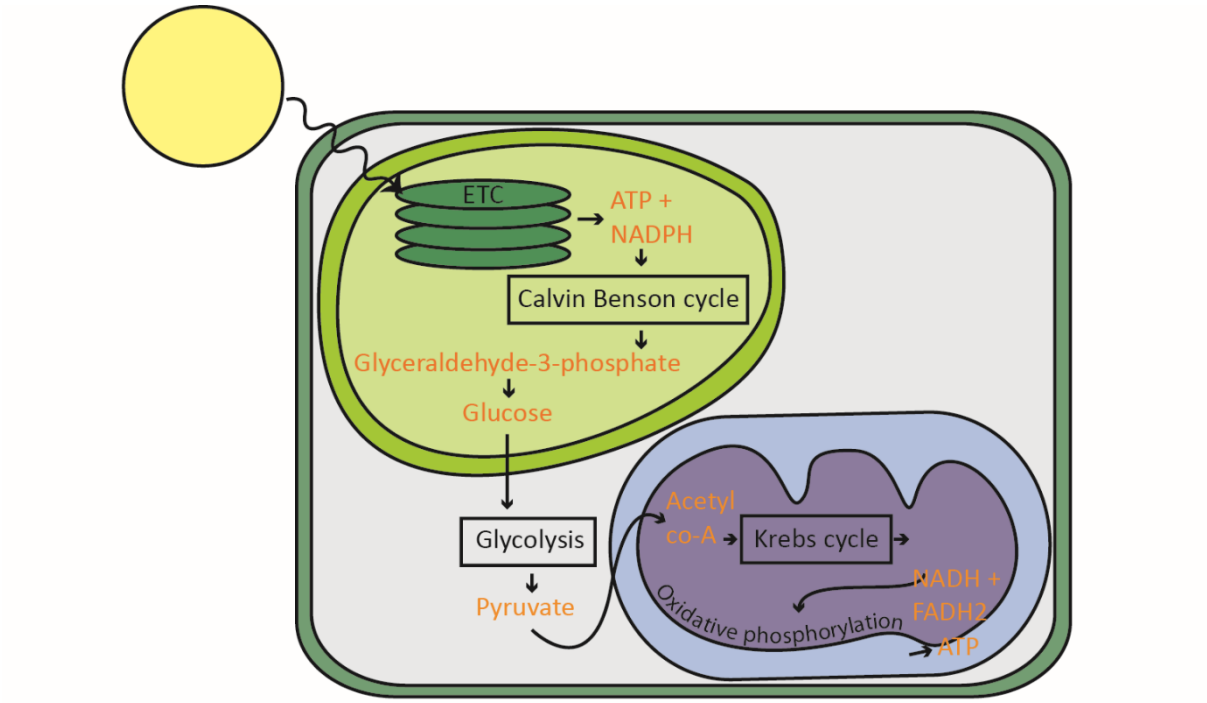

Figure 1. Schematic overview of the photosynthetic resactions in the chloroplast (green) and ATP-production in the mitochondria (purple). The electron transport chain (ETC) in the thylakoid membranes (dark green), Calvin Benson cycle in the stroma (light green), glycolysis in the cytosol (grey), Krebs cycle and oxidative phosphorylation with ATP production in the mitochondrial matrix (purple). Light blue represents the intermembrane space of mitochondria, where protons accumulate before passing the ATP synthase.

The second part of the photosynthetic reactions, the Calvin Benson cycle, uses the NADPH and ATP produced in the light reactions. Carbon dioxide enters the leaf through stomata and diffuses within the leaf and into the chloroplast. In the stroma, carbon dioxide is captured by ribulose-1,5-bisphosphate (RuBP), a reaction catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and both enzymes operate in the Calvin Benson cycle. During a series of reactions, NADPH and ATP are consumed, the carbon dioxide is fixed, and glyceraldehyde-3-phosphate is synthesized (in C3 plants). This can subsequently form other carbohydrates, e.g. glucose that makes up starch in the stroma or sucrose in the cytosol. These are the photosynthetic reactions, which all reside in the chloroplast (figure 1). However, to sustain all cellular processes, additional ATP is required. This can be accomplished by a breakdown of glucose from photosynthesis, resulting in pyruvate which in turn can be converted to acetyl Co-A. In the matrix of mitochondria, acetyl Co-A enters Krebs cycle (also named the citric acid cycle and tricarboxylic acid cycle) to generate NADH and $FADH_2$, subsequently used in the electron transport chain of mitochondria. It resides in the inner membrane of mitochondria and is known as oxidative phosphorylation. As the electrons are transported in the chain, protons are transferred from the mitochondrial matrix to the intermembrane space. The proton gradient is then used by an ATP synthase that, when transferring protons back to the matrix, produces ATP which is essential for a multitude of reactions in the cell (figure 1) [14, 16, 17].

The reactions of photosynthesis have been thoroughly investigated, but less is known about the biogenesis of the structures harboring the photosynthetic machinery: the thylakoid membranes. Lipids, constituting both the envelopes and thylakoids, are amphipathic molecules with both hydrophobic and hydrophilic ends. Due to this, lipids are not likely to pass the stroma spontaneously but rather arranged in a way that isolates the hydrophobic parts from the water-based surrounding. As the thylakoid lipids are produced at the envelopes, they have to pass the stroma. Hypotheses about how this transport is accomplished have been suggested over the years, ranging from invaginations of envelope membranes and direct contact sites between envelope and thylakoid membranes, to lipids being transferred as small spheres, i.e. vesicles. Indeed, vesicles have been observed using electron microscopy (EM) but although repeatedly observed, not much focus has been given to how vesicles in the chloroplast could be regulated. Vesicle transport is known from the cytosol of both animals and plants. There, vesicles with protein cargo shuttle different compartments and the process is highly regulated by different sets of proteins. The aims of this thesis are to demonstrate the presence of vesicles, not only in chloroplasts but also in other plastids and at various conditions (**paper I**), and to address the question of how the vesicles operate. Do they resemble cytosolic vesicles and what proteins are involved in the chloroplast processes? This is discussed in **papers II**, **III** and **IV**.

2. Chloroplasts

A typical plant cell ranges between 20 and 100 µm in size, and chloroplasts are generally considered to be ~5-10 µm large [17-19]. In chloroplasts of lettuce (*Lactuca sativa*), granum was shown to be 200-600 nm high and have a diameter of ~300 nm (~300-600 nm in Arabidopsis) [20]. A single layer in the grana stack was measured to be 20±2 nm, similar to stroma lamellae [20, 21]. The thylakoid membranes are separated from the envelope by stroma, with a distance of 50-100 nm (**paper I**)[18, 22].

The lipid composition of chloroplast membranes differs from other membranes of the cell. Chloroplasts mostly contain glyco- and sulpholipids, in contrast to extraplastidial membranes which main components are phospholipids [23, 24]. The composition of chloroplast membranes is very similar to the thylakoid membranes of cyanobacteria, reflecting its endosymbiotic origin [3].

2.1. The membranes

Chloroplasts of higher plants have double bilayer envelope membranes, where the outer envelope is often considered to originate from the endosymbiotic host, while the inner envelope is a remnant of the cyanobacterium itself (see e.g. [25]). However, this may be a simplification as the cyanobacterium was suggested to have had three surrounding layers at the time of endosymbiosis (both peptidoglycan and envelope membranes), gaining a fourth

membrane from the host as it was engulfed [3]. The host membrane (sometimes referred to as the food vacuole membrane) was then likely lost, together with a peptidoglycan layer. This left the chloroplast with only two surrounding layers, both considered remnants of the cyanobacteria [3]. Regardless of the origin of the remaining membranes, host or cyanobacterial, it is clear that today's outer envelope of the chloroplasts is different in composition compared to the inner envelope membrane and the thylakoids [23]. All chloroplast membranes have a high content of galactolipids in their membranes but the outer envelope membrane also has a significant proportion of phospholipids. This makes it more similar to extraplastidial membranes as glycerophospholipids are the main constituent of eukaryotic membranes, and differentiates it from the inner envelope membrane and the thylakoids [23, 24]. The outer envelope membrane also has a relatively high lipid:protein ratio (2.5-3), compared to the inner envelope membrane (0.8-1) and the thylakoids (0.4) [26].

The major galactolipids in chloroplast membranes are monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG); MGDG has a head group with one galactose whereas DGDG has two [23, 26, 27]. This difference provides different properties. The small head group of MGDG generates a cone-like geometry of the lipid and it can therefore not form bilayers in water. The two galactose molecules of DGDG on the other hand, produce a more cylindrical geometry that enables bilayer formation in water [28]. Both of these lipids are uncharged and the only neutral lipid class in the thylakoids. In contrast are the chloroplast exclusive bilayer forming sulfoquinovosyldiacylglycerol (SQDG) [23] and phosphatidylglycerol (PG), which are negatively charged at physiological pH [28]. The phospholipid phosphatidylcholine (PC) is one of the major constituents of eukaryotic membranes but also a large part of the outer envelope membrane (figure 2). It can form bilayers with its cylindrical geometry and is produced in the endoplasmic reticulum (ER) and Golgi [24, 29].

MGDG, DGDG and SQDG are all assembled in the envelope of the chloroplast [23, 30, 31]. MGDG in Arabidopsis is produced by three synthases, MGD1, 2 and 3. MGD1, producing most of the MGDG, is located in the inner envelope membrane, whereas MGD2 and 3 are found in the outer envelope membrane [28]. The synthases of DGDG (DGD1 and DGD2), are both located in the outer envelope membrane of the chloroplast, where DGD1 produces most of the DGDG. It has been shown that DGD1 carries a long N-terminal extension that is required for insertion of the synthase into the outer envelope membrane and enables transfer of galactolipids between the envelope membranes [28, 32]. SQDG is also synthesized in plastids, by SQD1 and SQD2 [23, 31].

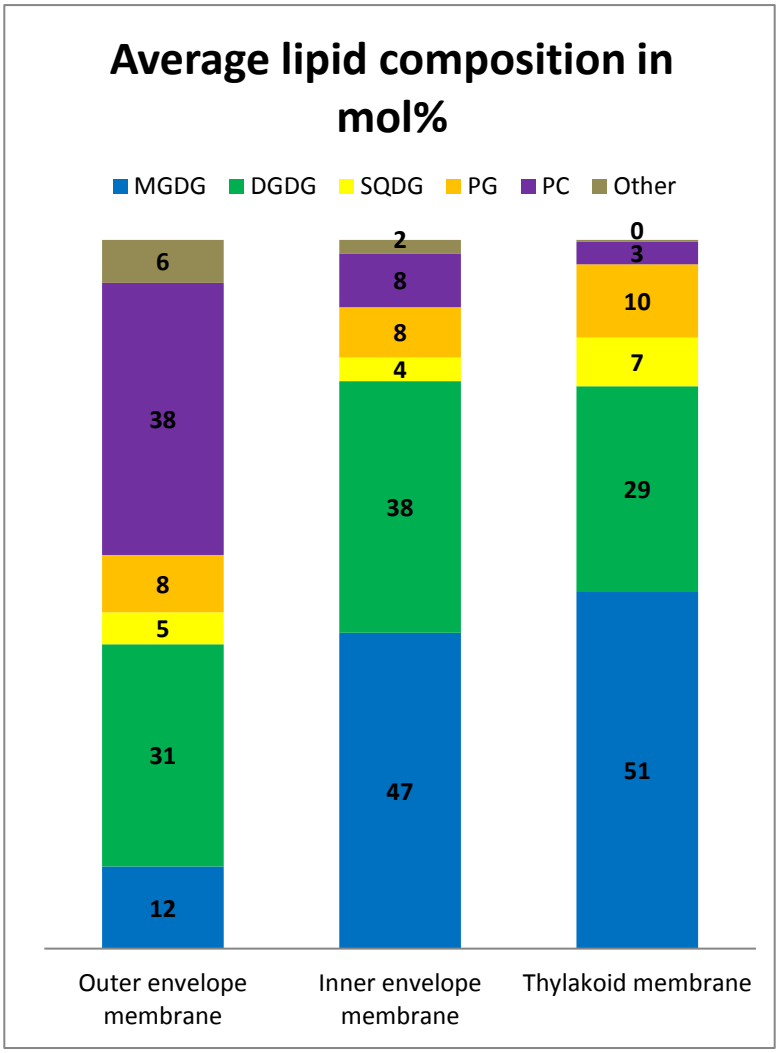

Figure 2. Lipid composition. Average values in mol% in different chloroplast membranes given by studies within [23]. Lipids represented are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), phosphatidylglycerol (PG) and phosphatidylcholine (PC).

