Vesicle Transport in Chloroplasts with Emphasis on Rab Proteins

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Abstract
Chloroplasts perform photosynthesis using PSI and PSII during its light-dependent phase. Inside the chloroplast there is a membrane called thylakoid. The thylakoid membranes are an internal system of interconnected membranes that carry out the light reactions of photosynthesis. The thylakoid membranes do not produce their own lipids or proteins, so they are mainly transported from the envelope to the thylakoid for maintenance of e.g. the photosynthetic apparatus. An aqueous stroma made hinder between the envelope and thylakoid for the lipids to move between the two compartments. Vesicle transport is suggested to transport lipids to thylakoids supported by biochemical and ultra-structural data. Proteins could potentially also be transported by vesicles as cargo but this is not supported yet experimentally. However, proteins targeted to thylakoids are mediated by four pathways so far identified but it has been proposed that a vesicle transport could exist for proteins targeted to thylakoids as well similar to the cytosolic vesicle transport system.

This thesis revealed similarity of vesicle transport inside the chloroplast to the cytosolic system. A novel Rab protein CPRabA5E (CP= chloroplast localized) was shown in Arabidopsis to be chloroplast localized and characterized to be important for thylakoid structure, plant development, and oxidative stress response. Moreover, CPRabA5e complemented the yeast homologues being involved in vesicle transport, and the *cprabA5e* mutants were affected for vesicle formation in the chloroplasts. Another Rab (CPRabF1) was also identified inside the chloroplast and could possibly play a role in vesicle transport. Interestingly, CPRabF1 has previously been characterized for its involvement in vesicle transport in the cytosol and thus its localization in chloroplasts might indicate dual targeting of CPRabF1. No phenotype was observed despite usage of several applied factors e.g. high light and osmotic stress.

A previous bioinformatics study predicted several Rab related proteins inside chloroplasts linked to a suggested COPII vesicle transport system. We analyzed the gene expression for the Rab related genes at several developmental stages covering the life span of Arabidopsis e.g. from seedlings to senescence. The data indicated a rather homogenous expression pattern among the genes studied being around 20-60% expressed for all developmental stages except for senescence were the expression pattern was more discrete. At senescence chloroplast degradation occurs indicating less need for vesicle components. The idea of a COPII vesicle system inside chloroplast raised the question of a COPI or clathrin coated vesicle (CCV) system in chloroplasts. Through a bioinformatics approach we found several homologues of cytosolic COPI and CCV related vesicle transport components inside chloroplast. However, many of them already had a clear function other than vesicle transport or were having an unknown function. Moreover, many necessary subunits to build a functional COPI and CCV system were not even identified to be chloroplast localized and so we concluded that vesicle transport in chloroplast do not have strong similarities with a COPI or CCV system, rather being more linked to a COPII system as recently suggested.

Keywords: chloroplast, clathrin, COPI/II, lipid, protein, Rab, transport, vesicle

Gothenburg, March 2014 ISBN: 978-91-85529-66-7

To my father and mother,
members of my family,
and my wife Aisha

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

Paper I *Karim S[1], **Alezzawi M[1]**, Garcia-Petit C, Solymosi K, Khan NZ, Lindqvist E, Dahl P, Hohmann S, Aronsson H (2013) A novel chloroplast localized Rab GTPase protein CPRabA5e is involved in stress, development, thylakoid biogenesis and vesicle transport in Arabidopsis. Plant Mol Biol DOI 10.1007/s11103-013-0161-x

Paper II Yin C, Karim S, **Alezzawi M**, Zhang H, Aronsson H (2014). Localization of ARA6/RabF1 in Arabidopsis chloroplasts and characterization of *cprabF1* mutants under different stress conditions. Manuscript

Paper III **Alezzawi M**, Karim S, Khan NZ, Aronsson H (2014) Gene expression pattern for putative chloroplast localized COPII related proteins with emphasis on Rab related proteins. Plant Sign Behavior Accepted

Paper IV **Alezzawi M[1]**, Lindquist E[1], Aronsson H (2014) COPI and clathrin related vesicle transport proteins are not evident in chloroplasts as predicted using a bioinformatics approach: An *Arabidopsis* model. Submitted

[1]First authorship shared between these authors.

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ABBREVIATIONS

ADL	Arabidopsis dynamin-like
AP	Adopter protein
ARF1	ADP-ribosylation factor 1
BFA	Brefeldin A
BiFC	Bimolecular fluorescence complementation
CCV	Clathrin coated vesicle
ceQORH	Chloroplast envelope Quinone Oxidoreductase Homologue
COPI/II	Coated protein I/II
CP	Chloroplast localized
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
ER	Endoplasmic reticulum
FZL	Fuzzy-onions like
G3P	Glycerol-3-phosphate
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
LPA	Lysophosphatidic acid
LHCP	Light harvesting complex protein
MGDG	Monogalactosyldiacylglycerol
PA	Phosphatic acid
PC	Phoshatidylcholine
POR	NADPH:protochlorophyllide oxidoreductase
Rab	Ras-related protein in brain
SAR	Secreted-associated and Ras-related
Sec	Secretory
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SRP	Signal recognition particle
Tat	Twin arginine translocation
TGDG	Trigalactosyldiacylglycerol
THF1	Thylakoid formation 1
TIC	Translocon at the outer envelope membrane of chloroplasts
TOC	Translocon at the outer envelope membrane of chloroplasts
TRAPP	Transport protein particle
VIPP1	Vesicle inducing protein in plastids1
Ypt	Yeast protein transport

1. INTRODUCTION

Plants convert light energy from the sun into food useful for animals and people. Plants also provide shelter, shade and safety for animals and other organisms and thus they are the primary habitat for thousands of organisms. Plants can help moderate the temperature and also affect climate change, such as in tropical rainforests when removed, thus the abundance of plants can actually change the rainfall patterns over large areas of the earth's surface. In addition, the roots of plants help hold the soil together, which reduces erosion and conserve the soil structure. When plants die, their decomposed remains are added to the soil making the soil rich with nutrients. Thus, the societal value and importance of plants in different aspects of life cannot be understated.

The oxygen (O_2) that we breathe from the air to keep our cells and bodies alive is produced by plants thanks to solar energy and the release of oxygen from water. This process is part of the photosynthesis reaction and the solar energy also produces ATP and NADPH molecules used to help incorporate CO_2 into carbohydrates, which are also backbones to, e.g., proteins and lipids. Photosynthesis occurs in plants, photosynthetic protists (e.g., brown algae), and some bacteria. In plants and other algae, it takes place within the organelle called the chloroplast.

The chloroplast is surrounded by two envelope layers covering the interior of the chloroplast, which consists of an aqueous environment, the stroma, which in turn houses an inner membrane called thylakoids. Photosynthesis occurs in these thylakoids and most proteins and lipids building up thylakoids (and thus the photosynthetic apparatus) are transported from the envelope membrane. This transport through the stroma may seem to be straight forward, but the aqueous environment of the stroma is a hindrance between the envelope and the thylakoids since it will make hydrophobic molecules (such as lipids and insoluble proteins) face difficulties in moving freely to the thylakoids. Four different protein targeting pathways have been identified to date for the insertion into the thylakoid membrane or transport to the interior of the thylakoid (i.e., to the membrane enclosed lumen): the Secretory (Sec) pathway, the Signal Recognition Particle (SRP) pathway, the Twin Arginine Translocation (Tat) pathway, and the spontaneous pathway (Jarvis and Robinson 2004; Keegstra and Froehlich 1999). However, the transport of lipids to thylakoids is still less well defined, but one theory supported by the literature makes use of the so called vesicle transport (Andersson et al. 2001; Morré et al. 1991).

The chloroplast vesicle transport system is suggested to be similar to the well characterized cytosolic secretory system (Morré et al. 1991; Westphal et al. 2001) mainly identified using yeast (Dacks and Field 2007). Several putative chloroplast localized components have been proposed to fill the puzzle of a complete vesicle transport system inside chloroplasts (Andersson and Sandelius 2004; Khan et al. 2013). Moreover, several putative cargo proteins destined for the thylakoid have been proposed using a bioinformatics approach, the majority being involved in building up the photosynthetic apparatus (Khan et al. 2013).

In this thesis facts about vesicle transport in chloroplasts are discussed, i.e., transport between the inner envelope and thylakoids using *Arabidopsis thaliana* as a model plant. The focus is on the relations between the chloroplast components and those components found in the vesicle transport system in the cytosol, such as the coat protein complex I (COPI), the coat protein complex II (COPII), and the clathrin coated vesicles (CCV). The emphasis will be on Rab small GTPases being part of any intracellular

membrane transport as key regulators, thus also involved in chloroplast vesicle transport. In **Paper I** Arabidopsis CPRabA5E (CP = chloroplast localized) (Rab = Ras-related proteins in brain), a homologue of yeast (*Saccharomyces cerevisiae*) Ypt31/Ypt32 proteins involved in vesicle transport, was studied and shown to be chloroplast localized and have an effect on thylakoid structure as well as vesicle transport within the chloroplast. **Paper II** describes the characterization of another Rab protein localized to chloroplasts, CPRabF1, and discusses its possible role in chloroplasts. In **Paper III** the gene expression pattern of putatively chloroplast localized COPII related proteins were studied using a publicly available database (Genevestigator) with emphasis on Rab related proteins within the COPII dataset. In **Paper IV** a bioinformatics approach was used to identify if there was any evidence for a COPI or clathrin related vesicle transport system inside chloroplasts as has been predicted for COPII. The data presented here argue against COPI and clathrin related vesicle transport components inside chloroplasts. Although a few components could be found possibly linked to these pathways, it is an insufficient number to cover all expected aspects of a vesicle transport.

