UNIVERSITY OF GOTHENBURG

Vesicle Transport with Emphasis on Chloroplasts

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Abstract: The plants on which we depend for food and oxygen need photosynthesis to prepare their own food. Photosynthesis takes place in the chloroplast. Inside chloroplasts is a specialized membrane called the thylakoids in which the photosynthesis activity takes place. The thylakoid membrane does not produce its own lipids, so instead they are transported from the envelope membrane to the thylakoid. Similarly, most of the proteins needed for maintenance of the photosynthetic apparatus and thylakoids are imported into the chloroplast across the envelope membrane and transported to the thylakoid. The lipids produced in the envelope membrane were suggested to be transported in three ways; through diffusion, through contact between thylakoids and the envelope or with the help of vesicles. The thylakoids and envelope membrane are well separated from each other by an aqueous solution, the stroma, which makes it hard for the lipids to move between the two compartments. Biochemical and ultrastructure data show vesicle transport inside chloroplasts. One of the vesicle functions in chloroplasts is to transport lipids from the envelope to the thylakoid to maintain its membrane structure.

Proteins transported from the envelope to the thylakoids take four routes (Sec, Tat, SRP and Spontaneous pathways). Only a few proteins have been shown or hypothesized to follow these pathways. For many proteins it is unclear how they are transported to the thylakoids. It has been shown that vesicle transport in the chloroplast is similar to the cytosolic secretory system, which transports both lipids and proteins between different compartments in the cytosol. This hypothesis became more likely when putative protein components of the COPII transport pathway i.e. Sec23/Sec24, Sec13/Sec31 and Sar1 (which operate between the endoplasmic reticulum and the Golgi apparatus) were suggested to exist in chloroplasts.

This thesis reports that indeed vesicle transport inside the chloroplast is similar to that of the cytosolic secretory system. The Sar1 homologue CPSAR1 (CP = chloroplast localized) has been characterized and shown to be important for embryo development and thylakoid biogenesis. Other studies have already shown that proteins such as VIPP1, THF1, ADL and FZL in the chloroplast do have an impact on vesicle transport and are also involved in thylakoid maintenance and biogenesis. This gives an indication that CPSAR1 could be involved in vesicular transport as well as collaborating with these proteins. Indeed it has been shown that CPSAR1 is found in low temperature induced vesicles and there are indications that CPSAR1 and THF1 may interact. CPSAR1 could be involved in several functions. Previous data shows its involvement in ribosome biogenesis, which is also indicated by genes co-expressed with CPSAR1 (on the publically available ATTED-II database) that have roles in protein synthesis.

If there is a functional vesicle transport system in chloroplasts we expect there to be more components that are similar to vesicle transport in the cytosol. A bioinformatics approach predicted components like tethering factors, SNAREs, Rab GTPase, etc., to be present in chloroplasts. It was also proposed that the transport of cargo proteins in vesicles from the envelope to thylakoids would occur in a similar way to the secretory system in cytosol.

One of the Rab GTPases, CPRabA5e has been found in the chloroplast and is localized in the stroma and thylakoids. It has been suggested that it binds to the thylakoid in its active form and has a role in vesicle tethering and fusion similarly to its homologue in yeast. Ultrastructure analysis of CPRabA5e mutant chloroplasts shows accumulation of vesicles at low temperature compared to wild type indicating a role in vesicle transport. Furthermore, CPRabA5e has been shown to have a role in seed germination, oxidative stress and maintaining the size of plastoglobuli.

There has been clear evidence of vesicle transport inside chloroplasts and this transport is related to the secretory system in the cytosol. Two proteins in the chloroplast similar to proteins found in the secretory system are CPSAR1 and CPRabA5e, whose roles have been further characterized in chloroplast vesicle transport. At the same time other predicted components need confirmation of their localization. Finally, the cargo protein transport using vesicles need experimental verification to fill the model of vesicle transport inside chloroplasts.