The outer envelope membrane mostly contains PC and DGDG, followed by MGDG (figure 2). PC and other phospholipids are present in inner envelope membrane and thylakoids as well, but in much smaller proportion. Occurrence of PC in these membranes is occasionally discussed as contamination, although most studies do report presence of PC [23, 33, 34]. The inner envelope membrane is instead dominated by MGDG, followed by DGDG (figure 2). Due to the organization of thylakoid membranes into grana, the thylakoids constitute the bulk of lipids in a green leaf [23]. The main component of thylakoid bilayer membranes is MGDG, followed by DGDG (figure 2), with enrichment of MGDG in the outer leaflet of the membrane and DGDG in the inner [35]. PG and SQDG are present in all chloroplast membranes but less in envelopes than thylakoids (figure 2). The composition of the thylakoid membranes is highly regulated, as the ratio of non-bilayer forming lipids:bilayer forming lipids is of importance to intracellular trafficking, protein folding and insertion to membranes [28].

Thylakoid lipids are shown to be required for photosynthetic processes and function as structural components of PSII and PSI complexes [31]. Mutants deficient of MGDG show different effects depending on the size of the reduction; at ~40% decrease of MGDG level, PSII was not affected, but if reduced by ~80% the PSII activity was strongly impaired. At a reduction of 90%, the plant experienced complete loss of the photosystem (PSII). In vitro it has also been shown that MGDG serves a photoprotective role and is required for oligomerization of light harvesting complex II (LHCII) and dimerization of PSII. The phospholipid PG also shows importance to PSII, but not to PSI. Degradation of PG impairs PSII activity, causing dissociation of PSII dimers, LHCII trimers and PSII-LHCII

complexes. Furthermore, DGDG is also important for structure, function and stability of the photosystems, but reduced levels of SQDG in Arabidopsis do not seem to have any major effects on the photosystems, when grown at sufficient nutrient conditions. However, in *Synechococcus* sp. PCC7942 was shown to be required for the activity of PSII [31].

The precise mechanism providing the extreme curvature of grana has not been known, although recently CURVATURE THYLAKOID1 (CURT1) protein family was suggested to be involved in the process [36]. They are conserved in plants and cyanobacteria and affect grana morphology, as absence results in flat, lobe-like grana stacks. Overexpression of CURT1 results in an increase of layers in grana stacks, with higher stacks but smaller diameter as a result. In Arabidopsis, four CURT1 proteins are found in the thylakoids (A, B, C and D) and curt1ac mutant shows accumulation of vesicles and tubules. Thylakoid layer organization is known to depend on phosphorylations, and a decrease in thylakoid phosphorylations has a similar effect on grana as a decrease of CURT1 protein levels. However, the effect of CURT1 on grana stacking was shown to override the PSII core phosphorylation effects [36]. The thylakoids are stable bilayers due to integral carotenoids and transmembrane spanning proteins [23], more than 70% of the spinach thylakoid membrane area is occupied by protein complexes [34]. The thylakoid membranes are continuous and enclose a single luminal space [21, 37]. Although its name implies it to be spacious, it is densely packed [38, 39] and mostly occupied by the oxygen evolving complex [20].

2.2. The stroma

The stroma is full of water-soluble proteins, amino acids, nucleic acids and ribosomes. Due to its content, stroma is considered to be very viscous, with as much as ~300 mg RuBisCo proteins/ml [40, 41]. The stroma's low mobility of water is comparable to water mobility in a 50% bovine serum albumin solution. The high viscosity has been demonstrated using green fluorescent protein (GFP), showing that diffusion rate in stromules was about 50 times slower than in the cytosol (having a protein concentration of up to ~200 mg/ml) [41].

Approximately 200 proteins have been identified in the stroma of Arabidopsis. The large functional categories of these were protein synthesis, targeting, folding and degradation (26%), unknown functions (16%) and primary carbon metabolism (12%). Although the number of proteins involved in carbon metabolism only accounted for 12% of total proteins, they constitute 76% of the total protein mass in the stroma [42].

3. Protein transport

As the majority of proteins residing in the chloroplast are produced in the cytosol (~95%), a protein import mechanism is essential for chloroplast development and function [6, 7]. Retrograde signaling allows the plastid to communicate with the nucleus, to adjust expression levels of nuclear genes according to the chloroplast's needs [2].

3.1. Into the chloroplast: TOC-TIC complexes

Most proteins are imported to the chloroplast by the translocons of the outer and inner envelope membranes of chloroplasts (TOC-TIC complexes) and most have a peptide sequence in its N-terminus (transit peptide) directing it. The cytosolic proteins enter the chloroplast by passing through the TOC-TIC complexes in an unfolded state [43] and once reaching the stroma the transit peptide is cleaved by stromal processing peptidases (SPPs) [6]. If the protein is destined to the thylakoid lumen there is a second transit peptide exposed as the first is cleaved, directing it further to its sub compartment [7, 44]. Transit peptide sequences vary in length and have little amino acid conservation, but are overall positively charged. They are rich in serine, threonine and basic amino acids, at the expense of acidic residues [6, 7]. Interestingly, proteins to be imported to mitochondria also have target sequences, but these are referred to as pre-sequences and not transit peptides. Some proteins can be imported to both organelles, a phenomenon known as dual targeting [6, 7].

The TOC-TIC complexes are composed of both ancient proteins, originating from cyanobacteria and adapted to its present function, and novel proteins [5, 6, 45]. This may be less surprising as the need for an import machinery developed after endosymbiosis and subsequent gene transfer [6], and the composition of TOC-TIC complexes differs between species. Cytosolic proteins are guided by chaperones to reach the TOC complex in an import-competent state. Toc159 and Toc34 are GTP dependent and recognize the proteins before passing them to the Toc75 channel in the outer envelope membrane [7]. Together these three TOC components form the TOC core complex [6, 7]. There are two models on how the import proteins interact with the receptors of the outer envelope membrane, depending on which receptor is considered primary. In the first model, Toc34 is considered to be primary receptor and turned from GDP- to GTP-bound state as the import protein associates. By this, Toc159 is attracted and facilitates further transport to Toc75 as GTP is hydrolyzed [44]. In the second model, Toc159 is regarded as the primary receptor and may bind import proteins by acidic domains, before these are transferred to Toc34 and Toc75. GTP cycling and dimerization of the receptors would then control transport of the import protein, before reaching the Toc75 channel [44]. However, it has been shown that GTP-binding to receptors is important but not essential for import activity levels [7]. Toc75 has beta barrel domains which forms a channel [7, 44] and estimations of the diameter of this varies between 14 and 23 Å in diameter (1.4-2.3 nm) [44].

Tic22 is suggested to provide a link between TOC and TIC complexes, aiding passage through the intermembrane space; the space between outer and inner envelopes. Tic22 is considered an intermembrane space component, as it is peripherally attached to the surface of the inner envelope and protrudes into the intermembrane space [6, 7]. Evidence of soluble components of the translocon residing in the intermembrane space is scarce [6].

Tic110 has been considered to form a channel in the inner envelope membrane [6], but this is now being challenged [7]. A 1 MDa complex has been found to have channel activity and hence suggested as the general TIC translocon. The complex consists of Tic20 and Tic21, with translocating proteins, as well as the newly identified Tic56, Tic100 and Tic214 components. Tic20 alone is able to form a channel, as based on electrophysiological analysis, with a pore size of 8-14 Å (0.8-1.4 nm). Tic110 was only found in smaller complexes of 200-300 kDa and instead suggested to be part of a motor complex or other stromal events. However, as Tic20 is less abundant than other translocon components, its suggested role as main TIC channel has been questioned [7]. Tic110, on the other hand, is considered the most abundant protein of the inner envelope membrane [6, 44], forming a pore either by beta barrel domains or alpha helices, with a diameter of 15-31 Å (1.5-3.1 nm) [7]. It may be that Tic110 forms the major channel and Tic20 complements this by specializing at a specific subset of proteins [44].

Even though most proteins use TOC-TIC complexes for re-location, not all do, and it is likely that more than one additional pathway is yet to be discovered [6, 7, 44]. Cytosolic proteins might use vesicles of the endomembrane system to reach their chloroplast destination. This has been known in algae [46] and is now suggested also in plants, as proteins lacking transit peptide locates to the chloroplasts after passing ER and Golgi [47]. Exactly how is not unraveled but models have been presented; once vesicles from Golgi fuse to the outer envelope, proteins are relocated within chloroplasts either by using the TIC complex, an unknown translocase or new vesicles (formed by the inner envelope membrane) [46]. Regardless of how proteins are imported, all destined to the thylakoids or lumen need further assistance reaching there (see section 3.2).

3.2. Within the chloroplast

To be inserted into, or translocate across the thylakoid membrane, four known pathways are known: spontaneous, signal recognition particle/Albino3 (SRP/Alb3), twin arginine translocation (Tat) and the Secretory (Sec or Sec1) [45] (figure 3).

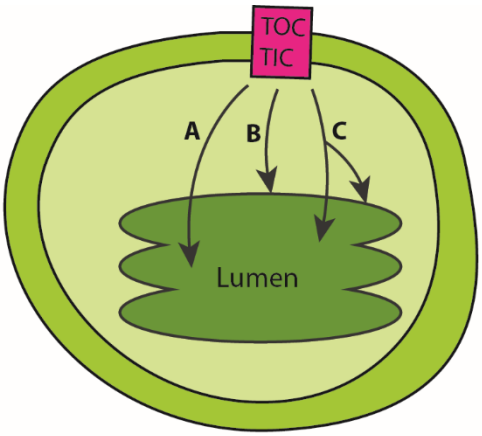

Figure 3. Pathways for protein transport within the chloroplast and their destinations. A, Sec1 & Tat B, SRP & spontaneous C, Novel pathways including Sec2 and vesicles

The spontaneous pathway includes insertions of proteins to thylakoid membranes without any additional energy supply or interaction with known protein translocases. In contrast to TOC-TIC machinery, SRP/Alb3, Tat and Sec pathways are all ancestral translocases conserved from the prokaryotic endosymbiont, but may vary in composition between species [3, 45]. A second Sec pathway (Sec2) has recently been discovered in chloroplasts and although its substrates are not yet definitively identified they are likely different from Sec1's, and the pathway was shown to be essential for plastid biogenesis [45] (figure 3).

3.2.1. Transport to lumen: Sec1 and Tat pathways

Luminal proteins (~80-100 proteins) are aqueous, soluble proteins, which need complete translocation across the thylakoid membrane [45, 48]. If a protein has 1-2 transmembrane domain(s), in addition to one or several large hydrophilic tails or loops, the protein is likely to use Sec1 or Tat pathways [45]. To enter these pathways a transit peptide is required, which has to contain a twin arginine motif if using the Tat pathway [43-45]. Based on the presence of this motif it has been estimated that 50% of the luminal proteins use the Tat pathway, whereas 50% uses the Sec1 pathway [45].

The Sec system in chloroplasts is minimal compared to *Escherichia coli*'s (*E. coli*), lacking non-essential protein components but still mechanistically similar [43, 45]. In plants, the Sec system consists of the ATPase SecA and the channel forming proteins SecE and SecY [44]. It translocates unfolded proteins and requires energy, supplied by nucleoside triphosphates (NTPs) [43, 44]. Besides being present in plant and algae chloroplasts, the Sec system functions in eukaryotic ER and archaeal and eubacterial plasma membranes [43].

The Tat translocon is composed of TatC, Hcf106 and Tha4 in chloroplasts, where Hcf106 and TatC form a receptor complex and Tha4 the translocation pore [44]. In difference to Sec pathway, it can transport folded proteins and was originally considered to be ΔpH dependent. However, its activity is likely also correlated to the membrane potential (ΔΨ) [43-45]. The size of the substrates using the Tat pathway differ, from about 2 kDa to

more than 100 kDa (or 2-7 nm in diameter), and pore forming proteins are able to adjust its opening [45].

3.2.2. Transport to thylakoid membranes: spontaneous and SRP/Alb3 pathways

The thylakoid membrane holds more than 100 proteins, anchored in the bilayer by alpha helical transmembrane domains [44, 45]. A protein with one or two such domains may insert spontaneously (without using any of the known translocases and without additional energy added). Examples of proteins that insert spontaneously are the single spanning proteins Tha4 and PsbY, and the double spanning protein PsAK [44, 45].

Besides spontaneous insertions to thylakoid membranes, proteins can utilize the SRP/Alb3 pathway. The SRP/Alb3 pathway requires GTP, but is further stimulated by ATP and ΔpH [44, 45]. About one third of the thylakoid proteins are light harvesting chlorophyll a/b binding proteins. These are nucleus encoded and subsequently inserted to the thylakoid membrane using the SRP/Alb3 pathway; bound and targeted by SRP54, SRP43 and FtsY, and translocated by Alb3 [37, 43-45].