2. CYANOBACTERIA

Cyanobacteria comprise a morphologically and genetically very diverse group of prokaryotes (Nielsen et al. 1999; Nielsen and Krogh 1998). They had an essential role in the development of life on Earth as they were the first organisms to perform oxygenic photosynthesis, which changed the atmospheric chemistry and thus led to the evolution of aerobic eukaryotes (Nielsen and Krogh 1998). Thus, eukaryotes are believed to have evolved from prokaryotes, explained by the endosymbiotic theory starting with endosymbiosis of mitochondria-like bacteria and thereafter chloroplast-like bacteria to create eukaryotes. This is supported by various structural and genetic similarities between cyanobacteria-derived chloroplasts of higher plants and algae as eukaryotes and cyanobacteria as the ancestral prokaryotes (Matsuda et al. 2005; Petsalaki et al. 2006) Photosynthesis entered eukaryotes via primary endosymbiosis, where cyanobacteria were captured by a heterotrophic protist and converted into a photosynthetic organelle (Matsuda et al. 2005). Chloroplasts were once free-living cyanobacteria that became endosymbionts, but the genomes of plastids encode only approximately 5-10% as many genes as those of their free-living cyanobacteria (Horton et al. 2006). It has been estimated that 800-2000 genes in the Arabidopsis genome might come from cyanobacteria, demonstrating that several genes were either lost from plastids or transferred to the nucleus during the course of plant evolution (Horton et al. 2006).

The plant chloroplast thylakoid membrane lipid composition resembles that of the cyanobacteria cell membrane, also supporting the idea that plant chloroplasts evolved from the endosymbiotic ancestral cyanobacteria (Reyes-Prieto et al. 2007). Lipids play an important role as structural constituents of most cellular membranes, and they also have a vital role in tolerance to several physiological stressors in a variety of organisms including cyanobacteria (Singh et al. 2002) as cyanobacteria can be found in different environments (Sharathchandra and Rajashekhar 2011).

The lipid profile of cyanobacterial membranes are composed of the uncharged lipids mono- and digalactosyl diacylglycerol (MGDG and DGDG, respectively), anionic lipid sulfoquinovosyl diacylglycerol (SQDG), and phospholipid phosphatidyl

diacylglycerol (PG). The lipid composition can change due to environmental factors such as temperature, light, salt stress and drought (Hölzl and Dörmann 2007; Sato and Wada 2010; Schmid and Ohlrogge 2002; Somerville ; Zepke et al. 1978).

3. CHLOROPLASTS

Chloroplasts, one type of plastid, are largely responsible for the maintenance and perpetuation of most of the major life-forms on earth, because of the photosynthesis reaction that occurs in photosynthetic eukaryotes. Except for photosynthesis, which delivers generation of ATP and NADPH, chloroplasts also synthesize amino acids, fatty acids, etc and are the host of sulfur, carbon, nitrogen, etc metabolism. The envelope, consisting of outer and inner envelope membranes with a soluble intermembrane compartment in between, surrounds chloroplasts. Inside the envelope is an aqueous stroma interior with a complex mix of enzymes and water. The Calvin cycle is hosted in the stroma and fixes carbon dioxide into stable carbohydrates. Embedded in the stroma is the thylakoid membrane, a complex network of stacked sacs (grana), linked with each other by flattened sacs (stroma lamallae). The thylakoid membrane comprises a series of photosystems and associated proteins and pigments. Inside the thylakoid membrane is the third soluble compartment in chloroplasts, the lumen (Cooper 2000).

3.1. Chloroplast lipids

3.1.1. Lipids in chloroplast membranes

Membranes of eukaryotic cells have many roles, from providing the boundaries of cells and organelles to the conversion of light into chemical energy in the photosynthesis. Thus, it is not surprising that different subcellular membranes have very different protein and lipid compositions that meet the functional requirements of the respective specialized cell membrane (Benning 2009). The envelope membrane itself surrounding chloroplasts is involved in several processes, e.g., fatty acid and pigment synthesis, transport of ions and proteins, as well as being a selective boundary (Joyard et al. 2010).

The chloroplast is of cyanobacterial origin, thus its membrane lipid composition is more similar to that found in cyanobacteria than that found in animals, fungi or non-photosynthetic bacteria, supporting the idea that plant chloroplasts evolved from the endosymbiotic ancestral cyanobacteria (Reyes-Prieto et al. 2007). Chloroplast membranes contain a large proportion of galactoglycerolipids in the form of MGDG (36 mol %), DGDG (29 mol %), SQDG (6 mol %), and oligogalactoglycerolipids such as trigalactosyldiacylglycerol (TGDG) with a very low 0.8 mol % (Xu et al. 2008). Phospholipids are represented by phosphatidylglycerol (PG, 9 mol %) and phosphatidylcholine (PC, 18 mol %).

The thylakoid membrane consists of approximately 60 mol % MGDG, 35 mol % DGDG, 7 mol % SQDG, and 10 mol % PG (Andersson and Dörmann 2008; Awai et al. 2006; Benning 2009; Block et al. 1983; Browse and Somerville 1991). Cyanobacterial membranes are similar in lipid composition to chloroplasts in higher plants and algae that have an outer and inner envelope membrane. Moreover, the thylakoid membrane and the inner envelope membrane of plastids are more similar regarding lipid composition, whereas the outer envelope membrane of plastids is more similar to extraplastidial membranes, e.g., the endoplasmatic reticulum membrane. In addition, the outer envelope

membrane has a higher MGDG to DGDG ratio (Block et al. 1983), more phospholipids, and a higher lipids to protein ratio compared to the inner envelope membrane, and thylakoid membranes (Andersson and Dörmann 2008; Block et al. 1983).

3.1.2. Two pathways for the assembly of thylakoid lipids

For the assembly of thylakoid lipid precursors, fatty acids are synthesized in plant chloroplasts with an interplay with parts of the cell outside the chloroplasts, e.g., the endoplasmic reticulum (ER) (Ohlrogge et al. 1979). Many land plants use two pathways for this fatty acid synthesis: the prokaryotic pathway (also referred to as the plastid pathway), where the glycerolipids are synthesized in chloroplasts, and the eukaryotic pathway (also referred to as the ER pathway), where the glycerolipids are synthesized in the ER and transported back to the chloroplast (Figure 1) (Roughan et al. 1980). The glycerolipids produced by the ER pathway have a different molecular composition (i.e., 18-carbon fatty acids in the *sn*-2 position of the glycerol backbone) than those produced by the plastid pathway (which have 16-carbon fatty acids in the *sn*-2 position of the glycerol) (Heinz and Roughan 1983). In Arabidopsis approximately equal amounts of chloroplast lipids are produced by the two pathways (Warwick et al. 1986).

On the basis of the fatty acid composition plants can be divided in two groups. The first group includes the 16:3 plants, such as *Spincia oleracea* (spinach), *Nicotiana sylvestris* (woodland tobacoo) and Arabidopsis, that have 16-carbon fatty acids in the *sn*-2 position of MGDG of glycerol in thylakoids. The other group includes the 18:3 plants, such as *Pisum sativum* (pea), *Avena sativa* (oat), *Vicia faba* (broad bean), and *Zea mays* (maize), that exclusively contain 18-carbon fatty acids on the *sn*-2 position of the glycerol of MGDG and DGDG (Heinz and Roughan 1983; Mongrand et al. 1998).

The biosynthesis of MGDG and DGDG starts with fatty acid synthesis in the plastid (Figure 1) using an acyl carrier protein and NADPH. The major fatty acids are synthesized in stroma of the chloroplast, 18:1 and 16:0 (Ohlrogge 1995; Rawsthorne 2002). In the ER and chloroplast glycerol-3-phosphate (G3P) is converted to form lysophosphatidic acid (LPA) and phosphatic acid (PA). The prokaryotic PA pathway has C16 at the *sn-2* position and in most cases C18 at the *sn-1* position and is converted to DAG which will be further converted into MGDG in the intermembrance space and DGDG in outer envelope membrane.

In the eukaryotic pathway PA contains C16 or C18 at *sn-1* and C18 at *sn-2*. PA is converted to phosphatidylcholine (PC). DAG, PC or lyso–PC are transported from the ER to the chloroplast where MGDG and DGDG are synthesized in the intermembrane space and the outer envelope membrane, respectively, and can be further transported to thylakoid membranes (Andersson and Dörmann 2008; Benning 2009). Plastid associated membranes (PLAMs) have been shown to exist as contact sites between the ER and chloroplasts which may be involved in the interplay between the ER and the chloroplast regarding fatty acid biosynthesis destined for chloroplasts (Andersson and Dörmann 2008).

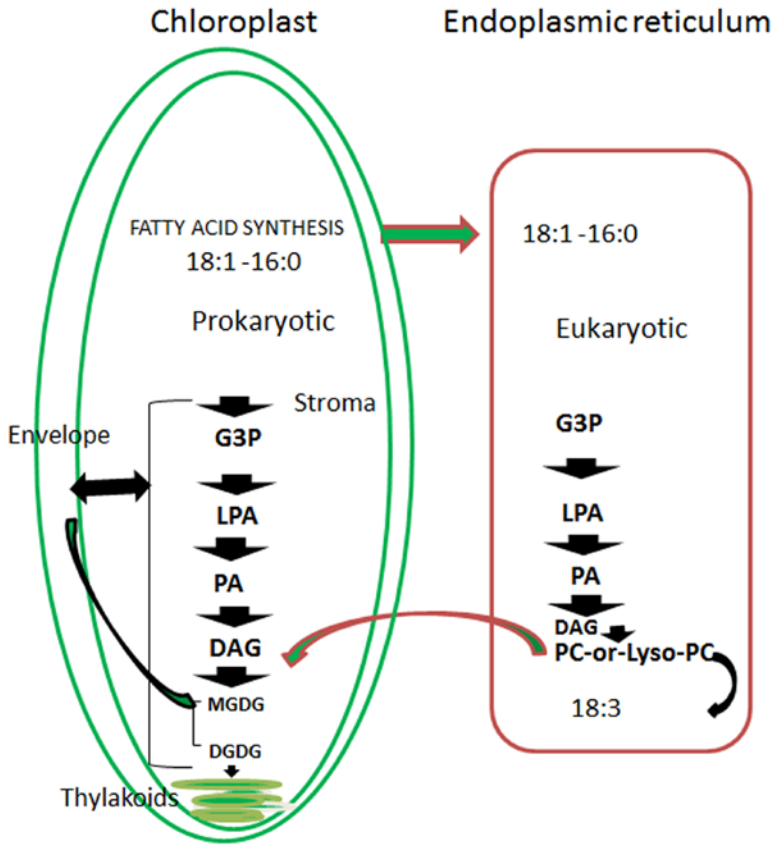

Figure 1. **Synthesis and trafficking of galactoplipids**. FAS, fatty acid synthesis; ACP, acyl carrier protein; LPA, lyco phosphatide acid; PA, phosphatidic acid; PC, phosphatidylcholine; DAG, diacylglycerol; G3P, glycerol-3-phosphate; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