Keywords: cargo, chloroplast, CPRabA5e, CPSAR1, lipid, protein, transport, vesicle

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To our kids
Varda and Mazin

Vesicle Transport with Emphasis on Chloroplasts

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

(I) Garcia C, **Khan NZ**, Nannmark U, Aronsson H. (2010) The chloroplast protein CPSAR1, dually localized in the stroma and the inner envelope membrane, is involved in thylakoid biogenesis. *Plant Journal*, *63*, 73-85.*

(II) **Khan NZ**, Garcia C, Aronsson H. (2010) Genes co-expressed with CPSAR1 identified using ATTED-II. *Plant Signaling & Behavior*, *5*, 1141-1143.*

(III) **Khan NZ**, Lindquist E, Aronsson H. (2013) New putative chloroplast vesicle transport components and cargo proteins revealed using a bioinformatics approach: An *Arabidopsis* model. *Submitted.*

(IV) Karim S, Alezzawi M, Garcia-Petit C, **Khan NZ**, Solymosi K, Lindquist E, Dahl P, Hohmann S, Aronsson H. (2013) A novel chloroplast localized Rab GTPase protein CPRabA5e involved in stress, development, thylakoid biogenesis and vesicle transport in Arabidopsis. *Manuscript.*

Publications not included in this thesis:

Sjögren LLE, Tanabe N, **Khan NZ**, Rodermel SR, Aronsson H, Clarke AK (2013) Characterization of the chloroplast molecular chaperone ClpC/Hsp93 in Arabidopsis. *Submitted.*

Kasmati AR, Töpel M, **Khan NZ**, Patel R, Ling Q, Karim S, Aronsson H, Jarvis P. (2013) Evolutionary, molecular and genetic analyses of Tic22 homologues in Arabidopsis thaliana chloroplasts. *Submitted.*

Khan NZ, Garcia-Petit C, Aronsson H. (2013) Understanding the chloroplast vesicle transport phenomenon, from a secretory pathway perspective. Review. *Submitted.*

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Abbreviations

ADL	Arabidopsis dynamin-like
AP	Adopter protein
ARF	Adenosine diphosphate ribosylation factor
ATP	Adenosine triphosphate
ATTED-II	Arabidopsis thaliana trans-factor and cis-element prediction database II
CCV	Clathrin coated vesicle
COPI/II	Coated protein I/II
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
ER	Endoplasmatic reticulum
FZL	Fuzzy-onions like
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
LHCP	Light harvesting complex protein
MGDG	Monogalactosyldiacylglycerol
Obg	SpoOB-associated GTP-binding protein
PA	Phosphatidic acid
PC	Phoshatidylcholine
Rab	Ras-related 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
THF1	Thylakoid formation 1
TIC	Translocon at the inner envelope membrane of chloroplasts
TOC	Translocon at the outer envelope membrane of chloroplasts
TRAPP	Transport protein particle
VIPP1	Vesicle inducing protein in plastids 1
Ypt	Yeast protein transport

1. INTRODUCTION

Plants are the backbone of our daily life, which affect our life directly or indirectly. We grow plants not only to increase the beauty of our surroundings, but because without them we will not survive. Plants are the main source of our food, they protect and fertilize the soil, many are used in medicine, in making clothes and producing energy. Plants are also important for animals, for which plants are their primary source of food and also used for shelter. We breathe just because of plants, because they not only clean up the environment from CO_2 but also produce O_2 while making food for them as well to survive. Briefly, the plants take up CO_2 and in the presence of water and sunlight produce the food to survive and release oxygen to the environment – a process known as photosynthesis. If we as human beings want to get all the benefits from plants, they have to perform photosynthesis efficiently. For this process plants have a specialized organelle which is not found in animals, called the chloroplast.