3.2.3. Novel pathways: Sec2 and vesicles

There are proteins in the thylakoid membrane, such as NADPH:protochlorophyllide oxidoreductase (POR), which has preferences that do not fit with any of the existing pathways. POR requires ATP and NADPH for association to membranes, implying that there are likely more pathways to be discovered in the future [44, 49].

The SRP/Alb3 pathway translocate the light harvesting chlorophyll a/b binding proteins, but which translocase that integrate other multispanning thylakoid membrane proteins (e.g. TatC and SecY1) is not known [45]. In *E. coli*, TatC is translocated by the Sec1 system, but analyses in plants do not show any support for TatC using either of the four known pathways [45]. However, a second Sec pathway (Sec2) was recently described (consisting of SecA2, SecY2 and a putative SecE2) [50]. These Sec2 components are distantly related homologs to the Sec1 system and an RNAi mutant of SecY2 showed reduced levels of SecY1, TatC, Tic110 and Tic40. This implies that they are substrates using the Sec2 pathway for translocation, although this remains to be confirmed [45].

Based on these findings, a speculative model has been presented in which the TOC-TIC machinery collaborates with the Sec2 pathway at the inner envelope membrane. Assuming that TatC and SecY1 are indeed true substrates, the systems working together could integrate these and other multispanning proteins into the inner envelope membrane. If so, the thylakoid-localized proteins TatC and SecY1 would be present at the inner envelope membrane for a period of time, before reaching their final destination. The model proposes thylakoid formation either by invagination of the inner envelope membrane or vesicles. As the proteins would be attached to the inner envelope membrane they would regardless of

formation method follow and position to thylakoid membranes [45]. If assuming that the model is correct and invaginations form the thylakoid membranes, then there must be an uneven distribution of the multispanning proteins. Alternatively, some other sorting mechanism must exist as not all proteins of the inner envelope membrane are to be found in the thylakoids. If instead vesicles form thylakoids these may provide such a sorting mechanism, as our bioinformatics study suggests cargo-selecting proteins to be present within chloroplasts (**paper II**). Vesicle could therefore provide a fifth (or sixth if counting Sec2) translocation pathway, although this needs experimental verification. Vesicle-like structures have been observed repeatedly in plastids, but there are different hypotheses about when these are most prominent; some suggest vesicles to function primarily in maintenance of existing thylakoids (see e.g. [19, 51]), whereas others suggest most activity in early plastids when the need for material from the inner envelope membrane is high (see e.g. [52-54]).

The idea that chloroplast vesicles could transport more than just lipids is not new (see e.g. [52]). Interestingly, the PSII associated LHCB4 and LHCB6 have been suggested as possible cargos in chloroplast vesicles (**paper II**) and LHCB1 and LHCB3 were found to interact with a chloroplast protein suggested to function in vesicle transport (CPRabA5e) in a yeast two-hybrid assay (**paper IV**). Although light harvesting complex proteins are considered to be SRP/Alb3 travelers [37], it was recently found that a disulphide isomerase named snowy cotyledon 2 (SCO2) interacts with LHCB1 both *in vitro* and *in vivo*. SCO2 is suggested to be involved in protein folding and mutants show impaired thylakoid biogenesis with accumulation of vesicles in chloroplasts. However, no interaction between SCO2 and SRP54 or FtsY of the SRP/Alb3 pathway was identified and SCO2 was hypothesized to mediate vesicle transport of light harvesting complex proteins in cotyledons, leaving the SRP/Alb3 pathway dominant in rosette leaves [53]. Even if SRP is known to transport several LHCB proteins, the presence of another pathway for these proteins has been suggested as homozygous single and double mutants of the SRP/Alb3 pathway are still viable [53]. In addition to this, LHCB proteins have been suggested to be transported by vesicles in the single cell green alga *Chlamydomonas reinhardtii*, as the proliferation of vesicles coincides with transport of these proteins [53, 55-58].

Furthermore, a protein similar to the vesicle component Secretion associated Ras related GTPase 1 (Sar1) was found to localize to chloroplasts. The protein was named CPSAR1 and was found in both envelope and stroma, where it co-localizes with vesicles. POR, which is imported in an unknown way, has been found as interacting partner to CPSAR1, in a co-immunoprecipitation experiment (unpublished observation, Khan NZ, Aronsson H). What this means remains to be elucidated, but it could be speculated that (also) POR could use vesicle transport within the chloroplast.

4. Different plastids and chloroplast biogenesis

Proplastids can differentiate into a multitude of plastids, depending on the conditions and tissue in which they are present. In light exposed meristematic cells, proplastids differentiate to chloroplasts [19, 52, 59]. During differentiation, the poorly developed internal membrane system of proplastids with its many vesicles develops into thylakoid membranes with grana and stroma lamellae [19, 37, 52]. In absence of light, proplastids instead differentiate into etioplasts, with a characteristic membrane network (prolamellar body and prothylakoids). Upon light, etioplasts have the ability transform into chloroplasts, as the prolamellar body and prothylakoids are substituted for thylakoid membranes. There are also other plastids, which main role is not photosynthesis. Chromoplasts are ecologically very important. They repel herbivores and attract both pollinators and seed dispersing animals by providing yellow, orange and red coloration of flowers and fruits. Other plastids can serve as storing units, like the starch storing amyloplast. These have one or several large starch grains in stroma and are especially common in roots and tubers [19].

Mature plastids divide by binary fission mediated by specific proteins. As plastids divide, four contractile rings surrounding the chloroplast are formed and after contraction two daughter plastids are formed [60-62]. There are two external rings, located to the cytosolic side of the chloroplast, and two internal within the chloroplast. The external rings are the Replication of Chloroplasts 5 (ARC5)/Dynamin-Related Protein 5B (DRP5B) ring (ARC5/DRP ring) and the outer plastid dividing (PD) ring, composed of polyglucans. On the stromal side, the inner PD ring is formed but its composition is unknown. The filamenting temperature sensitive Z (FtsZ) protein forms the second internal ring (FtsZ ring). The FtsZ ring and ARC5/DRP5 ring are interconnected through the envelopes by membrane spanning proteins. Together with additional proteins, these assure proper localization and coordinates constriction of the FtsZ and ARC5/DRP5 rings, but possibly also the PD rings [60, 61]. Analyses of an Arabidopsis FtsZ mutant also open up for existence of a second plastid division mechanism, with budding of vesicles from the chloroplast into the cytosol [60]. To which extent chloroplasts form vesicles leaving the chloroplast is not known, but has been observed in both proplastids and chloroplasts by EM. Observations have also been made concerning vesicle-like structures formed during stromule tip breakage [60], although further research will be needed to verify their presence and function in plant cells.

Thylakoids extend through the contractile zone during early phases of chloroplast division, but separates from the zone in an unknown process before the two daughter plastids are formed [60]. That thylakoid membranes can be found in both daughter plastids after division [60, 61] is likely important as membranes almost exclusively are formed by growth and division, or fusion of already existing membranes [3].

5. Thylakoid biogenesis

Three non-exclusive models are considered regarding lipid transfer from envelope to thylakoids during thylakoid membrane formation: (1) soluble lipid transfer proteins (LTPs) through stroma, (2) direct contact between the membranes and/or through invaginations of the inner envelope and (3) vesicle transport [37, 59, 63] (figure 4). Although these three models are commonly mentioned, the support for them varies.

5.1. Soluble lipid transfer proteins

Lipid transfer proteins (LTPs) are proteins (~9 kDa in size) that can bind and transfer lipids. Previously, LTPs were considered to be involved in lipid transfer within the cell, but have now been suggested to mediate cuticular lipid transfer instead, as many LTPs locate to plasma membranes, cell walls and surface waxes [59]. LTPs are rarely detected in chloroplasts with one report observing a LTP in a chloroplast of rough lemon (*Citrus jambhiri* Lush). However, this LTP was rather speculated to function in biosynthesis and transport of lipids, chloroplast repair and protection [64], than transferring the bulk of lipids during thylakoid biogenesis. Thus, clear evidence supporting LTPs as main lipid transporters during thylakoid biogenesis is lacking and consequently not discussed in detail in recent reviews (such as [37] and [63]).

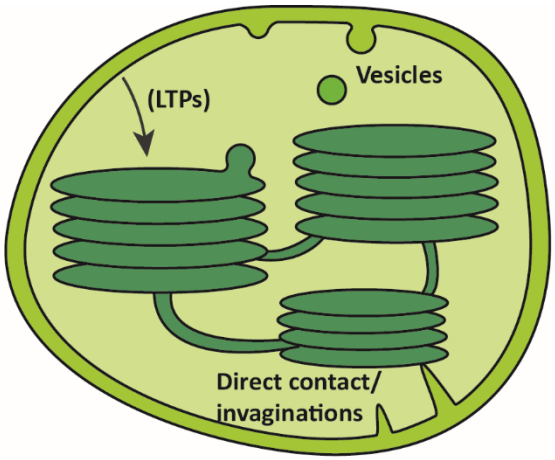

Figure 4. Lipid transfer from the envelope to the thylakoids.

5.2. Direct contact of membranes

Invaginations have been repeatedly observed by EM, suggested to be found exclusively in young undifferentiated chloroplasts and proplastids, and to be the general lipid transfer mechanism during thylakoid assembly [23, 52]. It has also been assumed that invaginations do not occur in mature chloroplasts of plants and cyanobacteria, due to the lack of observations [52, 59]. Although invaginations may be more prominent in early stages of plastid development, this view may be too simplified as rare observations have indeed been made also in mature chloroplasts. Connection of stroma lamellae to the inner envelope membrane has been noted in lettuce (*Lactuca sativa*) [21] and invaginations, or tubular structures, have also been observed in mature pea chloroplasts [51]. Interestingly, the invaginations in pea co-existed with vesicles. Although rarely observed, it suggests that the two mechanisms are non-exclusive and can occur simultaneously (**paper I**)[59]. Despite

several observations of invaginations of inner envelope membranes, no protein components regulating or mediating this process has yet been proposed [59].

5.3. Vesicles

Vesicle formation is not a spontaneous event [65] but requires protein interactors. In contrast to LTPs and invaginations, there are several proteins suggested to mediate vesicle transport from the inner envelope membrane to thylakoids (**papers II, III** and **IV**) [53, 66-68] and vesicles are therefore considered the most substantiated model although much remains to be studied [59]. Vesicles have been observed by EM in chloroplasts (**paper I**)[51, 67, 69, 70] (figure 5) but also in other plastids, e.g. pro-, etio-, chromo- and amyloplasts (**paper I**) and are most often considered to be a mechanism to maintain thylakoid membranes in mature plastids [23, 52, 63]. However, vesicles are occasionally discussed as a lipid transfer mechanism in developing plastids [53, 54, 71, 72] and vesicles are indeed often observed in proplastids (**paper I**)[19, 52]. It is likely that more than one lipid translocating pathway is present in chloroplasts (**paper I**)[54, 59], as both invaginations and vesicles have been observed in both young and mature plastids. Thus, these two mechanisms may co-exist, independently of plastid developmental stage.

In addition to electron micrographs and the proteins discussed in section 6, lipid transport experiments support a vesicle transfer mechanism. It has been observed that movement of galactolipids from envelope to thylakoids seizes at low temperature. This is a phenomenon known from cytosolic vesicle transport and low temperature treated chloroplasts display similar result with accumulation of vesicles in the stroma [51, 54]. This indicates similarities between cytosolic and chloroplastic vesicles, although it could be questioned why vesicles in the chloroplasts are not observed in other temperatures as well, if being a lipid transport mechanism. However, in **paper I** it was shown that vesicles are indeed present not only in cold treated plants but also in plants grown at ambient temperatures. This indicates that vesicles in chloroplasts are not artefacts induced by low temperature treatment, but persistent features present regardless of temperature. Moreover, it has been shown that galactolipid release from isolated envelopes requires stromal protein(s). The release is stimulated by ATP and GTP and together these requirements further support vesicle transport [54, 71]. Within stromules, a directional ATP-dependent transport with batches of GFP has been observed and was suggested as vesicles, moving with a speed of 0.12 μm/s [41]. If the batches represent vesicles and assuming similarity to envelope-to-thylakoid vesicles, they would be transported from the envelope to the thylakoid membranes within a second(s). Although speculative, this may explain why plastid vesicles are not frequently observed (see table in **paper I**), as (1) the process would be very fast and (2) the need of vesicles may not be constant but vary with development and conditions.