3.1.3. Mechanisms of lipid transport to thylakoids

The lipid precursors originating from the chloroplast or the ER that will become glycerolipids synthesized in the envelope membrane will also have to be transported to the thylakoid membrane. There are different ways proposed for how this transport could occur. Physical contact sites between the inner envelope membrane and the thylakoids could facilitate the transfer of lipids as a transient fusion (Rawyler et al. 1995). Lipids could also be transported by diffusion in the stroma with the help of soluble proteins or with a gradient to establish some kind of polarity for directional transport. So far there is no evidence for the transport of lipids using contact sites or by diffusion from ultra-structural or biochemical studies. Another suggested transport mechanism of lipid transfer is vesicle transport where ultra-structural and biochemical studies support the idea. The earliest support for vesicle transport in the chloroplast stroma was observed at low temperature using transelectron microscopy (TEM) (Morré et al. 1991). The observation of vesicles at low temperature has been interpreted as a slower or blocked fusion processes at the thylakoid membrane, i.e., the vesicle are on their way to the thylakoids and not vice versa. However, the fission (at the budding stage) of vesicles at the envelope is not blocked and obviously not the fusion of the vesicle itself given that it

is observed in the stroma, i.e., after leaving the inner envelope. Thus, vesicles accumulate in the stroma and the lipid transfer is decreased at low temperature (Andersson et al. 2001) since vesicle fusion is inhibited at the thylakoid membrane. These observations are similar to the ER-Golgi transport vesicles at low temperature, where also vesicle fusion, but not budding, is inhibited (Moreau et al. 1992).

Chloroplast vesicle transport is stimulated by nucleotides (ATP and GTP) and stromal proteins, and are similar in size to those formed on the ER (Morré et al. 1991; Räntfors et al. 2000). Vesicle transport in the cytosol also requires nucleotides and soluble proteins (Bonifacino and Glick 2004) indicating similarity between the two transport mechanisms. Furthermore, a known inhibitor of vesicle formation in the cytosol, brefeldin A (BFA), has shown a negative effect also on chloroplast vesicle transport (Westphal et al. 2001). BFA causes a morphological and functional change of the Golgi thereby redistributing its content and membranes to the ER (Feng et al. 2003). BFA induces rapid release of the ADP-ribosylation factor 1 (ARF1) from Golgi membranes but has less effect on the organization of the trans-Golgi network. The decrease of activated ARF1 on the Golgi membrane leads to a general collapse of the Golgi apparatus (Feng et al. 2003).

Based on these observations it was initially suggested that chloroplast vesicle transport might be a late evolutionary feature in order to better cope with challenges linked to a changing surrounding environment (Westphal et al. 2003) and thus it originated from eukaryotes, since no clear evidence existed for vesicle transport in cyanobacteria. Nevertheless, there are some indications of vesicles inside photosynthetic organisms such as cyanobacteria (Nevo et al. 2007; Schneider et al. 2007), which would then also imply a possible prokaryotic origin. This latter explanation would be in line with findings that proteins suggested to be part of the vesicle transport in chloroplast not only originate from eukaryotic sources but also from bacterial ancestors, e.g., the identified proteins vesicle inducing protein in plastids (VIPP1) and CPSAR1 (Garcia et al. 2010; Kroll et al. 2001).

3.2. Chloroplast proteins

3.2.1. Import of proteins to the chloroplast

Plastids entered the eukaryotic lineage through endosymbiosis and are thought to be of monophyletic origin. They have evolved from an ancient photosynthetic prokaryote that are similar to cyanobacteria found today (Leister 2003; Palmer 2000). The majority of plastid proteins is nuclear-encoded and translated into precursors in the cytosol and thus must be imported from outside of the chloroplast. Proteins are directed to the chloroplast by an amino-terminal transit peptide acting as a targeting signal. The import itself is an active post-translational process mediated by a coordinated action of protein translocon complexes in the outer and inner envelope membranes called TOC and TIC (translocon of the outer/inner envelope membrane of chloroplasts) (Chen et al. 2000; Keegstra and Cline 1999).

3.2.2. Targeting of envelope membrane proteins

Proteins targeted to the envelope use one of two so far identified mechanisms (Keegstra and Cline 1999). One mechanism include that the protein is using the TOC/TIC pathway into the stroma and thereafter gets transported back to envelope membrane (Li and

Schnell 2006; Lübeck et al. 1997; Tripp et al. 2007), whereas in the other mechanism the protein only passes through the TOC complex before it enters the envelope membrane i.e. without passing the TIC complex (Brink et al. 1995; Knight and Gray 1995; Tripp et al. 2007). To be noted, proteomics data indicates that protein can enter the envelope without a transit peptide (Kleffmann et al. 2004) e.g. Tic32, chloroplast envelope Quinone Oxidoreductase homologue (ceQORH), and glutamate receptor GLR3.4 (Armbruster et al. 2009; Bergantino et al. 2003).

4. TARGETING OF PROTEINS TO THE THYLAKOID MEMBRANES

The thylakoid membrane contains both soluble and transmembrane proteins e.g. both necessary for the photosynthesis machinery consisting of the four complexes: PSII, PSI, cytochrome *b6/f*, and the ATP synthase holding several different proteins. Soluble proteins are found on the inside of the thylakoid membrane, the lumen. Lumen thylakoid proteins contain an additional address tag, thus except for the transit peptide for crossing the envelope membrane to stroma they have another one for luminal targeting (Hageman et al. 1986).

Two pathways, the twin-arginine translocation (Tat) pathway and the secretory (Sec) pathway are proposed to be responsible for lumen protein targeting. Tat directs proteins that have a two-arginine residue motif, while the Sec pathway takes care of proteins having a lysine residue close to the H-domain. According to proteomics studies there are at least about 100 proteins that reside in the thylakoid lumen of which the majority are nuclear-encoded (Peltier et al. 2002; Schubert et al. 2002).

Two other pathways exist, the spontaneous pathway and signal recognition particle (SRP) pathway that target transmembrane proteins to the thylakoid membrane. These proteins have no extra target signal for the thylakoid; instead they might have a targeting signal within the mature protein (the protein as it stands after the transit peptide been cleaved off) (Aldridge et al. 2009; Celedon and Cline 2012; Jarvis and Robinson 2004). In addition, a recent bioinformatics study suggests that protein transported to the thylakoid membrane might be mediated by vesicle transport, i.e. cargo proteins but hard core evidences are still to be provided (Khan et al. 2013).

4.1. The twin-arginine (Tat) pathway

The Tat pathway requires neither stromal factors nor ATP but is instead energized by the trans-thylakoidal proton gradient (Figure 2). Translocation is dependent on the thylakoid ΔpH and transport can occur with protein in the folded state (Clark and Theg 1997; Cline et al. 1992). The Tat pathway is proposed to transport luminal proteins (Gutensohn et al. 2006) and so the signal peptide of proteins using the Tat pathway contains an amino terminal twin-arginine motif just upstream of a hydrophobic region (Chaddock et al. 1995). In chloroplasts the Tat pathway consists of three integral membrane proteins Tha4 (Mori et al. 1999), Hcf106 (Settles et al. 1997), and cpTatC (Mori et al. 2001). Hcf106 and cpTatC forms a receptor complex, and the Tha4 oligomer forms a separate complex making a channel like structure (Cline and Mori 2001; Mori and Cline 2002). The proteins to be transported binds to the receptor complex, which stimulates assembly of the Tha4 oligomers with the receptor complex, and the protein is transported to the lumen in presence of a proton gradient (Aldridge et al. 2009).

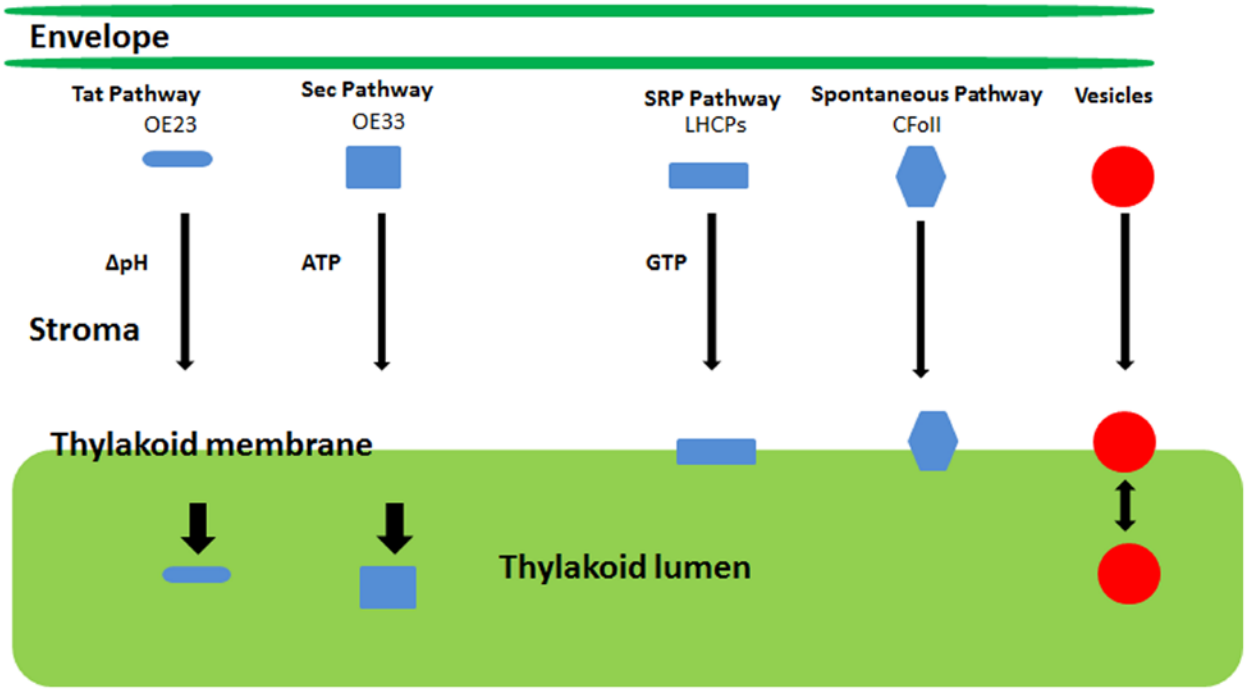

Figure 2. Overview of protein transport to thylakoids. The figure shows four different transport routes shown to deliver different proteins (in blue) to thylakoids: the Tat and Sec pathway for delivery to the lumen, and the SRP and spontaneous pathway for delivery to the membrane. All pathways have different energy requirement as indicated. In red vesicle transport shown that possible could transport protein as well to thylakoids.