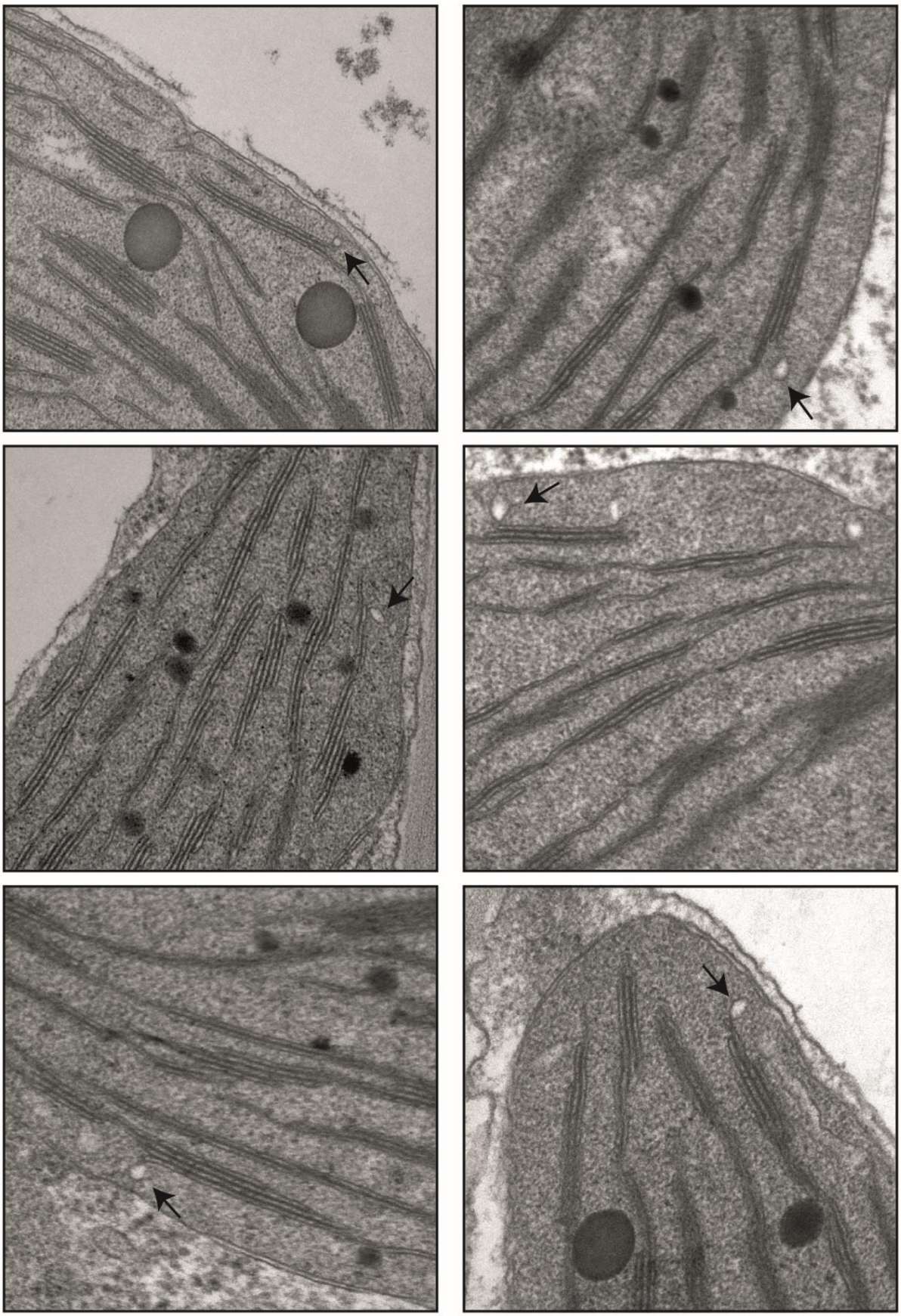

Figure 5. Vesicles in Arabidopsis chloroplasts of CPRabA5e mutants, with examples indicated by arrows. Prepared by Dr. K. Solymosi, Eötvös University, Hungary.

Electron micrographs are often two dimensional, meaning that spherical structures may represent cross-sections of tubules rather than vesicles (discussed in **paper I**). Although this is a possibility, spherical vesicles have been observed by 3D imaging using dual-axis transmission electron microscopy (TEM) and scanning-TEM tomography [73]. Tubules were also reported, but the diameter differed between the two [73], a pattern consistent with the findings of **paper I**. Plastid structures interpreted as tubules had a smaller diameter (~35-40 nm) than vesicles (~50 nm). In addition to 3D imaging, vesicles in chloroplasts have been demonstrated in 2D by serial sectioning using EM. As vesicles appeared in one slide but not the subsequent one it was concluded that the structures were solitary vesicles and not tubules [69]. These experiments clearly show that there are indeed vesicles in chloroplasts. It is therefore not very fruitful to claim that all spherical structures observed in micrographs are cross-sectioned tubules. Having said this, the apparent existence of tubules shows the need of analysis in several dimensions.

Similar to chloroplasts, cyanobacteria have an internal membrane system but without extensive stacking of grana lamellae [52]. Photosynthetic membranes of cyanobacteria in *Synechococcus elongatus* PC 7942 and *Microcoleus* sp. are interconnected and not singular sheets [74] and in *Synechocystis* the thylakoids are separate compartments without continuous connections to the plasma membrane [75-77]. The formation of thylakoids in cyanobacteria has been discussed and in *Synechocystis* no invaginations or vesicles were observed in 4-5 day old cultures. Despite the lack of observations in these cells, vesicles cannot be ruled out to exist during other growth conditions [75] or developmental stages. Vesicle transport is known from eukaryotes, but not prokaryotes. It has not been unambiguously shown or established in cyanobacteria [52, 63, 70, 75]. However, there are some indications that a vesicle system might exist also in cyanobacteria. Homologs to a protein suggested to be vesicle related in yeast was recently found by bioinformatics in *Synechocystis* [78]. If experimentally verified the result is interesting, although a preliminary bioinformatics study could not identify many of the vesicular core components in cyanobacteria (unpublished observation Lindquist E, Aronsson H). By EM, vesicles have been observed in *Microcoleus* sp., although the size of these structures was comparably larger than in chloroplasts: 150-300 nm in diameter compared to 30-70 nm [69, 74]. Notably, such structures could not be observed in the other investigated species (*Synechococcus elongatus* PC 7942) [74] or in any of the species studied in [70]. If vesicles were the general lipid transfer- and thylakoid formation mechanism in cyanobacteria, it would be reasonable to assume that vesicles would occur more abundantly. Considering this, previous experiments ([51, 69, 70]) and lack of additional observations, presence of vesicles in cyanobacteria remains to be confirmed and further elucidated.

The vesicle system in chloroplasts shows several eukaryotic traits. Chloroplast vesicles accumulate during treatment of cytosolic vesicle fusion inhibitors and low temperature and budding is likely controlled by GTPases; all characteristics of cytosolic vesicles [51, 69]. In a study by Westphal et al. [70] vesicles were found in land plants but not

in algae and cyanobacteria and was therefore hypothesized to be an adaptation to life on land, acquired from its endosymbiotic host. However, vesicles have been suggested in algae in other studies (e.g. [55-57, 79]). Regardless of whether vesicles are an adaptation to land or not, the eukaryotic traits persist although there are some suggested protein components of prokaryotic origin (see section 6).

6. Proteins involved in thylakoid biogenesis and vesicle transport

Several proteins have been suggested to be involved in thylakoid biogenesis, as mutants show accumulation or deletion of vesicles. Evidently, the precise role of several of these proteins has been hard to elucidate. Here, a selection of proteins with roles in thylakoid biogenesis and vesicle transport is presented.

6.1. VIPP1 – a simple story made complicated

Vesicle inducing protein in plastids 1 (VIPP1) has been found in organisms with oxygenic photosynthesis like plants, algae and cyanobacteria [52, 77, 80]. In plants, VIPP1 is nuclear encoded with a transit peptide directing it to the chloroplasts, where it has been considered to be peripherally attached to envelope and thylakoid membranes [68, 77]. It was originally suggested to transport lipids between these compartments, which was further supported by mutant analyses in Arabidopsis and cyanobacteria [68, 77, 81]. In Arabidopsis, mutants with reduced levels of VIPP1 have defective thylakoid biogenesis, deficient photosynthesis with a disturbed electron transport chain and lack vesicles [68]. Similarly, in the cyanobacteria *Synechocystis* a reduction of VIPP1 resulted in loss of thylakoid membranes and reduced photosynthesis [77, 81]. VIPP1 was therefore suggested to be involved in thylakoid biogenesis by enabling vesicle formation. The protein is considered to be of prokaryotic origin with a bacterial homolog in non-photosynthetic bacteria (the phage shock protein A, PspA), and to have evolved by gene duplication of cyanobacterial PspA [77, 81]. VIPP1 assembles into rings that can, at high concentrations, shape rod-like structures that have been suggested to resemble microtubules [23, 77].

However, the precise function of VIPP1 is challenged, as follow-up studies imply VIPP1 to have a membrane-stabilizing role and function similarly to PspA, rather than mediating lipid and/or vesicle transport (see e.g. [37, 77]). Mutants of VIPP1 have affected photosynthesis, but it is debated if it is due to incomplete assembly of photosystem components, as suggested in cyanobacteria and single cell algae [80, 82], or if it is due to its perturbed thylakoid formation *per se*, as shown in Arabidopsis [68, 83] and cyanobacteria [81]. VIPP1 has also been shown to enhance substrate binding to the Tat pathway and to interact with Alb3.2 [37, 80], but the implications of this need to be further elucidated. The

localization of VIPP1 is also questioned. The protein has now been suggested to be in equilibrium, both bound to membranes and in soluble form, similar to PspA [80]. The precise role for VIPP1 therefore remains elusive [77], although it is clearly a protein of great importance to the chloroplast.

6.2. THF1 – a protein with multiple roles?

Similar to VIPP1, Thylakoid formation 1 (THF1) protein was first suggested in vesicle transport inside chloroplasts. Mutants in Arabidopsis showed variegated leaf pattern with an accumulation of vesicles and a lack of thylakoid membranes in the white/yellow leaf patches of leaves, and THF1 was therefore suggested to have a role in vesicle fusion [72]. In the green leaf sectors, the inner structures of the chloroplast differed from disturbed to normal. This suggests a possibility for compensation of the inhibitory effect of THF1 [72]. Thylakoid organization was inhibited especially in young seedlings [72], which would imply vesicles to be important during this developmental stage. This is interesting to note, considering the discussion about thylakoid biogenesis and when vesicles/invaginations, are most dominant.

THF1 is a nuclear encoded protein, conserved in oxygenic photoautotrophs and present in thylakoids and stroma [72]. Recently, THF1 was shown to interact with LHCB proteins [84], which is interesting as vesicles have been speculated to transport such proteins (**paper II**)[53]. Although this interaction was shown, it was rather suggested as a way of regulating the PSII dynamics than to be a cargo of vesicles [84]. In addition to this, THF1 has been named Psb29 and suggested to play a part in PSII biogenesis, pathogen defense and sugar signaling [84-86]. As the localization of THF1 was further investigated, it was shown in the outer envelope membrane and stroma but notably not in thylakoids [86]. Its dual location might reflect different roles of THF1, with the outer envelope membrane protein being involved in sugar signaling and the stroma localized THF1 in vesicle transport [72, 86, 87]. Thus, the true role of TFH1 needs to be further elaborated in the future.

6.3. CPSAR1 – a protein located to chloroplast vesicles

In the cytosol of yeast, mammals and plants, the GTPase Secretion associated Ras1 (SAR1) is known to be involved in vesicle transport as it regulates the initial steps during vesicle budding. A protein with similarities to SAR1, the chloroplast localized SAR1 (CPSAR1), was suggested to have a similar role and identified as a homolog in Arabidopsis [66, 67]. It has intrinsic GTPase activity, is involved in thylakoid biogenesis, locates to chloroplast envelope and stroma, and is found adjacent to vesicles [67, 88]. GFP displays a punctuate pattern of CPSAR1 in chloroplasts. This is assumed to be due to dimerization [88], but the pattern could also be speculated to reflect CPSAR1 attachment to vesicles, as a similar pattern was shown in stromules and was there hypothesized to reflect vesicles [41]. The protein expression pattern shows that CPSAR1 is expressed throughout a plants life (although it is mostly expressed at young age) [67], which could support the notion of vesicles being present regardless of age but speaks in favor of young ages.

In mutants with reduced levels of CPSAR1, thylakoids were partially developed, whereas plants lacking CPSAR1 experienced arrested embryo maturation, resulting in lethality [67, 89]. In similarity to VIPP1 and THF1, the function of CPSAR1 has been challenged, reflected by its other names AtOBGL and AtObgC [88, 89]. Phylogenetic analyses show that CPSAR1 does not likely originate from the cytosolic SAR1, but from a bacterial Obg (SpoOB-associated GTP-binding protein) protein subfamily, and it has been suggested to function in protein synthesis and ribosome biogenesis within the chloroplast [89-91]. As the crystal structure of SAR1 was determined, it was suggested to possess a Sar1–NH2-terminal activation recruitment (STAR) motif, enabling interaction with the Guanine nucleotide Exchange Factor (GEF) Sec12. In its N-terminus, SAR1 also has a coat protein interacting alpha helix, followed by GTPase domains [92]. However, the STAR motif is composed of nine bulky hydrophobic amino acids that vary between species, but PROSITE (database of protein domains, families and functional sites, prosite.expasy.org) fails to identify the motif both in yeast and in Arabidopsis SAR1 amino acid sequences. Moreover, the STAR motif holds a combination of three different amino acids, combining either phenylalanine (F), isoleucine (I), leucine (L), tryptophan (W) or valine (V). An exception in yeast shows a combination of only two of the amino acids, isoleucine and leucine (IL) in the SAR1 protein's N-terminus [92]. In SARA1A and SARA1B of Arabidopsis, a combination of three amino acids that could be part of a STAR motif is found: phenylalanine, leucine and phenylalanine (FLF). They are found in the N-terminus and PROSITE identifies SARA1A and SARA1B as part of the small GTPase Sar1 family, similar to SAR1 of yeast. However, CPSAR1 belongs to the GTP1/Obg family and in this protein, no IL or FLF is to be found prior to the coiled coil domain, but rather an amino acid combination consisting of two leucines (LL). Hence, CPSAR1 is different from other SAR1 proteins in Arabidopsis. If LL could serve the role as a STAR motif, and if its coiled coil domain could provide the same function as the alpha helix in SAR1, remains to be shown.

The functions of Obg proteins are largely unknown. They have been suggested in e.g. ribosome activity and sporulation processes, where the latter also requires membrane trafficking [90]. Alignments of CPSAR1 and SAR1 show that CPSAR1 possess about 200 unique amino acids in its N-terminus. These may have been retained during evolution due to new cellular functions and may well specify its role in plant plastids [90]. Despite its differences, the fact that absence of CPSAR1 results in developmental arrest [67] shows that this protein doubtless has a very important role and its presence in close proximity to vesicles cannot be explained by a ribosomal role.

6.4. CPRabA5e – a homolog to the yeast vesicle related proteins Ypt31/32

Another GTPase suggested to be involved in transport is the chloroplast localized Ras-related in brain GTPase (Rab): CPRabA5e. It has a transit peptide, GTPase activity and locates to stroma and thylakoids (**paper II** and **IV**). The protein was originally suggested as a plant ARF1 homolog [66] but was unable to complement the *arf1Δ arf2Δ* mutant. Instead, CPRabA5e

was shown to have similarities to Rab proteins, anchoring to membranes by a geranylgeranylation in contrast to ARF proteins anchoring by myristoylation (**paper IV**)[93]. Rabs have numerous roles as they function as molecular switches and regulate effector proteins, but their prime function is membrane transport by controlling vesicles (see also section 7.4). By GTP/GDP-binding and hydrolysis Rabs modulate vesicle budding, cargo sorting, uncoating, movement, tethering and fusion – i.e. all the important steps during vesicle transport [93-95].

CPRabA5e was concluded as a Rab protein by sequence and domain similarities and its ability to complement yeast mutants deficient of the yeast Rab Ypt31/31 (**paper IV**). Ypt31/32 are known to regulate vesicle transport in exo- and endocytosis in yeast (**paper IV**). Based on gene expression data, CPRabA5e is mostly expressed during seed germination and seedling stages, but shows some levels throughout life (**paper IV**)[96]. This is similar to CPSAR1 and could support the conclusion that vesicles are likely present regardless of developmental stage (but with highest protein levels early in life). Similar to THF1 mutants, low temperature treated CPRabA5e mutants displayed accumulation of vesicles close to envelope and altered thylakoid membranes (lower grana stacks), in addition to delayed seed germination. This suggests a role for CPRabA5e in vesicle fusion (**paper IV**).

In a yeast two-hybrid screen, several possible protein interactors to CPRabA5e were identified and among these were CURT1A and proteins involved in photosynthesis. The implications of this is not yet known, although their interaction is interesting to note as CPRabA5e has been suggested to be involved in vesicle transport that may build and maintain thylakoids, and CURT1A induces curvature and affects grana morphology [36]. Additionally, LHCB1 and LHCB3 were identified as interactors to CPRabA5e (**paper IV**), which is encouraging as light harvesting complex proteins have been suggested as cargo proteins before (**paper II**)[53]. In an attempt to validate this idea, bimolecular fluorescence complementation (BiFC) was used. In this method, a yellow fluorescent protein (YFP) is split in two and one part is fused to a bait protein and the other part to a prey protein. If the bait and prey proteins interact, the split YFP is united and starts to exhibit fluorescence. Unfortunately, the method did not show any interaction for LHCB3 and CPRabA5e regardless of the positioning in the vector, which may indicate that LHCB3 is not transported by vesicles (unpublished data, Lindquist E, Karim S, Aronsson H). Thus, whether any interaction between CPRabA5e and other LHCBs exists, remains to be further investigated.

7. Cytosolic vesicle system

Intracellular transport of lipids and proteins by vesicles is a fast and selective system, taking only seconds to move from donor to acceptor membranes [97]. The coated vesicle systems include coat protein complex I and II (COPI and COPII) vesicles as well as clathrin coated vesicles (CCV). Regardless of which coat the vesicle carries, they all follow the same procedure. The vesicle is initiated, with coat and cargo collection, and buds from a donor membrane. The coat is shed and recycled before the vesicle is tethered and fused to the acceptor membranes. Although similar procedures, the three vesicle systems all require different sets of proteins enabling the processes [65, 97]. The coated vesicle systems are mainly known from yeast and mammals. These systems are generally considered to function also in plants [65], although some controversies are emerging which will be further discussed in section 8.

7.1. CCV

CCVs traffic both from plasma membranes to endosomes (endocytic pathway) and from Golgi to plasma membranes and endosomes, but additional pathways may be possible [97, 98]. The route of CCVs differs in plants and mammals, as they possess different internal compartments, further discussed in section 9.3).

The coating of CCVs includes clathrin triskelion structures, each composed of three “legs” and adaptors. There are several types of adaptors and several adaptor protein complexes. The adaptor protein complexes are labelled 1-5 (AP1-5), located at different positions within the cell and considered to bind both cargo receptors and clathrin [97, 99]. The composition of the AP complexes varies slightly between organisms as mammals have separate β1 and β2 subunits of AP1 and AP2, whereas plants have only one subunit that is considered to cover the function of both the β1 and the β2 subunit (β1/β2 subunit) [65]. Not all AP complexes interact with clathrin, as some are considered to form vesicles independently [65].

Adaptors are generally recruited to membranes by GTPases, e.g. ARF1 to AP1 complex at TGN [97, 99]. This enables clathrin to bind to the adaptors and its arrangement causes deformation of the donor membrane and the budding vesicle is formed. As the vesicle is ready to leave the donor membrane, accessory proteins might together with the GTPase dynamin mediate scission [97]; the same protein involved in chloroplast division (see section 4). Upon uncoating, the coat components are recycled. However, the uncoating of CCVs may not be initiated by GTPases (like in the other two coated vesicle systems) but rather by Hsc70 and auxilin proteins, before the naked vesicle fuses with acceptor membranes [97, 99].

7.2. COPI

COPI vesicles constitute retrograde transport as they traffic Golgi (or the ER-Golgi-intermediate compartment, ERGIC) to ER, and additionally within Golgi [65, 97, 99, 100]. The coat, or coatomer, of COPI vesicles consists of two sub complexes: (1) the ARF1 binding and cargo selective F-COP and (2) the cage forming B-COP [65, 99]. Each of the sub complexes is composed of several subunits and most of these are encoded by several genes in Arabidopsis. As ARF1 is membrane anchored through a myristoylation motif and activated by a GEF it recruits the coat consisting of the F-COP and B-COP complexes. Upon GTPase activating protein (GAP) interaction, GTP is hydrolyzed and ARF1 is inactivated and dissociates from the vesicle together with the coat. Brefeldin A (BFA) is a common inhibitor of vesicle transport, as it hinders activation of ARF proteins by targeting their GEFs [101].

The COPI system both has similarities and differences to CCVs. In COPI, the GTPase activity of ARF1 is considered to gather the coat once it is activated (GTP-bound), and its hydrolysis is suggested to induce coat dissociation, whereas CCVs coat dissociation could depend on other factors (see section 7.2.) [97].

7.3. COPII

COPII vesicles compose the anterograde transport, with transport of cargo from ER to Golgi. The small GTPase SAR1 is found in the cytosol, when GDP-bound and inactive. Upon activation by Sec12 (a GEF), SAR1 is instead GTP-bound and recruited to the ER membrane [97, 99]. As SAR1 targets to the membrane, the cargo binding Sec23/Sec24 complex arrives and thereafter the cage forming Sec13/Sec31 complex. Sec13/Sec31 deforms the membrane, forming the budding vesicle [101]. COPII vesicles are considered relatively unstable, as Sec23 functions as a GAP and is part of the coat [65, 99]. Upon hydrolysis, SAR1's GTP is exchanged for GDP. SAR1 dissociates together with the coat proteins and the vesicle is able to fuse with Golgi [97].

In plants, the COPII vesicle system likely functions similar to that found in other eukaryotes, with several homologs in both the cytosol and the chloroplast (**paper II**)[65-67]. Experiments show localization of Sec12, SAR1 and most coat proteins to the ER, supporting the presence of a COPII system in plants [65]. Despite this, COPII vesicles have recently been questioned in plants (see section 8).

7.4. GTPases, with focus on Rab proteins

GTPases are proteins that can function as molecular switches, known to regulate several processes including vesicle traffic. CPSAR1, SAR1 and CPRabA5e are all GTPases, organized in different groups. The superfamily of small GTPases include the family groups Rab, Arf, Rho, Ran and Ras GTPases. In Arabidopsis, members of all families but Ras GTPases have been identified [102]. The different families are known to be involved in different processes. ARF and Rab proteins function in membrane transport; ARFs control vesicle budding and Rabs

transport and docking [102, 103]. The largest family of small GTPases is the Rab family [93]. In Arabidopsis, 57 Rab proteins have been identified, including CPRabA5e (**paper IV**)[102]. In the Arf family of small GTPases, SAR1 and ARF1 are included, but notably not CPSAR1 which instead is part of the Obg protein subfamily (see section 6.3.) [88, 89].

The Rab proteins cycle between soluble and membrane bound states. A newly synthesized Rab is found associated with a Rab escort protein (REP) in the cytosol, where it is inactive and GDP-bound. As the REP presents it to a geranylgeranyl transferase (GGT), the Rab acquires geranylgeranyl groups that enables it to bind reversibly to membranes [93, 104]. In the cytosol, the Rab protein is held in its inactive state by a GDP dissociation inhibitor (GDI) that prevents GDP to GTP exchange. As the GDI is removed, likely by a GDI displacement factor (GDF), a GEF protein exchanges GDP for GTP and the Rab is activated [93]. Note that it is not the activation that positions Rab to the membranes (although active Rab is found there), but the membrane binding is enabled by the geranylgeranyl moieties and mediated by a GDF [104-106]. As the membrane located Rab is active, it can interact with other proteins referred to as effectors. The Rab is inactivated again as a GAP protein removes the GTP for GDP. Extraction from the membrane is mediated by GDI, which will also keep Rab inactive and soluble in the cytosol, ready for a new cycle [93, 104, 105].

The effector proteins are proteins that GTPases interact with and regulate [107, 108]. The variety of effectors and the possibility of a GTPase to interact with several effectors give Rab GTPases their multitude of roles and effects (pers. communication Karim S). In vesicle traffic, Rabs regulate virtually all steps [108], as they are involved in budding and control coat assembly, cargo selection, uncoating, motility, tethering, docking and fusion [93, 109].

8. Cytosolic vesicles in plants and other organisms

Both CCVs and COPI vesicles have been observed in plants using EM [79]. Clathrin mediated endocytosis is well established, although the precise function of all AP complexes is not yet elucidated [100, 110, 111]. As for COPI, it appears to be no doubt that the vesicles are formed and released at the periphery of Golgi cisternae, where also ARF1 is found [79]. While both these systems are well established in plants, the generally accepted idea about COPII presence in plants has recently been challenged. This is partly due to the lack of observations with chemical fixation using EM, but it is important to note that observations of COPII vesicles have been made in plants with EM with another fixation technique: ultra-rapid high-pressure freeze fixation [79, 101]. It is also established that COPII proteins in plants are essential for internal traffic between ER and Golgi and they have most (if not all) COPII proteins required, positioned at locations consistent with a role in vesicle transport [79, 101]. Mutant analyses also provide support of a COPII system in plants, e.g. was Sec16A found to have a regulatory role in the COPII coat assembly in Arabidopsis. Furthermore,

labeled COPII coat components appear as punctate structures. These move with Golgi stacks and are considered to indicate ER exit sites (ERES) [79].