4.2. The secretory (Sec) pathway

The chloroplast Sec pathway is similar to the Sec pathway system in the bacteria membrane. Proteins using the Sec pathway in chloroplast are e.g. plastocyanin, PsaF and the oxygen-evolving protein (OE33) (Figure 2) (Mant et al. 1994; Robinson et al. 1996; Yuan et al. 1994). More components for the Sec pathway exist in bacteria: SecA, the ATP–driven translocation motor; the membrane-bound SecE, SecG and SecY; and a SecB in addition to the SecDFYajC complex (Driessen et al. 2001). In chloroplasts, only homologues to SecA, SecE and SecY have been identified and shown to be involved in the protein transport to thylakoids (Laidler et al. 1995; Yuan and Cline 1994; Yuan et al. 1994). Thus, the Sec pathway in chloroplasts do not have SecB, SecG and the SecDFYajC complex and it is speculated that they are not essential components for the Sec transport (Du Plessis et al. 2011). Nevertheless, support exists that the SecAEY complex in chloroplasts is functionally and structurally similar to the bacterial Sec complex e.g. Sec transportation across the thylakoid membranes is ATP-dependent and sensitive to azide (Fröderberg et al. 2001; Mori et al. 1999; Yuan et al. 1994). The chloroplast Sec pathway might have evolved to suit its environment e.g. a different lipid composition and ATPase activity is stimulated by thylakoid signal peptide rather than the *Escherichia coli* signal peptides (Sun et al. 2007). The inability of the Sec pathway to

transport folded proteins is similar to bacteria as well (Hynds et al. 1998; Marques et al. 2004).

4.3. The spontaneous pathway

The spontaneous pathway (Figure 2) was initially described to explain the insertion of bitopic transmembrane proteins such as CFoII, PsbW. PsbX and PsbY (Kim et al. 1998; Michl et al. 1994). Proteins inserted spontaneously have a bipartite transit peptide i.e. for stromal and thylakoid targeting. The bipartite transit peptide sequence possesses two hydrophobic regions, one close to the N-terminal on the target peptide and another on the C-terminal. Those proteins do have a cleavage signal to be recognized by a luminal protease but no signal for the stromal processing protease, thus these proteins are targeted to thylakoids without the removal of the transit peptide (Gutensohn et al. 2006). Other multi-spanning proteins have also been suggested to insert spontaneously e.g. PsbS and ELIB2. To be noted, the Hcf106 and Tha4 subunits of the Tat pathway are suggested to use the spontaneous pathway (Schünemann 2007) .

4.4. The signal recognition particle (SRP) pathway

Classical SRP systems can be found in the cytoplasm of both prokaryotes and eukaryotes. All members of the abundant LHCPs family are translocated to thylakoids using the SRP pathway(Figure 2). LHCPs associates with SRP in the stroma to form a transit complex of which there are three factors having an effect on the thylakoid targeting of LHCPs: SRP54 (Franklin and Hoffman 1993; Li et al. 1995), SRP43 (Schuenemann et al. 1998) and FtsY (Kogata et al. 1999). SRP54 has GTPase activity, and suggested to have a role in thylakoid insertion (Franklin and Hoffman 1993). SRP43 have ankyrin repeats responsible for protein-protein interactions (Klimyuk et al. 1999). Binding between SRP43 and LHCP is mediated between ankyrin repeats and transmembrane domains of LHCP e.g. transmembrane 3 (Tu et al. 2000). SRP54 binds directly to LHCP at the same transmembrane 3 region (High et al. 1997; Li et al. 1995). Interaction also occurs between SRP54 and SRP43 via other domains, a methinone rich domain in SRP54 and chromodomain of SRP43 (Goforth et al. 2004; Sivaraja et al. 2005). FtsY is assumed to target the transit complex to the thylakoid membrane (Stengel et al. 2007). Finally, Albs3, an integral membrane protein, help insertion of LHCP into the thylakoid membrane (Moore et al. 2000).

4.5. Vesicle transport pathway

Vesicle transport inside the chloroplasts (Figure 2) is suggested to be similar to the cytosolic vesicle transport. The cytosolic vesicle transport system transport both lipids and proteins. Evidence of lipid transport via vesicles inside chloroplast exists. If the chloroplast system is similar to the cytosolic system one could suggest transport of cargo proteins as well when it transport lipids from the envelope to the thylakoid membrane (Andersson et al. 2001). A recent study found when studying knockout plants not having the snowy cotyledon 2 protein (SCO2) that transport of LHCPs to thylakoids might occur using vesicle transport in addition to the already established SRP pathway. Yeast two-hybrid analyses demonstrated that SCO2 directly interacted with the light-harvesting chlorophyll-binding 1 (LHCB1) proteins and this was also confirmed by using bimolecular fluorescence complementation (BiFC). Analysis of the snowy cotyledon 2,

sco2-1, mutant chloroplasts revealed that formation and movement of transport vesicles from the inner envelope to the thylakoids was negatively affected. The mutant has a disrupted chloroplast biogenesis at the cotyledon stage. It is suggested that SCO2 provides an alternative targeting pathway for LHCB1 proteins to the thylakoids via transport vesicles predominantly in cotyledons during germination possibly facilitating a faster way to form thylakoids and photosystems (Tanz et al. 2012).

A bioinformatics study suggests that many proteins could take an alternative route outside the four established pathways to the thylakoid and one would be using a vesicle transport pathway. By searching for signals housed by cargo proteins in the COPII transport system in the cytosol in chloroplast localized protein several hits were found e.g. LHCP which have been shown to be targeted to the thylakoid by the SRP pathway, and also PSII proteins which have been told to use the spontaneous pathway, and also transmembrane proteins using the SRP and Sec pathway could be transported via the vesicle pathway (Khan et al. 2013). In addition, the NADPH:protochlorophyllide oxidoreductase (POR) enzyme is a protein that requires NADPH and ATP for its correct association with the thylakoid membrane which is not in line with any of the four established thylakoid targeting pathways (Aronsson et al. 2001) but if it could use the vesicle pathway instead remain to be tested. In fact, POR has been found to interact with CPSAR1 during co-immunoprecipitation (in manuscript, C Yin, S Karim, NZ Khan, H Aronsson) which could support usage of vesicle transport for thylakoid targeting. Moreover, the identification of Rab proteins inside chloroplasts **(Paper I, II)** and their possible role in vesicle transport system from budding to fusion by recruiting effector protein during tethering etc. could help to explain the observed results for POR. However, it remains to be elucidated in the future.

5. GENERAL MECHANISM OF VESICLE TRANSPORT

5.1. Cytosolic vesicle transport

The vesicle transport pathways function relies on rounds of vesicle budding and fusion reactions, and those reaction mechanisms are highly conserved from yeast to humans. The budding step occurs when a COP complex assemble on a donor membrane surface. The complex can capture cargo proteins and polymerize into spherical cages thereby deforming the donor membrane into a bud. Eukaryotic cells hold two COP complexes: COPI which buds vesicles from the Golgi apparatus, and COPII which operates at the ER. In addition, a clathrin coated vesicle system is involved in budding from the plasma membrane, trans-golgi network and endosomal compartments (Figure 3) (Bonifacino and Glick 2004).

Thus, clearly vesicles and tubule transport containers move proteins and lipids from one donor membrane o another acceptor membrane using one of the coatamer mechanism (Figure 3). The coated vesicles start with a GTPase activation of a GTPase, e.g. Sar1 or Arf1, at the donor membrane followed by cargo and coat recruitment. Then the coat buds off from the donor membrane and uncoating of the vesicle starts, possibly through GTP hydrolysis. The uncoated vesicle continues towards the acceptor membrane where it is tethered with help of tethering factors. The final steps are then facilitated by SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) to help the vesicle to fuse with the acceptor membrane for delivering of lipids and possible

cargo proteins (Figure 4) (Bonifacino and Glick 2004). To note, uncoating does not necessarily start immediately after budding but can also be found interacting with the fusing machinery (Trahey and Hay 2010). Several COPI, COPII and clathrin components have been identified in the Arabidopsis, although the mechanisms are not been yet been studied in enough detail, unlike in yeast or mammals (Bassham et al. 2008).