An important difference between plants and animals is that Golgi in plants is mobile. As a result, the distance between ER and Golgi is narrower (less than 500 nm) and the need for vesicles therefore has been questioned. Instead, interconnecting tubules have been proposed to provide transport in higher plants, although the sole observations of tubules do not prove transport [79]. The tubules *per se* are also discussed as they have been claimed to be both *cis*-Golgi to ER spanning and to provide lateral connections between an undefined compartment (probably median Golgi) and ER [79]. Although the distance between ER and Golgi in plants is narrow, a similar distance is found in lower eukaryotes, e.g. in the alga *Chlamydomonas noctigama*. Interestingly, COPII vesicles have been observed in this alga [79] and the distance argument against vesicles could therefore be considered less strong.

Taken together, it is indeed puzzling that vesicles cannot be detected by chemical fixation in plants. It could, however, be due to the slow fixation process, combined with the rare occurrence and a rapid fusion of vesicles. If so, it could be argued that the fast freeze method is preferable and in such case, this will not remain a problematic issue [79]. To claim that COPII vesicles are not part of plants only due to this may be a mistake, considering that COPII vesicles have been observed with other methods and is supported by other experiments [79]. Having said this, it may similarly be ignorant to claim that tubules are non-existing.

9. The cells and the organelles: comparisons

The structures and compartments within the cells of plants and mammals share large similarities. However, there are differences and these differences are even larger when comparing the interior of the chloroplast to the cells. Considering vesicle transport and the compartments it shuttles, these differences may be of interest.

9.1. Cytosolic and chloroplastic vesicles

The vesicles of the cytosol often range between ~50-100 nm but both smaller and larger vesicles have been observed. CCVs are flexible in size (up to 200 nm) but tend to be smaller in plants and yeast (about 35-60 nm in diameter where the smaller number is without the coat) [112]. COPI vesicles have a diameter of ~60-100 nm in diameter [99] and COPII vesicles can vary in size depending on the cargo. Generally, they are considered to be ~60-90 nm in diameter but can expand up to 500 nm [101, 113]. The chloroplast vesicles (figure 6) are comparable to cytosolic vesicles, with a size of ~30-70 nm (**paper I** and **IV**) [67, 69].

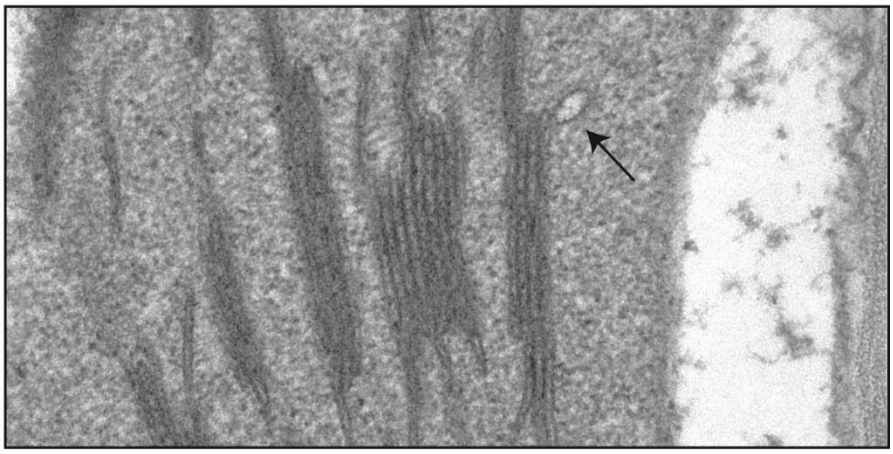

Figure 6. Vesicle about to fuse with thylakoid membranes in an Arabidopsis CPRabA5e mutant. The arrow indicates the vesicle. Prepared by Dr. K. Solymosi, Eötvös University, Hungary.

As discussed in section 5.3. cross-sectioned tubules, both in the cytosol [79] and in the chloroplast [73], could resemble vesicles in 2D micrographs. However, several lines of evidence show that there are indeed spherical vesicles present in the chloroplast (see section 5.3.). If also tubules are present in chloroplasts, as suggested by [73], these could not only be cross- but longitudinal sectioned. The appearance of such has not been established, although it is not hard to imagine longitudinal sections of tubules to resemble invaginations. At present, the thought about tubules and invaginations possibly being identical structures is just speculative, but again shows the importance of defining the 3D shape of studied structures. Vesicles and invaginations can be observed simultaneously (as in [51]) and if tubules and invaginations represent identical structures a co-existence could also be noted in [73].

If chloroplast vesicles are related to cytosolic systems, some degree of conservation or similarity considering the involved proteins would be expected [66]. Bioinformatics studies enable comparisons of proteins between species. In **papers II** and **III**, proteins known to be involved in the cytosolic vesicle transport system were used as models to search for similar proteins in the chloroplast. First, vesicle related cytosolic proteins were analyzed with regard to their amino acid sequence. Within an amino acid sequence are stretches that enable the protein to assemble super secondary structures (motifs) and fold into stable tertiary structures (domains) [114]. Domains are formed by different combinations of secondary structures elements and motifs, and certain domains are often associated with specific functions of a protein [114]. As domains and motifs are important for protein function, the cytosolic proteins' domains and motifs were searched for in chloroplast proteins. If proteins with relevant domains and motifs were found in the chloroplast it would imply that these could have the same function as they have in the cytosol (i.e. vesicle transport). Similarity between species can arise for several reasons. Analogy is defined as "the any two characters that have descended convergently from unrelated ancestors" (Fitch, W M), e.g. wings of bats and birds are functionally analogous

[115]. Homology is ”the relationship of any two characters that have descended, usually with divergence, from a common ancestral character” where characters can be “any genic, structural or behavioral feature (…)” (Fitch, W M). An example of this is the human arm and a bat wing. There are two subtypes of homology: orthology and paralogy. These concern the cause of the divergence, rather than considering function [116]. Orthologs have differences due to a prior speciation event, whereas paralogs have differences resulting from gene duplication [115, 116]. Concerning sequences, they are most often considered to be homologous, as it is less likely that such a degree of similarity results from convergence [115], and as we have not investigated the origin of sequence differences we refer to our findings as proteins homologous to the cytosolic proteins.

Analyses of cytosolic vesicle related proteins and findings of homologs resulted in lists of proteins putatively involved in the vesicle transport in chloroplasts (**paper II** and **III**). In **paper II**, suggestion of an almost complete vesicle system was proposed, with most homologs bearing resemblance to the cytosolic COPII system. Of the searched proteins, only Sec31, a Rab GEF and a Rab GDI was lacking. In addition, putative cargo proteins were identified, and when grouping these a large proportion was found to be related to photosynthesis, e.g. five light harvesting complex proteins (**paper II**). Interesting to note is that LHCB1 was not identified here, although previously suggested to be transported by vesicles (**paper IV**) [53]. In **paper III,** attempts were made to identify COPI and CCV homologs in the chloroplast, but as many of the core components were lacking it was concluded that the vesicle system is not likely to have any greater resemblance to COPI or CCV in the cytosol. Taken together, an almost complete COPII system combined with the identification of some COPI and CCV homologs, implies the chloroplast vesicle system to be unique to its organelle (**paper III**). In the studies, PFAM (database of protein families, xfam.org) and PROSITE were used to identify domains and motifs of cytosolic proteins, although in **paper III** an additional dimension was added as the identified proteins were there required to have the domains and motifs in the same order as its cytosolic counterparts. This was done to enhance the similarities and possibilities of the proteins to have similar functions, although in **paper II** the positioning of identified proteins in the membranes was studied for the same reason. The possible implications of this difference are to be revealed in future experiments; experiments that will be of great importance as bioinformatics data need verification. To date, two of the suggested proteins have been experimentally validated, CPSAR1 and CPRabA5e, see section 6.3. and 6.4.).

9.2. ER, Golgi and cytoskeletons

Plants and mammals have some fundamental differences concerning organelles and their organization that may be important to consider when discussing vesicle transport. In eukaryotes, ER initiates synthesis and folding of proteins, controls quality and functions in primary glycosylation [101]. After proteins are produced in the ER, most are transported to the Golgi where additional glycosylation of proteins and lipids take place. Golgi also serves as

a connecting platform for the secretory pathway and mediates signaling and sorting of proteins [101]. Fully folded proteins, both soluble and membrane bound, exit the ER from ERES by COPII vesicles. The number, size and dynamics of ERES varies between species and depend on the cargo type, size and volume to be transported [101]. ERES can be observed by fluorescence imaging of COPII proteins displayed as puncta [79, 101]. In most eukaryotes, Golgi is composed of stacked cisternae, organized into *cis-, medial-* and *trans*-Golgi cisternae. The *cis* side faces the ER and the *trans* side is directed towards the TGN. In TGN, the final sorting steps are conducted, before export to other destinations [101, 117].

In plants, Golgi is formed by flattened cisternae, arranged as individual stacks. It is closely associated with ER, likely by a tethering matrix, and the TGN can function independently of Golgi [101]. Moreover, the Golgi of plants is mobile (with a speed up to 4 µm/s), in contrast to both yeast and mammals [101, 118].

Golgi of mammals is different from yeast and plants as the structures are interconnected by tubules, forming a stationary Golgi ribbon. The distance between ER and Golgi is larger in mammals than in yeast and plants. However, mammals possess an ER-Golgi intermediate compartment (ERGIC), something that is lacking in the other organisms. ERGIC may be a way of facilitating long distance transport as it could serve as an intermediate stop for vesicles, as suggested in the stable compartment model [101]. There, COPII vesicles are considered to traffic the short distance between ERES and ERGIC independent of microtubules and the longer distance between ERGIC and *cis*-Golgi in a microtubule dependent way. However, another model (transport complex model) suggests ERGIC to move from ER to Golgi. This model includes vesicles forming tubular clusters tracking on microtubules to reach Golgi (figure 7).

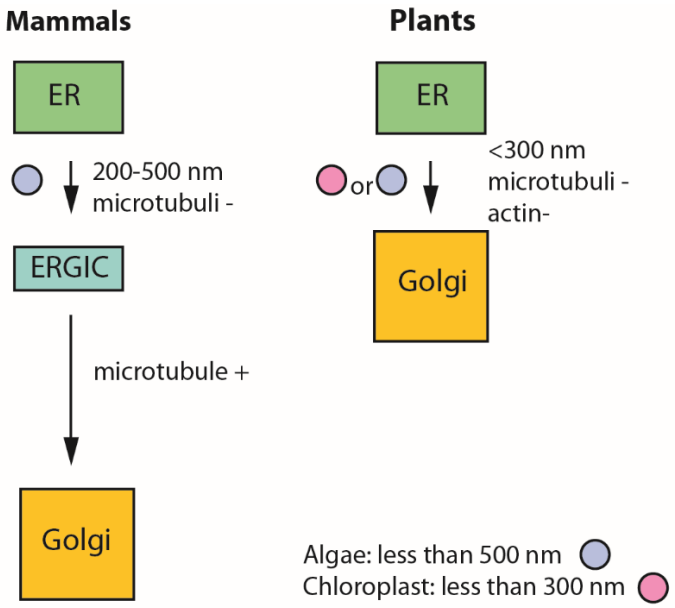

Figure 7. Distances between different compartments in different organisms, where mammals show the stable compartment model. Purple circles represent COPII vesicles and pink represents an undefined vesicle. Plus signs indicate dependence and minus independence.

As yeast and plants lack ERGIC they are instead considered to transport COPII vesicles between the ER and Golgi directly [101]. The distance between ER and ERGIC is considered to be 200-500 nm. Despite this small distance, vesicles have been repeatedly observed in the ER/ERGIC interface [79]. Interestingly, the distance between ER and Golgi in plants is ≤ 300 nm [101]. This means that the distance between ER and Golgi in plants is comparable to ER-ERGIC distance in mammals, and the idea about COPII vesicles not being present in plants due to the short distance may therefore not be valid. In addition to this is the fact that a similarly small distance is found in other organisms, but these too display COPII vesicles (see section 8). This further supports the notion that COPII vesicles can be present regardless of distance (figure 7).

Both microtubules and actin filaments of the cytoskeleton have shown to be of importance to vesicle transport in the cytosol, serving as tracks and providing motility [119, 120]. The cytoskeleton is comprised of three classes of filaments: intermediate filaments, actin filaments and microtubules. Intermediate filaments are lacking in plants but provide strength and resiliency to animal cells. In plants, this could be provided by the cell wall. Actin filaments are formed by the protein actin and are ATP dependent, whereas microtubules are hollow cylinders formed by tubulins and hydrolyze GTP. Both actin and microtubules are dynamic and can adjust to the environmental needs [14].

ER-Golgi transport in mammals rely on microtubules, but the stable compartment model suggests that observed COPII vesicles traffic the short distance between ER and ERGIC independently of microtubules [79, 101, 121]. In plants, the ER to Golgi transport is also independent of microtubules (and actin), and the compartments are estimated to be within the same distance as in mammals [101]. These similarities could imply that vesicles function independently of microtubules during such short-range transport.

Interestingly, a similar suggestion has been made where long range vesicles require microtubules but short range vesicles may instead use actin (or possibly even diffuse) [120]. However, if using actin for short range, plants would likely not experience actin independence for ER to Golgi transport. If requirement of microtubules is dependent on distance will have to be investigated further, although the distance between compartments is also of interest when considering chloroplasts. Distances between donor and acceptor membranes in chloroplasts are also small (<300 nm) (**paper I**) [18] and to date no cytoskeleton has been established. However, based on the speculation here the apparent lack of a cytoskeleton in chloroplasts may be of less concern, as the distances are so small.

It is interesting to note that cytoskeletons are not only a eukaryotic trait but also present in prokaryotes. Although a cytoskeleton as such not has been described in chloroplasts, there are reports of related proteins. Microtubule-like structures have been observed in various plastids [19, 122], where they have been suggested to mediate vesicle transport [122]. Microtubules consist of tubulin and in the chloroplast the plastid division

protein FtsZ is found, being a tubulin relative [14]. Actin has been found to co-sediment with a TOC component and VIPP1 [123], which may suggest an import and vesicle transport route for proteins directed to the thylakoids [124]. The VIPP1 proteins can also form structures that resemble microtubules and a function of VIPP1 as vesicle tracks would be consistent with the defected thylakoids found in VIPP1 mutants [122]. Considering this, a cytoskeletal structure within chloroplasts cannot be ruled out although its need for vesicle transport may be questioned.

9.3. Endosomes and lytic compartments

The endocytic pathway with CCVs involves plasma membrane, endosomes and lytic compartments but the pathway differs between animals and plants as it includes different compartments [98, 125-127]. Endosomes are a collection of organelles functioning in transport from the plasma membrane and Golgi to lytic compartments, and animals have early, late and recycling endosomes. In plants, on the other hand, only early and late endosomes have been clearly defined [126]. Early endosomes (EE) are characterized as the first compartment that receives endocytic cargo. It is in animals a separate organelle but in plants the TGN serves as this [125, 126]. The late endosomes (LE) in animals are called multivesicular bodies (MVBs) but in plants and yeast these are sometimes also referred to as prevacuolar compartments (PVCs) (figure 8). CCVs involved in endocytosis traffic between the plasma membrane and the EE in plants, but has also been assumed to shuttle the EE (TGN) and LE (MVB/PVC) [125, 128]. However, it may be that vesicles do not traffic EE and LE but instead the TGN matures into a MVB, which subsequently fuses with the lytic compartment (model by [129]). In animals the vacuole and/or lysosome constitutes the lytic compartment, whereas in plants the lysosome is generally absent in favor of the vacuole [126]. An intermediate compartment has been suggested to locate between the MVB and the vacuole in plants, named the late pre vacuolar compartment (LPVC). During maturation the MVB would then form late PVC before fusion to the vacuole [126] (figure 8).

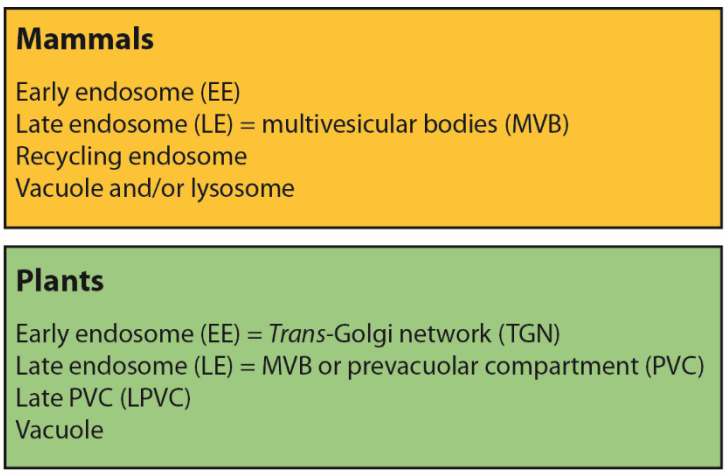

Figure 8. Suggested compartments concerning endocytosis in different cells.

Different Rab proteins locate to distinct endosomal compartments, e.g. are Rab5 marker of early endosomes, Rab7 of late endosomes and Rab11 of recycling endosomes [125, 126]. Interestingly, CPRabA5e is related to Rab11 in animal cells (**paper II** and **IV**). Recycling endosomes receives material from the EE and sends it back to the cell surface or to the TGN [126]. However, in plants, no recycling endosomes have been characterized and moreover CPRabA5e locates to the chloroplast. It is therefore apparent that, although related, Rab11 and CPRabA5e are distinct proteins that operate at different locations within animal and plant cells, but may not necessarily have different roles but mediate vesicle traffic.

10. Concluding remarks and future perspectives

Vesicles are present in chloroplasts, but also in other plastids as shown in **paper I**. These are present during different conditions, in ambient temperatures and without chemical inhibitors. How chloroplastic vesicles are regulated is largely unknown although they are strongly suggested to be of eukaryotic origin and appear to have similarities with cytosolic vesicle systems. We showed that chloroplast vesicles are likely most similar to the cytosolic COPII system, as homologs to the majority of the COPII proteins were found in the chloroplast (**paper II**). In contrast, homologs to COPI and CCV components were not identified to the same extent (**paper III**). One of the putative vesicle components was characterized in **paper IV** and was proposed a role in vesicle fusion.

The similarities in sequences of COPII and chloroplast proteins are considered to indicate homology, as such similarities are unlikely to have evolved separately of each other and are therefore implied to share a common ancestor [115, 116]. As the cytosolic proteins are vesicle related, the chloroplast homologs are likely to have a similar role, which is also supported by the current validations (**paper IV**)[67]. Despite sharing an ancestor, it is not known when and why chloroplasts acquired vesicles. However, its absence in cyanobacteria implies that the system was adopted from the cytosol and therefore originated after endosymbiosis [70] (although absence in cyanobacteria is somewhat ambiguous). If the chloroplast vesicle system was adopted from the cytosol of its endosymbiotic host, the evolutionary benefits of adopting such a system should be considered. Plastid vesicles have been suggested as an adaptation to land life [70]. If so, since the life expectancy of a plant is greater than that of cyanobacteria, plants would possibly benefit from the repair system that vesicles might provide. An imported vesicle system from the eukaryotic host may also provide capacity to remodel thylakoid membranes, due to circumstances and needs. A plant on land that is part of a community experiences differences in light intensities, due to shading and seasonal changes, and could be hypothesized to benefit from adaptable thylakoid membranes during beneficial photosynthetic conditions.

Despite the vesicle system's resemblance to COPII and that vesicles found in land plants can be inhibited by the same inhibitors as eukaryotic vesicles (**paper I**)[69, 70], proteins of prokaryotic origin have also been suggested to play parts in the vesicle transport, e.g. VIPP1 and CPSAR1. This, together with the fact that a few components of COPII remained unidentified, suggests that the vesicle system in chloroplasts is unique, constituted of both pro- and eukaryotic components. A system adopted from the cytosol could have required modifications and prokaryotic proteins may have evolved to fulfil such roles. Such an idea is not to be considered unlikely, as it is known that the import machinery of TOC and TIC possesses both pro- and eukaryotic protein components [5, 6, 45].

Attempts to isolate vesicles using density gradients of stroma, with subsequent lipid and protein analyses, have proven difficult. However, if successful, they would provide much of the information lacking to date and further efforts and development of the method could therefore be justified. The sole existence of lipids in stroma would not necessarily indicate vesicles, as fragments of broken envelopes and thylakoids could be present and are likely to form spheres due to their hydrophobicity. Complementing analyses are therefore required. However, with regard to lipid and protein composition, vesicles are likely more or less identical to envelope and thylakoid membranes, as these are the donor and acceptor membranes. One way to differentiate vesicles from membrane fragments could be by using marker proteins, although several of the potential marker proteins, e.g. coat proteins and CPSAR1, will be shed during uncoating. Therefore, using these for labeling and visualization of vesicles is not ideal, as it would not necessarily indicate presence of a vesicle but just the protein itself. Visualization of vesicles using EM has provided great benefits and its potential will likely be most important to examine further. Imaging in three dimensions would effectively solve the discussion about presence of tubules and most ideally, a time lapse should be produced, showing budding to fusion *in vivo* with labeled proteins. However, vesicles in the cytosol of plants are considered to be rather fast and rare, with extrapolations calculating one vesicle in one thin section [79]. Similarly, vesicles in the stroma are also not frequent (**paper I**), but only expected to be present in an extent needed to build and/or maintain existing thylakoid structures. When vesicles are most prominent in the chloroplast would be an interesting future project, which could provide clues to understanding their role. If combined with proteomic analysis, protein interactors may be revealed and hopefully coincide with the findings in **paper II** and **III**. Although much work remains before chloroplast vesicles are completely understood, the putative components suggested here provide a great start and opportunities for future research.

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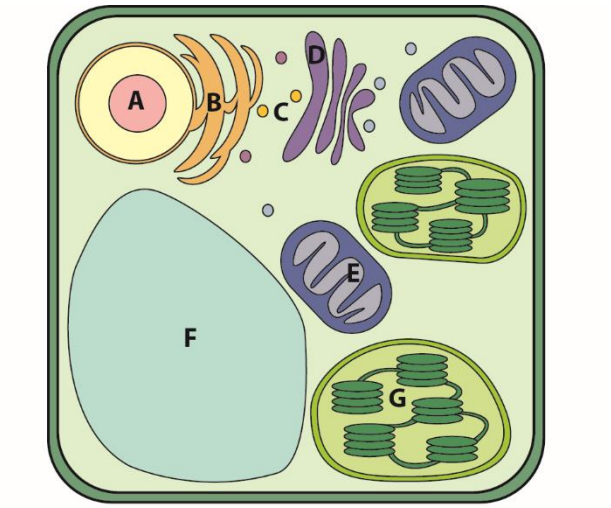
12. Populärvetenskaplig sammanfattning

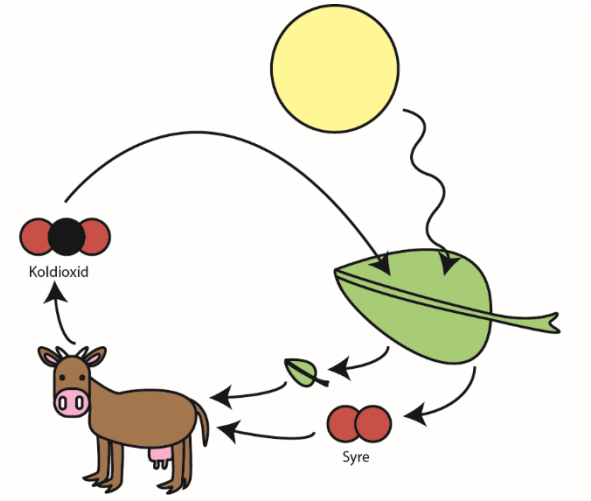
Bakgrund

En växt är uppbyggd av celler och olika delar av växten har olika funktioner. Bladens primära funktion är att utföra fotosyntes. Cellerna i bladet innehåller kloroplaster, som är små avskilda enheter, ungefär som organ, och det är i dessa fotosyntesen sker (figur 9).



Figur 9. En schematisk bild av en växtcell. A, kärnan B, Endoplasmatiska nätverket C, olika vesiklar D, Golgi apparaten E, mitokondrie F, vakuol G, kloroplast

Kortfattat så är fotosyntesen en samling reaktioner där växten gör om koldioxid till socker och syre. En växt tar in koldioxid (som finns i luften) genom små öppningar i bladen. Koldioxiden går sen in i cellerna och vidare till kloroplasterna. I kloroplasten sker reaktioner som omvandlar koldioxiden till socker, samtidigt som syre bilas. Vi får alltså både syre att andas och mat från fotosyntesen (antingen genom att vi äter växterna eller äter djur som har ätit växter) och skulle inte överleva om fotosyntesen upphörde. Läs bilagan om du vill lära dig mer om hur fotosyntesen funkar! Människor och djur behöver andas in syre och ut koldioxid, växterna tar in koldioxiden och släpper ut syre. På så sätt blir det ett kretslopp (figur 10).