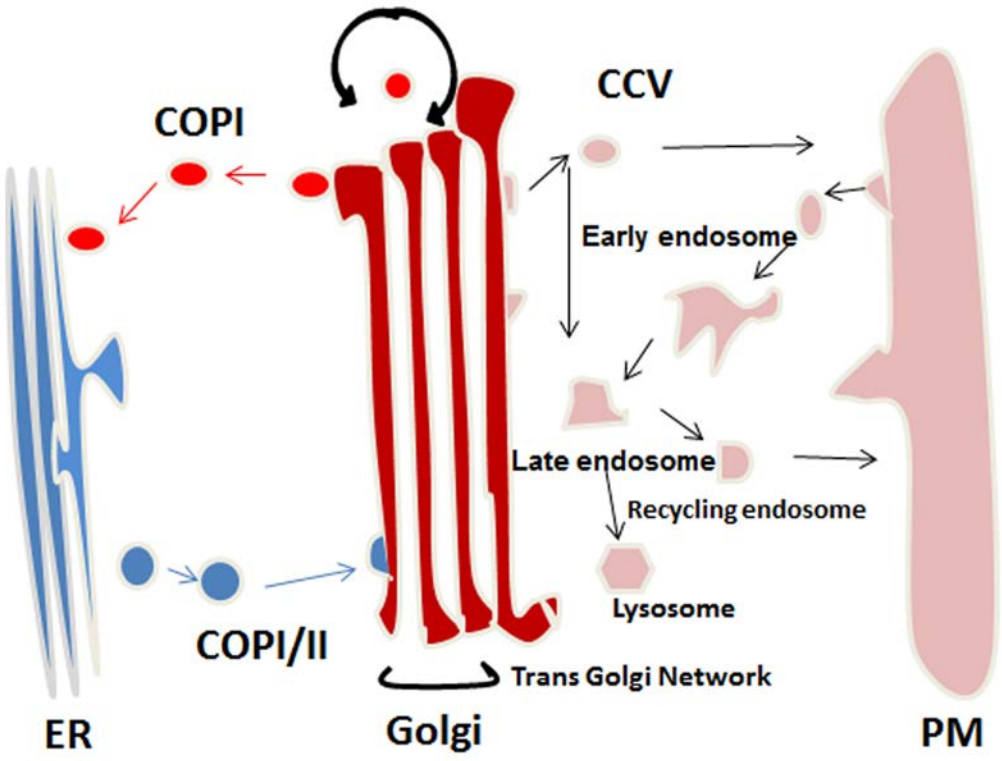

Figure 3. **Overview of cytosolic vesicle transport.** COPI vesicle transport occurs from the Golgi to the endoplasmic reticulum (ER) and from the ER to the Golgi and between the Golgi cisternae. COPII vesicle transport operates in one direction from the ER to the Golgi. Clathrin coated vesicle (CCV) transport take place between the Golgi and the plasma membrane (PM) and in the endocytosis pathway.

5.1.1. COPI vesicle transport

COPI is involved in transport between the ER and the Golgi in both directions e.g. intergrade and retrograde (Figure 3). ARF1 activation is essential for recruitment of a heptomeric COPI complex from the cytosol (Orci et al. 1993). Which consists of two main sub-complexes, the F-COPI sub-complex including several subunits (β, γ, ∂, ζ) and the B-COPI sub-complex also including several subunits (α, β -, ε) (Fiedler et al. 1996). The activation of ARF1 is stimulated by the Sec7 family of guanine nucleotide exchange factors (GEFs) (D'Souza-Schorey and Chavrier 2006). Golgi-associated BFA-resistant

GEF 1 (GBF1), and the known GEF being localized in cis-Golgi, plays an important role in mediating protein transport between the ER and the cis-Golgi (Claude et al. 1999; Kawamoto et al. 2002). Stimulation of GTP hydrolysis of ARF1 to promote coat disassembly is not mediated by a subunit of the coat but by a separate ARF GTPase activating protein (GAP). In yeast, the Golgi-localized GAPs Glo3 and Gcs1 have functions in COPI coat disassembly (Dogic et al. 1999). In Arabidopsis between nine and twelve ARF1 GTPases are encoded (Jürgens and Geldner 2002; Vernoud et al. 2003), whereas eight homologues of ARF-GEFs exist (Anders and Jürgens 2008), and 15 ARF-GAPs (Vernoud et al. 2003).

The sub-complex subunits in general have multiple genes encoding them except for the F-COPI subunits δ-COP and γ-COP. Otherwise there are two isoforms for α-COP, β-COP and ε-COP as well as three for β′-COP and ζ-COP (Bassham et al. 2008). The COPI isoforms might reflect different classes of COPI-coated vesicles. In Arabidopsis two COPI-vesicle populations exist being different in size: the COPI a population derived from the cis-cisternae, and the COPI b population derived from the medial and trans-cisternae. This suggests that the transport from the cis-cisternae to the ER is performed by the COPI a population, and transport from the trans to medial and finally to cis-cisternae is mediated via the COPI b population (Donohoe et al. 2007).

5.1.2 COPII vesicle transport

The first stage in protein secretion from eukaryotic cells is facilitated by COPII vesicles which transport proteins from the rough ER to the Golgi apparatus (Figure 3) (Lee and Miller 2007). The COPII coat is responsible for direct capture of cargo proteins and for the physical deformation of the ER membrane that drives from the COPII vesicle formation (Sato and Nakano 2007). The COPII vesicle formation starts by the activation of the small GTPase Sar1 by a SEC12 protein acting as a GEF (Barlowe and Schekman 1993). This activation causes the recruitment of coated proteins. The budding process is mediated by the coat proteins Sec23 and Sec 24, and cargo proteins is enriched by binding to Sec24 (Bi et al. 2002). Finally, two more coat proteins, Sec13 and Sec31, form the outer layer of the budding vesicle and assist in the invagination of the donor membrane (Lederkremer et al. 2001).

5.1.3. Clathrin coated vesicle transport

There are two major routes for clathrin coated vesicle transport: one from the plasma membrane to the early endosome and the second from the Golgi to the endosome (Figure 3). Clathrin coated components are named light and heavy chain proteins and are collectively called triskelions (Fotin et al. 2004). Adapter proteins (APs) are components of clathrin coated vesicles making up complexes that associates with the TGN and the plasma membrane. There are two kinds of APs: AP-1 is found on the TGN and endosomes, and AP-2 is found on the plasma membrane (Keen 1990). Additional adaptor complexes, AP-3 and AP-4, have been identified. AP-3 and AP-4 are both found on TGN and endosomal membranes, with AP-3 localized more to endosomes and AP-4 more to the TGN (Robinson and Bonifacino 2001).

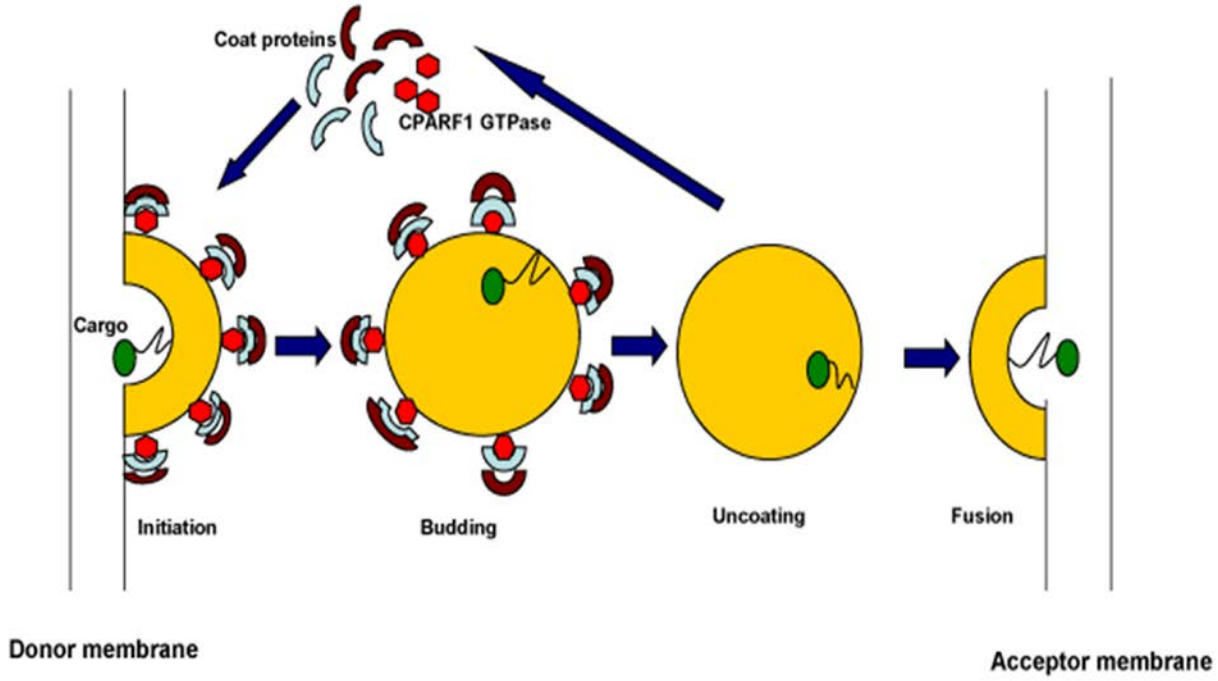

Figure 3. **Vesicle transport from a donor to an acceptor membrane.** Initiation, a GTPase is activated by a GEF, causing it to attach to the membrane and start curvation. Budding, coats are recruited as well as different cargos and it buds from the donor membrane. Uncoating, soon after budding the vesicle loses its coat. Fusion, the uncoated vesicle moves to the acceptor membrane, and becomes tethered to the acceptor membrane by the combination of Rab and a tethering factor, v- and t-SNAREs assemble into a tight bundle and the cargo is transferred to the acceptor membrane. Yellow, vesicle; red, coat GTPase; light blue, first coat complex; brown; second coat complex; green, cargo.

The Arabidopsis genome encodes homologues of APs and triskelions found in mammals and yeast. Plants in general have multiple genes encoding for clathrin coated vesicle proteins except for the AP-2 mu and δ subunits, and AP-3, AP-4 (Bassham et al. 2008). In addition, other partners or accessory proteins are found in association with clathrin coats at the plasma membrane e.g. amphyphysin, epsin, synaptojanin and Eps15. They not only interact with clathrin but also have binding sites for AP adaptors, and for proteins such as the large GTPase dynamin which is involved in the budding step, and even for specialized lipids such as phosphoinositides (PI). The function of many of these proteins are not known yet, but it seems that they are part of a network of complex molecular switches that can regulate various aspects of clathrin-mediated traffic (Kirchhausen 2000).

6. CHLOROPLAST VESICLE TRANSPORT

Vesicles inside chloroplasts were observed at low temperature (Morré et al. 1991). Isolated chloroplasts were treated with specific vesicle fusion inhibitors and thus vesicles

were not observed and so it indicated similarities to the vesicle transport in the cytosol where also vesicle fusion is known to inhibited with the same inhibitors (Westphal et al. 2001). Several chloroplast localized proteins have been predicted to play a role in vesicle formation, fusion, budding, scission etcetera using a bioinformatics study (Khan et al. 2013).