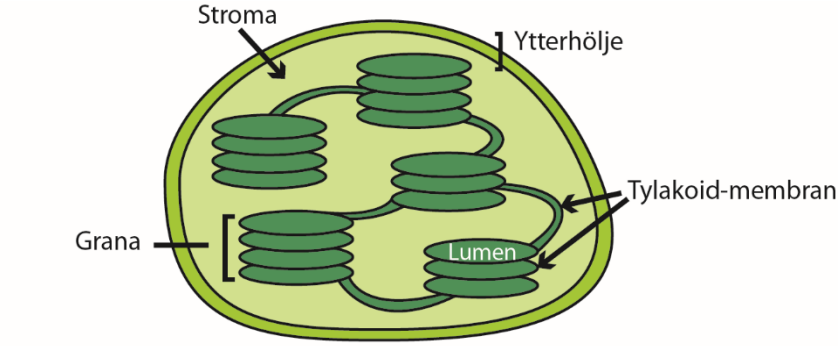


Figur 10. Växten producerar syre som kon andas in. När den andas ut koldioxid tar växten upp den och det bildas ett kretslopp.

I en bladcell finns flera kloroplaster, hur många beror både på art och på miljö men en typisk växtcell uppskattas innehålla 100-300 stycken[1]. Kloroplasten är som en oval boll, där ytterhöljet utgörs av membraner (figur 11). Ett membran är en avgränsande struktur som består av fett. Det är som en hinna, som oftast också har proteiner i sig. Inuti kloroplasten finns också membran, där hinnorna har bildat små platta säckar som är staplade och sammanbundna med varandra. Dessa inre membraner kallas tylakoider och innehåller både proteiner som utför fotosyntes och fetter. Staplarna kallas grana

[1] Pogson, B.J. and V. Albrecht, *Genetic Dissection of Chloroplast Biogenesis and Development: An Overview*. Plant physiology, 2011. **155**: p.1545-1551.

och inuti dessa finns vatten och proteiner. Mellan det inre systemet och det omgärdande höljet finns det också en vattenmassa med proteiner, denna kallas stroma (figur 11). I verkligheten är dimensionerna lite annorlunda än i figurerna här. Om en cell är stor som en fotbollsplan så är kloroplasten lika stor som en lätt lastbil. En hög med grana har då storleken av ett handbagage man får ha med sig när man flyger och en vesikel är som en innebandyboll i storlek.

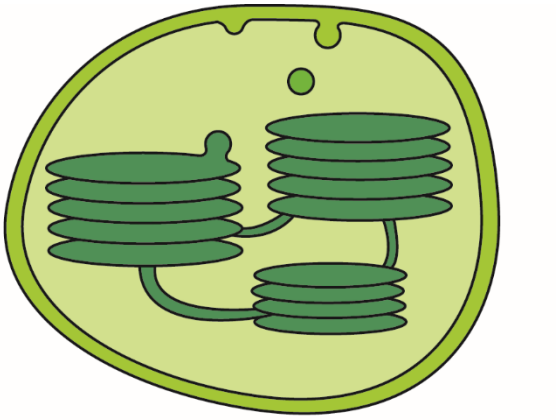


Figur 11. En kloroplast med dess olika strukturer.

Vad jag jobbat med

Membranen består av fetter och proteiner och både kloroplastens ytterhölje och de viktiga strukturerna inuti kloroplasten består av membran. Mellan dessa finns stroma, som är en vattenbaserad vätska. Vi vet två saker: (1) fett löser sig inte i vatten. Om man blandar olja och vatten så bildas det bara bubblor, det blir ingen jämn blandning och (2) de fetter som utgör tylakoid-membranen produceras i kloroplastens ytterhölje. På grund av att fett inte kan lösa sig i vatten kan inte fettet som produceras i ytterhöljet förflyttas till tylakoiderna utan problem. De måste transporteras från ytterhöljet, genom vattenmassan (stroma), till tylakoiderna (figur 12). I vår forskargrupp driver vi idén om att fetterna går från ytterhöljet till innerstrukturerna (tylakoiderna) genom att bilda just små bubblor, som olja i vatten. Dessa små bubblor kallas vesiklar och syns i figur 12. När vesiklarna bildas vid ytterhöljet så tror vi att det är proteiner som samverkar och gör så att detta sker. Vi tror också att det är proteiner som hjälper till att ta emot vesiklarna när de når tylakoiderna. Exakt vilka protein det är gör detta jobbet i kloroplasten har jag försökt ta reda på. Vi vet förresten en sak till, och det är att samma sak händer ute i den stora cellen; där bildas det vesiklar på ett ställe som förflyttas till ett annat och dessa har dessutom med sig last som de levererar till slutdestinationen. Vesiklarna i cellen regleras av proteiner men där vet man redan vilka proteiner som är inblandade och det verkar som att systemet inuti kloroplasten liknar systemet ute i cellen.

Våra upptäckter

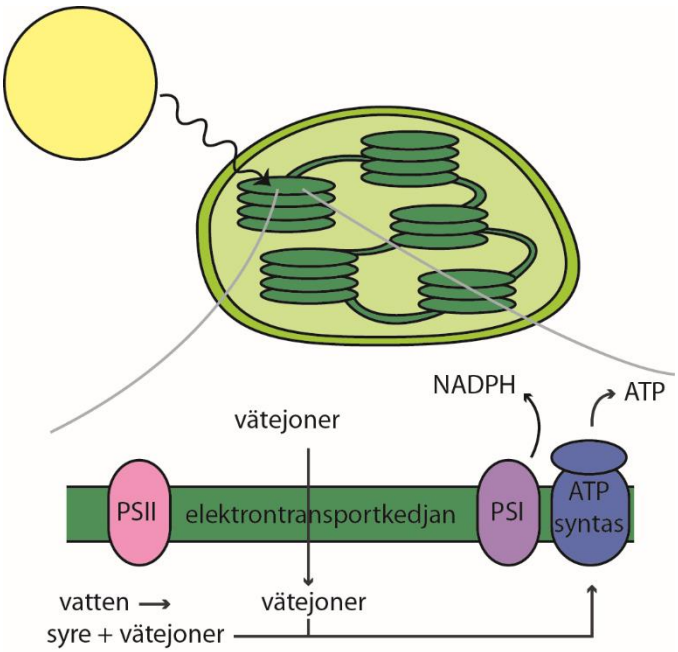


Figur 12. En kloroplast med vesiklar som avgår från ytterhöljet, färdas genom stroma och smälter samman med tylakoiderna.

I elektronmikroskop har man många gånger sett vesiklar, både i cellen och i kloroplasten. Vi har jämfört de proteiner som finns inuti kloroplasten med de proteiner som finns i den stora cellen och sett att det finns liknande proteiner på båda ställen. Eftersom proteinerna liknar varandra kan de förmodligen utföra samma arbete, dvs bilda och ta emot vesiklar inuti kloroplasten (**artikel II** och **III**). Detta stödjer idén om att ett vesikelsystem inuti kloroplaster finns, men man måste genom experiment testa vilken roll de föreslagna proteinerna i kloroplasten har. Detta har bara gjorts för ett fåtal proteiner hittills, varav vi har beskrivit ett vesikelrelaterat protein i **artikel IV**. En plastid kan beskrivas som en typ av organ inuti en cell och kloroplaster som utför fotosyntes är en typ av plastider. Men det finns fler typer av plastider, som exempelvis kromoplaster och amyloplaster. Dessa ger blomblad dess färg och fungerar som sockerlagringsenheter. Vi har kunnat visa att det finns vesiklar även i dessa, och att vesiklar inte bara förekommer under de förhållanden och i de arter och organ man sett innan (**artikel I**). Det betyder att det är troligt att vesiklar finns och fungerar på det sätt vi tror, men i framtiden måste även resten av de föreslagna proteinerna testas för att verifiera detta.

13. Bilaga

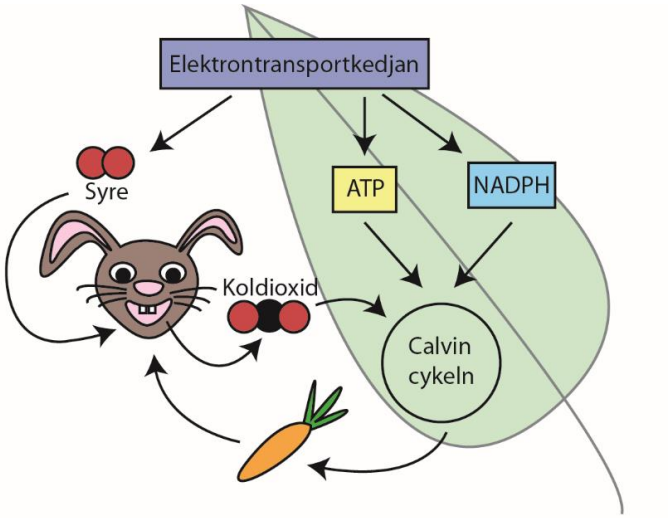
I tylakoiderna inuti kloroplasten sitter det en mängd olika proteiner och många har som roll att hjälpas åt vid fotosyntesen. När ljus träffar kloroplastens tylakoid-membran så leds energin från denna ner till ett område med en samling proteiner (reaktionscentrum i fotosystem II och I). Samtidigt som detta händer så sönderdelas vatten inuti tylakoiderna (dvs i lumen) och elektroner frigörs. Då bildas även syrgas, som är en viktig del av luften vi andas. Ljusenergin från solen gör att en elektron i speciella klorofyllpar som sitter i reaktionscentrumen exciteras, och elektronen flyttas från sin ursprungsposition till att gå från protein till protein (som sitter fast i membranet). I denna process transporteras alltså elektronen igenom en kedja av proteiner och det kallas därför elektrontransportkedjan (figur 13). Under tiden elektronen flyttas och vandrar igenom dessa proteinkomplex, så händer flera saker som respons. Dels så pumpas det in vätejoner från stroma in i lumen. Dessa, tillsammans med de vätejoner som kommit från att vatten sönderdelats, genererar ett överskott av vätejoner (H^+) i lumen. Detta överskott utjämnas genom att vätejonerna går ut till stroma igen men för att komma dit måste de ju passera över det avgränsande membranet och det gör vätejonerna genom att gå igenom ett proteinkomplex som kallas ATP-syntas. När jonerna går igenom detta så bildas det en molekyl som kallas ATP. Man kan tänka på ATP som en energibärare – ett batteri med energi som kan transporteras till de delar i cellen som behöver energi till att utföra ett arbete eller en reaktion. Förutom ATP så bildas också en annan molekyl som kallas NADPH. Både ATP och NADPH behövs sen för att socker ska kunna bildas (figur 13). Eftersom allt detta som sker beror av ljuset brukar dessa reaktioner kallas de ljusberoende reaktionerna. Utan solen skulle inga av dessa reaktioner ske och produkterna från elektrontransportkedjan är alltså dels syre som vi kan andas, dels ATP och NADPH som används i påföljande reaktioner.



Figur 13. De ljusberoende fotosyntesreaktionerna.

De påföljande reaktionerna är inte beroende av ljus för att fortgå och kallas därför ljusoberoende reaktioner och innefattar Calvin cykeln (figur 14). Calvin cykeln är namnet på en hel rad reaktioner som sker i kloroplastens stroma och som genererar sockerarter. Koldioxid som växten tagit in i bladet, går alltså in i bladcellerna och hela vägen in till kloroplastens stroma. Där träffar koldioxiden en annan molekyl som binder fast den. I

de reaktioner som sker därefter används produkterna från de ljusberoende reaktionerna (ATP och NADPH). Det bildas en molekyl som kallas glyceraldehyd-3-fosfat. Denna kan i sin tur bilda sockerarter: stärkelse som är uppbyggt av glukos kan bildas i kloroplastens stroma, och sukros kan bildas ute i cellens vätska, cytosolen. Sockret använder sen växterna för att bilda mer ATP som behövs i andra reaktioner. En del arter gör mycket stärkelserika rötter och knölar, som exempelvis morot och potatis, som vi kan äta. Genom fotosyntesen får vi därför både syre och mat, mat åt oss direkt eller åt andra djur och båda sakerna är basala för vår överlevnad. Därför är fotosyntesen viktig!



Figur 14. I bladets kloroplaster sker elektrontransportkedjan och Calvin cykeln. Elektronstransportkedjan sker i kloroplastens tylakoider och ger syre att andas, ATP och NADPH.
Calvin cykeln sker i kloroplastens stroma och använder ATP och NADPH samt koldioxid för att bilda socker. Sockret kan sen lagras i växten och blir mat till djur.

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