6.1. COPII related transport in chloroplasts

The transport from ER to Golgi is mediated by COPII vesicles (Figure 3). The chloroplast vesicle transport system showed similarity to the cytosolic secretory system (Morré et al. 1991; Westphal et al. 2001) and bioinformatics tools indicates several homologues of COPII e.g. Sec23/Sec24, Sec13/31, Sar1 and RabA5e to exist in chloroplasts (Andersson and Sandelius 2004). Supporting those initial data and expanding the data now also covering candidates for vesicle initiation, budding, tethering and fusion indicates a COPII vesicle transport in chloroplasts (Khan et al. 2013). Many components required for vesicle initiation such as budding are related to COPII but SNAREs and tethering factors predicted for the fusion of the vesicles at the acceptor membrane (in this case the thylakoids) are also similar to the ones facilitating COPI and CCV components during docking and fusion.

6.1.1. CPSAR1

CPSAR1, similar to the small GTPase Sar1 in the cytosol, is confirmed to be localized *both in the stroma and in the inner envelope (Figure 4) (Garcia et al. 2010). CPSAR1* contains a long N-terminal stretch and this stretch was suggested to help in binding to the envelope. CPSAR1 has been shown to be co-localized with vesicles in the stroma at low temperatures, supporting the idea that CPSAR1 has a role in vesicle transport (Garcia et al. 2010) CPSAR1 has been characterized under different names such as CPSAR1 (Garcia et al. 2010), AtObgC (Bang et al. 2009), and AtObgL (Chigri et al. 2009) and has been shown to be important for thylakoid and embryo development. The Obg superfamily, which Sar1 is predicted to belong to, include proteins with unknown functions but also several different functions have been suggested that could be involved in stress response, sporulation and ribosome synthesis (Kobayashi et al. 2001). The highest expression of CPSAR1 occur at the early stages of development (Garcia et al. 2010) and the absence of CPSAR1 gives lethal plants indicating a vital function such as the proposed vesicle transport for maintaining thylakoids (Garcia et al. 2010).

6.1.2. CPRabA5e

Rab GTPase proteins switch between an active GTP bound form and an inactive GDP bound form. Rab GTPase in its active form is reversibly associated with a membrane by a hydrophobic geranyl-geranyl group, which is attached to one or two carboxy-terminal cysteine residues of Rab. This enables the Rab proteins to regulate membrane transport, e.g. in eukaryotic cells Rab proteins are involved in vesicle budding, uncoating and fusion (Stenmark 2009).

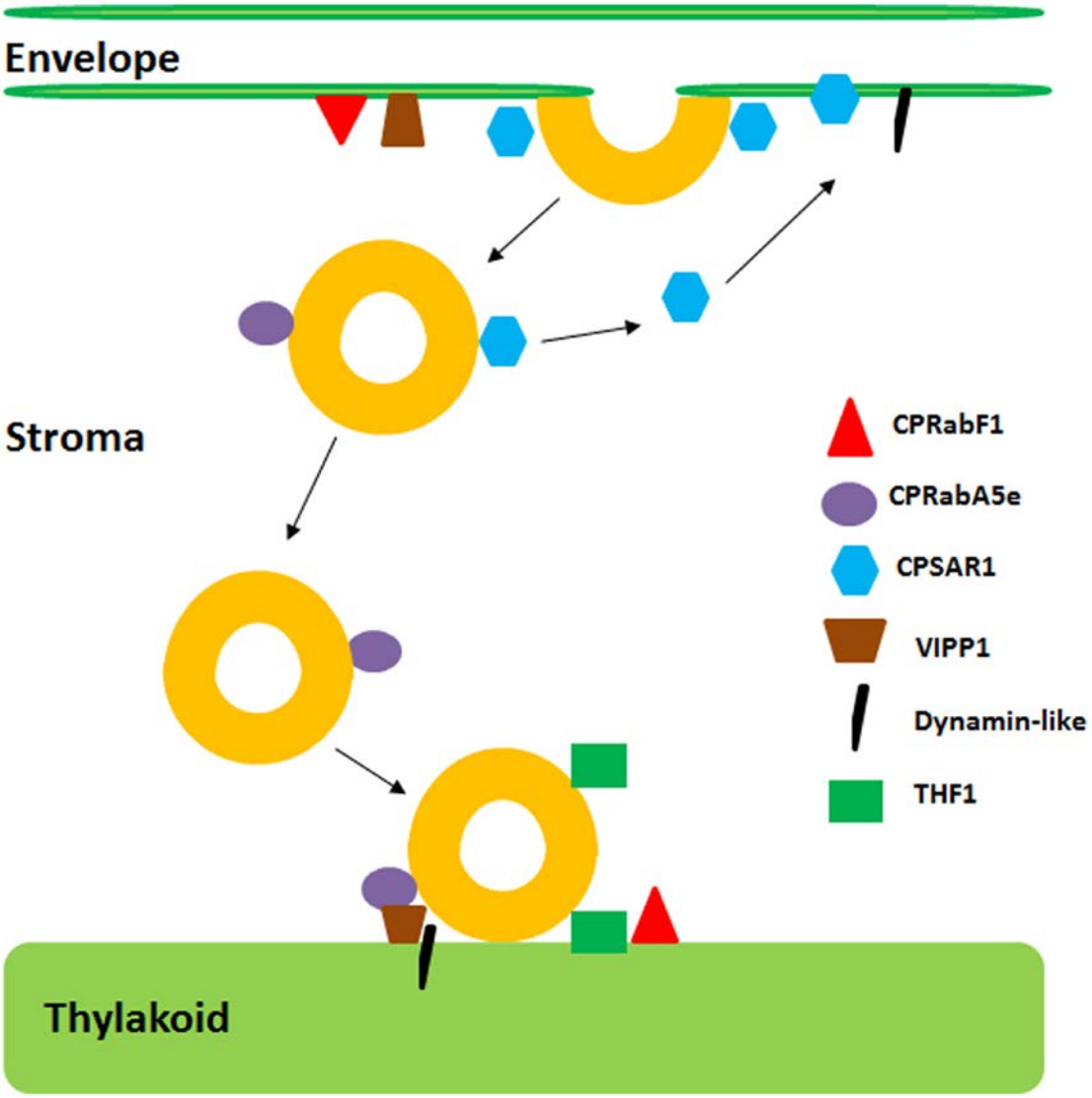

Figure 4. Vesicle components and localization in chloroplasts. Proteins being suggested to be involved in chloroplast vesicle transport, and verified to be chloroplast localized. VIPP1 (brown), dynamin-like proteins (black), and CPRabF1 (red) are all located at the donor envelope and the thylakoids, and could play roles for fission and fusion of vesicles. CPSAR1 (blue) is located at the envelope, the vesicle and the stroma suggesting to help form vesicles, and to recycle back to the envelope for next round. THF1 (green) and CPRabA5e (purple) are both located in the stroma and the thylakoid and could possibly interact with the vesicle before the fusion step and onwards.

Arabidopsis contains 57 different Rab GTPases that are divided into eight distinct subfamilies. The subfamilies are designated A to H, and corresponds to the mammalian Rab GTPase classes 11, 2, 18, 1, 8, 5, 7, and 6, respectively (Table 1) (Nielsen et al. 2008; Rutherford and Moore 2002; Vernoud et al. 2003). Each class is proposed to

regulates distinct paths in the membrane transport system (Pereira-Leal and Seabra 2001). Three Rabs in Arabidopsis are predicted to be within chloroplasts: RabA5e, RabB1c, and RabF1 (Khan et al. 2013). RabA5e been shown to be localized in chloroplasts by immunochemistry and GFP tagging **(Paper I)**. RabF1 has also been localized to chloroplast by immunochemistry **(Paper II)**. These two proteins are therefore designated with a prefix of CP (CP = chloroplast localized) to indicate their localization, i.e. CPRabA5e and CPRabF1. So far no direct experimental data for the third putative chloroplast localized Rab, RabB1c, is present. Initially CPRabA5e was predicted to be an Arf1 protein (Andersson and Sandelius 2004), but due to the presence of typical Rab characteristics e.g. cysteine residues at the C-terminal and Rab domain CPRabA5e was predicted as typical Rab GTPase. Moreover, support for its link to Rab also comes from the fact that CPRabA5e cannot complement the yeast Arf1 homologues, but can complement the yeast RabA5e GTPase homologues Ypt31/Ypt3. This indicates not only that CPRabA5e can work as a Rab GTPase but also implement a possible role in vesicle transport since Ypt31 and Ypt32 are involved in vesicle transport in yeast **(Paper I)** (Segev 2001).

Table 1. Arabidopsis subfamilies of Rab proteins and the relation to mammalian and yeast Rab classes.

Arabidopsis subfamily	Mammalian and yeast RAB GTPase classes	Number of Arabidopsis Rab proteins in each subfamily
RAB-A	RAB-11/YPT31/32	26
RAB-B	RAB-2	4
RAB-C	RAB18	3
RAB-D	RAB-1/YPT1	4
RAB-E	RAB-8/YPT2	5
RAB-F	RAB-5/YPT5/10	3
RAB-G	RAB-7/YPT7	8
RAB-H	RAB-6/YPT6	3

CPRabA5e is localized in thylakoids and in the stroma but not in the envelope membranes (Figure 4). The activity of Rab GTPases is also to regulate downstream effector molecules. Results in **Paper I** supports the idea that its soluble inactive form is in the stroma but its active GTP-bound form at the thylakoid membrane, similar in action to other Rab GTPases switching between a soluble and membrane phases **(Paper I)** (Grosshans et al. 2006). The Rab can function in close connection with tethering factors and SNAREs, e.g. tethering factors can work as a Rab GEFs or Rab effectors to regulate downstream reactions (Cai et al. 2007). Bioinformatics predicted several putative chloroplast localized tethering factors e.g. COG complex, ExoH70 and AtCASP (Khan et al. 2013) which then could work as Rab effectors (Grosshans et al. 2006). However, in yeast the tethering complex called TRAPP works as a GEF for Ypt31/Ypt32 (Jonas-Straube et al. 2001), and no TRAPP or GEF were found for Rab in the chloroplast (Khan

et al. 2013). How Rab GTPases located in chloroplasts are activated remains to be investigated.

Chloroplasts from *cprabA5e* mutants pre incubated at 4ºC had more vesicles close to the envelope membranes compared to wild-type chloroplasts **(Paper I)**. Under oxidative stress and still pre incubated at 4ºC, more vesicles were observed in mutants than it was in wild-type chloroplasts indicating that vesicles had problems to fuse at the thylakoid membrane. The sizes of plastoglobuli, that can store thylakoid membrane, are slightly increased in *cprabA5e* mutants which could infer a role of Rab for maintenance of the thylakoid membrane by regulating the plastoglobuli **(Paper I)**. The ultrastructure analysis of mutant chloroplasts indicated that CPRabA5e might have a link to vesicle transport and thylakoid structure. In yeast and mammals the Rab homologues work with post-Golgi transport by helping tethering factors and SNAREs during fusion, and to interact with the cytoskeleton to facilitate vesicle motility. If CPRabA5e also fulfill these functions or has evolved different roles in chloroplasts is still to be elucidated.

CPRabA5e also has a putative role in seed germination and oxidative stress **(Paper I)** as the germination efficiency in *cprabA5e* mutants was delayed and mutants showed a more vulnerable effect to Rose bengal, a chemical mirroring oxidative stress. The highest gene expression of CPRabA5e according to Genevestigator is at the germinated seed stage **(Paper III)** which could explain the effect seen at this stage. Furthermore, study to confirm the role of CPRabA5e in other stresses and the effect on photosynthesis activity during cold and light senescence were tested without any significant outcome A yeast-2-hybrid screen using CPRabA5e as the bait found 13 interacting proteins localized in chloroplast, five of them involved in stress responses, and seven were found to be closely linked to the light reactions of the photosynthesis, and three were involved in Calvin cycle related processes **(Paper I)**.

6.1.3. CPRabF1

RAB5 GTPase is involved in regulators of endosomal membrane transport in yeast, plants, and animals. RAB5/ARA6-like proteins could have different functions in spite of the high similarity between characean green algae that is a close relative of land plants, and flowering plants (Hoepflinger et al. 2013). In Arabidopsis the RAB5 group includes RHA1 (RABF2a), ARA7 (RABF2b), and ARA6 (RABF1) (Nielsen et al. 2008). In chloroplasts several putative components involved in the vesicle transport have been identified using a bioinformatics approach, one of them being RabF1 also known previously as ARA6. ARA6/RabF1 has previously been shown to be in the cytosol and to have an important role in the cytosolic vesicle transport system. Being predicted to be in chloroplasts experiments were performed to find and detect localization of RabF1 inside chloroplast. By immunochemistry CPRabF1 was determined to be localized to the envelope membrane and the thylakoid membranes but not being present in the stroma fraction (Figure 4). Knockout mutants for CPRabF1, *cprabF1-1* and *cprabF1-2*, were tested for possible visible phenotypes versus wild-type plants by using different stress condition but so far no clear phenotype has been observed **(Paper II)**.

The gene expression pattern according to Genevestigator is rather similar for CPRabF1 compared to CPRabA5e, being between 20-60% for most developmental stages. CPRabF1 have its highest gene expression at the senescence stage being between 67-83% and CPRabA5e has instead its highest gene expression at the germinated seed

stage (Paper III). If CPRabF1 has a role in senescence is currently not known but it is interesting to note that one gene being co-expressed with *CPRabF1* according to the public available co-expression database ATTED-II (http://atted.jp) is a protein involved in autophagy called autophagy 3 (APG3) (unpublished observation, M Alezzawi, Aronsson H). Autophagy involves degradation of chloroplasts and if CPRabF1 is involved autophagy it could be either at the cytosol localization or at its chloroplasts localization.

6.2. COPI related transport in chloroplasts

A study was performed to find possible involvement of COPI proteins for fusion and docking, and for proteins transport from the envelope to the thylakoids and vice versa using a COPI related mechanism. Previous results raise the probability that a COPII type vesicle transport system exists in the chloroplast thus the question remained to be answered if also a possible COPI type vesicle transport system exist. A bioinformatics approach was used but no strong evidence for a COPI based vesicle transport was observed **(Paper IV).** According to the prediction tools used indeed several COPI components were localized in chloroplasts but several had already been shown to have other functions not related to vesicle transport and some were with unknown function that could indicate a role in vesicle transport but overall there was to many components not identified to be able to support a COPI vesicle transport system in chloroplasts **(Paper IV)**. In the performed study, eight proteins were found to be similar to the β' subunit of the B-COPI, and two proteins were found to be similar to the F-COPI subcomplex ζ, four being similar to the Coat GTPases, and two proteins had common occurring domains similar to F-COPI γ and F-COPI coat δ, respectively **(Paper IV)**. Even if the results were negative one has to remember that even if a COPI related vesicle transport is not supported by looking at similarity it can still be a vesicle transport not only relying on a COPII related transport system but could involve components being totally different form the known ones in the cytosol. Indeed, some suggested components for vesicle transport in chloroplasts are not having clear vesicle transport homologue in the cytosol (Figure 4).

6.3. Clathrin related transport in chloroplasts

Just as for COPI the question is asked if there could be a CCV related system inside chloroplasts since a COPII system has been suggested. Clathrin triskelion consists of heavy and light chain proteins, and one protein was identified for each of the proteins. Five proteins similar to the γ subunit of the AP1 complex were found and five proteins similar to the β2 subunit were found for the AP2 complex. For AP3 and AP4 only one hit each were found with common occurring domains. In addition, three other proteins shared a common occurring domain for almost all AP1-4 protein subunits **(Paper IV)**. Both COPI and CCV if working as in the cytosol would infer that transport would occur not only in one direction but in two directions i.e. also from the thylakoid to the envelope. This kind of transport could e.g. involve transfer of pigments as chlorophyll is known to be degraded not in the thylakoids but in the envelope. However, if vesicle transport is involved is yet not known. But it is interesting again to note that chlorophyll breakdown is strongly linked to senescence i.e. when autophagy also occurs.

6.4. Other proteins related to vesicle transport

6.4.1. Vesicle inducing protein in plastids 1 (VIPP1)

VIPP1 is believed to play a role in thylakoid biogenesis and is conserved in photosynthetic organisms (Zhang et al. 2012). VIIP1 is involved in vesicle budding at the inner envelope of the chloroplast, and transport of cargo proteins from the inner envelope membrane to thylakoids (Figure 4). VIIP1 has also been suggested to be involved in reorganization of the structure of the thylakoids and to facilitate protein transport of the Tat pathway (Lo and Theg 2012). VIPP1 form a ring-like structure on the inner envelope to bind to the membrane (Aseeva et al. 2004; Otters et al. 2013), and this structure could facilitate vesicle fission similar to a dynamin GTPase (Vothknecht et al. 2012). However, VIPP1 is not suggested to be required for lipid accumulation (Kroll et al. 2001). That opens up the possibility that VIPP1 might be involved in transport of proteins as a cargo from the inner envelope membrane to thylakoid. VIPP1 has been found in samples when using mass spectroscopy for analysis of interactors with TIC and TOC components. Thus, an actin-TOC-TIC-VIPP1 complex was suggested and could provide a means of channeling cytosolic stuff to the thylakoid membranes (Jouhet and Gray 2009). In addition, using a bimolecular fluorescence complementation system indicated involvement with Hsp90 in chloroplast biogenesis i.e. Hsp90 may help in the disassembly of VIPP1 for thylakoid membrane formation and/or maintenance (Feng et al. 2013). Thus, VIPP1 could be involved in several functions linked to vesicle transport.

6.4.2. Dynamin-like proteins

Dynamins is a large group of GTPases being involved in many processes including budding of transport vesicles, division of organelles also involved in the scission of vesicles, and for organelles they play roles in fission, fusion and cytokinesis (Praefcke and McMahon 2004). The chloroplast has been indicated to be the compartment for some dynamin-like proteins. Dynamin-like proteins identified in Arabidopsis chloroplasts are FZL (Fuzzy-onions-like protein). FZL, is both in the inner envelope membrane and the thylakoid membrane and is important for chloroplast morphology. FZL may function in membrane fusion (Figure 4) (Gao et al. 2006) as well as vesicle transport from the envelope membrane (Park et al. 1998). Another dynamin–like protein 1 (ADL1a) has been suggested to be localized in thylakoid membranes and involved in thylakoid biogenesis (Park et al. 1998). In addition, ADL2a has been shown to be involved in vesicle formation, particularly the vesicle scission at the chloroplast inner envelope membrane (Figure 4) (Kim et al. 2001).

6.4.3. Thylakoid formation 1 (THF1)

Thylakoid Formation 1 (THF1) protein controls PSII–LHCII dynamics during dark-induced senescence and light acclimation suggesting that THF1 is required for dynamics of PSII–LHCII supramolecular organization in higher plants and involved in chlorophyll degradation during senescence (Huang et al. 2013). THF1 is important for leaf and chloroplast development by maintaining thylakoid stacks. It has been shown to be localized in the envelope, and the stroma (Figure 4) (Wang et al. 2004). In THF1 knockout plants the non-green areas in leaves accumulate vesicles when lacking thylakoids, suggesting that vesicle fusion does not occur due to the absence of an organized thylakoid structure (Wang et al. 2004).

7. CHLOROPLAST AUTOPHAGY

Autophagy seems important during leaf senescence and nutrient recycling (Thompson and Vierstra 2005) and degradation of e.g. organelles in plants is mediated mainly by autophagy. Autophagy plays an important role in nitrogen remobilization and seed filling (Guiboileau et al. 2012). However, it can also play a role in degradation of oxidized proteins during oxidative stress, and removal of damaged proteins and organelles during normal growth conditions (Bassham 2007). Autophagy is usually divided into two major pathways, microautophagy and macroautophagy (Bassham et al. 2006). Microautophagy occurs in some species during seed germination for degradation of starch granules and storage protein in vacuoles, whereas macroautophagy functions in suspension cultures and whole plants in response to sucrose and nitrogen starvation. Also important during senescence as described for several plant species (Hanaoka et al. 2002) since chloroplasts contain about 80% of the total leaf nitrogen is important for recycling of nitrogen during leaf senescence (Wada et al. 2009). While degradation of the cytosol and organelles in plants is mediated by autophagy, its role in chloroplast catabolism is yet not understood. Data accumulated indicates a role of autophagy during senescence, and degradation of stromal proteins requires autophagy-dependent processes. However, stromal protein degradation also relies on autophagy-independent pathways which could be proteolysis within the chloroplast (Lee et al. 2013). Rubisco, a stroma localized protein, is degraded during leaf senescence, and released nitrogen from Rubisco can be recycled to growing organs and developing seeds (Friedrich and Huffaker 1980). Rubisco and chloroplasts are both degraded in the vacuole, which contains more than 95% of a cell's proteolytic activity against Rubisco (Lin and Wittenbach 1981). Rubisco can be mobilized to the vacuole via Rubisco-containing bodies (RCBs) by autophagy. They are located in the cytoplasm and occasionally in the vacuole of naturally senescent leaves of wheat (*Triticum aestivum).* Similar structures were also confirmed as RCBs but instead called senescence-associated vacuole (SAV) in senescent leaves of soybean (*Glycine max*) and Arabidopsis (Otegui et al. 2005). If the observed bodies also include known vesicle transport components or vesicle like features will be interesting to evaluate in the future.

8. CONCLUSION AND FUTURE PERSPECTIVES

It is obvious that in chloroplasts many (putative) proteins are indicated to be involved in vesicle transport (e.g CPSAR1, VIPP1, THF1, FZL, CPRabA5E and CPRabF1 etc.) being important for e.g. thylakoid biogenesis and maintenance through the transport of lipids or proteins from the envelope to the thylakoid as well as involved in many developmental and physiological processes in Arabidopsis. These led us to think about the genetic and biochemical mechanism of the protein components involved in chloroplastic vesicle transport and how they are involved in different steps from vesicle budding, coating, tethering, fusion and finally to delivery. In this work we tried to build up the notion of a functional vesicle transport system inside the chloroplast very similar to the cytosolic system. Through scrutinizing the bioinformatics data we managed to identify and characterize two novel small GTPases Rab proteins in Arabidopsis for their role in vesicle transport and plants development **(Paper I, II)**. The first Rab CPRabA5E was shown to be localized in stroma and thylakoids and experimentally demonstrated its

role, for altering thylakoid structure, plant development, and stress response. It confirmed its role in vesicle transport by complementing the yeast Rab homologues those were involved in vesicle transport as well as by showing the *cprabA5e* mutants were affected for vesicle formation in the chloroplasts **(Paper I)**. The second Rab (CPRabF1) was also identified inside the chloroplast envelope and thylakoids and most likely to play a role in vesicle transport. However, CPRabF1 was previously characterized for its involvement in vesicle transport in the endosome. Thus we predicted that its localization in chloroplasts might indicate dual targeting of CPRabF1. So far no phenotype was observed in the *cprabF1* mutants under the treatments of different conditions e.g. high light and osmotic stress **(Paper II)**. Based on the results from a previous bioinformatics study to predict several Rab related proteins inside chloroplasts linked to a suggested COPII vesicle transport system we also predicted the gene expression for the Rab related genes at several developmental stages covering the life span of Arabidopsis e.g. from seedlings to senescence. The data indicated a rather homogenous expression pattern among the genes studied being around 20-60% expressed for all developmental stages except for senescence where the expression pattern was more discrete indicating less need for vesicle components at senescence stage when chloroplast degradation is more evident **(Paper III)**. Moreover, the presence of a putative COPII vesicle system inside chloroplast encouraged the work to study the possibility of a putative COPI or clathrin coated vesicle (CCV) system in chloroplasts. However, in that work a bioinformatics approach found several homologues of cytosolic COPI and CCV related vesicle transport components inside chloroplast distinctive for specific functions other than vesicle transport or unknown functions without having any clear link to vesicle transport in chloroplast **(Paper IV)**. Thus, thesis has shown more possible components linked to a putative COPII system involved in vesicle transport of proteins and lipids inside chloroplast similar to the cytosolic vesicle transport system.

The following aspects and methods can be studied for the future clarification and to answer demanding questions to decipher the intriguing riddle that lies behind the vesicle transport within the chloroplast. It will be interesting to continue this work for further development of knowledge of the components involved as well as the real mechanism of this transport system. We need to know the knowledge behind the lipid components involved and possible cargo proteins and the phase when vesicles appear. Moreover, the interaction among the components involved in chloroplast vesicle transport will answer many questions. To know all these answers one has to examine the aspects carefully by performing thorough molecular and biochemical approaches such mutant analysis, gene expression and proteome analysis, protein-protein interaction studies as well as fluorescent based localization studies of the components and ultramicroscopic analyses for their functions and pinpoint localization.

9. ACKNOWLEDGEMENT

All praise to the Almighty Allah. Over the past years during my time at Botan there are many people who have helped me and encourage me for my study and research. I wish to extend my gratitude to them. First of all thanks for the excellent guidance and support given to me by my supervisor Associate Professor Henrik Aronsson. Thank you for your endless enthusiasm, regarding science in general and vesicle transport system in particular. In our Wednesday's group meetings you have encouraged me in scientific discussions, and in journal club helping me to develop my scientific thinking. Thank you for all your support in editing and proofreading posters, manuscripts and my thesis.

Special thanks for my examiner Professor Adrian Clarke, and my second supervisor Professor Olof Olsson for your insightful comments and during annual meetings.

I would also like to thank all collaborators directly involved in my work; especially Dr Sazzad Karim for lab-supervision, providing useful hints and advice and for fruitful discussions and proof reading of my thesis, and Dr Nadir Zaman Khan for helping me a lot during the years! I wish to say for both of you - bark Allah fek!

Many thanks to all members in our group for great help and joyful time throughout my time here - Emelie Lindquist, Congfei Yin, and Milton Karlsson as well as previous lab members Christel Garcia and Patrik Boij.

During my time at the department I also received valuable information and assistance from Anders Tryggvesson, Dr Anders Nilsson, Dr Azeez Bebo, Oskar Johansson, Andrei Herdean and Kemal Sanli, thanks for everything!

Thanks to Professor Cornelia Spetea Wiklund and Dr Mats X Anderson for all answers to my questions, and to Dr Bernard Pfeil for useful comments of my thesis. To all the staff at the Department of Biological & Environmental Sciences, in Gothenburg University, from whom I received valuable support throughout the years, I would like to extend my special thanks. In particular I would like to thank Sven Toresson for your help with lots of different things (to many to mention) and Niclas Siberg for computer support.

Finally thanks to all the funding sources making it possible to run lab work and go for conferences etcetera – the Libyan Higher Education, the Adlerbertska Foundation, the Olle Engkvist Foundation, the Carl Tryggers Foundation, the Swedish Research Council, the Royal Society of Arts and Sciences in Gothenburg, the Theme Life Science.

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11. POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Kloroplasten fångar in solljuset med hjälp av olika fotosystem. Dessa sitter inne i kloroplasten i speciella membraner, tylakoider, som bl.a. består av lipider och proteiner. Dessa delar tillverkas på andra ställen i cellen och måste transporteras till tylakoiderna oftast via det membran som omsluter kloroplasten, envelopet. Mellan envelopet och tylakoiderna finns en lösning, stroma, som utgör ett hinder att ta sig direkt till tylakoiderna. Små blåsor, vesiklar, kan bidra till transport av lipider vilket stöds av experiment och mikroskopstudier. Eventuellt kan även protein färdas med dessa vesiklar men det är inte bevisat ännu. Det finns redan fyra andra färdvägar identifierade för proteiner till tylakoiderna. Men vesikeltransport i andra delar av celler transporterar både lipider och proteiner så det kan eventuellt också förekomma i kloroplaster.

Denna avhandling har visat likheter mellan vesikeltransport i kloroplaster och den som sker i cytosolen. Ett Rab protein, CPRabA5E (CP = kloroplastlokaliserat), har hittats i kloroplaster hos modellväxten Arabidopsis (backtrav på svenska). CPRabA5e verkar vara viktig för strukturen hos tylakoider, utvecklingen av växten, och vid oxidativ stress. CPRabA5e visade sig kunna ersätta ett liknande protein i jäst som är involverat i vesikeltransport och växter utan CPRabA5e påverkade bildningen av vesiklar. Ett annat Rab, CPRabF1, har vi också hittat i kloroplaster som skulle kunna spela en roll i vesikeltransport. CPRabF1 har tidigare visat sig medverka i vesikeltransport i cytosolen och att den nu hittades i kloroplaster kan tyda på att den kan färdas till två olika ställen i cellen. Inge tydlig skillnad syntes hos växter som saknade CPRabF1 trots försök under olika stressbetingelser ex. hög ljusintensitet och osmotisk stress.

Tidigare data har förutspått flera Rab-relaterade proteiner inuti kloroplaster som skulle vara kopplade till ett vesikeltransportsystem som finns i cytosolen, COPII systemet. Vi analyserade uttrycket för dessa Rab relaterade gener i flera stadier under Arabidopsis livscykel ex. från frö till åldrande. Det vara ett ganska likartat uttryck för de studerade proteinerna, ca 20-60 % uttryck via alla stadier utom vid åldrandet där uttrycket var mer varierat. Vid åldrande bryts kloroplaster ner och det indikerar minskat behov av protein för vesikeltransport. Idén om ett COPII relaterat vesikelsystem inuti kloroplaster bidrog till att vi ställde frågan om också ett COPI- och klatrinrelaterat vesikelsystem finns i kloroplaster. Våra bioinformatikstudier hittade flera möjliga proteiner i kloroplasten som skulle kunna ha liknande funktion som dessa i cytosolen som tillhör COPI eller klatrinsystemet. Men många av dessa proteiner hade redan en tydlig funktion annan än vesikeltransport eller hade en okänd funktion. Dessutom kunde många av de nödvändiga proteinerna för att bygga ett funktionellt COPI och klatrin vesikelsystem inte ens identifieras i kloroplasten. Vi konstaterar att vesikeltransport i kloroplasten inte har stora likheter med ett COPI eller klatrin vesikelsystem, snarare är det mer kopplat till ett COPII vesikelsystemet som nyligen föreslagits.