The chloroplast is protected by two envelope layers; inside the chloroplast is an aqueous environment housing the stroma and thylakoids. Photosynthesis takes place in thylakoids and most of the lipids and proteins that build up thylakoids and the photosynthetic apparatus are transported from the envelope membrane. Although it seems straightforward, actually it is not. The hindrance between the envelope and thylakoids is the stroma, which, as an aqueous environment, will make hydrophobic molecules such as lipids and insoluble proteins face difficulties in moving to the thylakoids. For transport of proteins to the thylakoids four pathways has been proposed: the Signal Recognition Particle (SRP) pathway, the Secretory (Sec) pathway, the Twin Arginine Translocation (Tat) pathway and the spontaneous pathway (Jarvis and Robinson 2004; Keegstra and Froehlich 1999). For lipid transport to the thylakoid there are different theories but the most likely scenario so far supported by the literature is vesicle transport (Andersson et al. 2001; Morré et al. 1991b).

Chloroplast vesicle transport is similar to the secretory system (Morré et al. 1991a; Räntfors et al. 2000; Westphal et al. 2001) in the cytosol. Some putative candidate proteins have been identified through a bioinformatics approach that are related to COPII-type vesicle transport in the secretory system (Andersson and Sandelius 2004).

The aims of this thesis are to (1) explore the vesicle transport system inside the chloroplast using *Arabidopsis thaliana* as a model plant and (2) characterize the components suggested as being involved in vesicle transport related to the secretory system in the cytosol. In Paper **I** we characterized the COPII GTPase Sar1 (Secreted-associated and Ras-related 1) homologue CPSAR1 (CP = Chloroplast). In paper **II** possible co-expressed genes with CPSAR1 were identified by using the public available database ATTED-II to elucidate putative roles of CPSAR1 by identifying possible partner proteins. In Paper **III**, a renewed bioinformatics approach was used to explore if there are more components involved similar to the secretory vesicle transport, if so, then whether there is any possibility for chloroplast vesicles to transport cargo proteins between envelope and thylakoids. In paper **IV** we characterized CPRabA5e (Rab= Ras-

related in brain), homologue of Ypt31/32 (Yeast protein transport 31/32) GTPases to see its role in chloroplasts and in vesicle transport.

2. PLASTIDS

Plastids are found in plant and algal cells and is one of the features that differentiate them from animal cells. Some plastids contain pigments that can harvest light energy that then can be converted into stable chemical energy by incorporating CO_2 into carbohydrates, such as starch. Plastids are surrounded by a double envelope membrane layer and were derived from cyanobacteria through an endosymbiotic event into plant and algae cells. Primary plastids, which evolved from the direct engulfing of photosynthetic cyanobacteria by the eukaryotic host, have two membranes, which is comparable to the outer and inner envelope of cyanobacteria, and are found in algae and plant cells. Secondary plastids, which evolved by the engulfing of primary plastids already present in algae by other eukaryotes, are surrounded by more than two membranes, and are found in plankton such as diatoms and dinoflagellates (Figure 1A) (Chan and Bhattacharya 2010; Keeling 2004).

In higher plants plastids are divided into different groups based on their pigment content, structure and developmental stage (Figure 1B) (Solymosi and Keresztes 2012; Wise 2006).

1) Proplastids are colorless and found in meristematic tissues. They have variable shapes and contain starch granules and lamellae. All other plastids develop from proplastids.
2) Chloroplasts are the most prominent member of the plastids, contain chlorophyll (which gives them the green color) and are specialized for photosynthesis having a thylakoid membrane.
3) Chromoplasts are red, orange or yellow in color and are found in fruits and petals. The colors are due to carotenes and xanthophyll pigments. Chromoplasts are derived from chloroplasts.
4) Etioplasts are found in leaves when grown in darkness. They are yellow in color and are converted to chloroplasts when exposed to light, a process called greening. The yellow color comes from carotenoids.
5) Leucoplasts are colorless and can be divided to subgroups e.g. amyloplasts containing starch and found in storage tissues, elaioplasts filled with oil and found in epidermal tissues, and proteinoplasts that store proteins. They are located in roots and other non-photosynthetic tissues.
6) Desiccoplasts are found in desiccation-tolerant plants upon dehydration. They contain several, large plastoglobuli and only few thylakoids arranged often in concentric vesicle layers.
7) Gerontoplasts appear only in senescent cells as a result of plastid aging and always develop from mature, senescing chloroplasts.

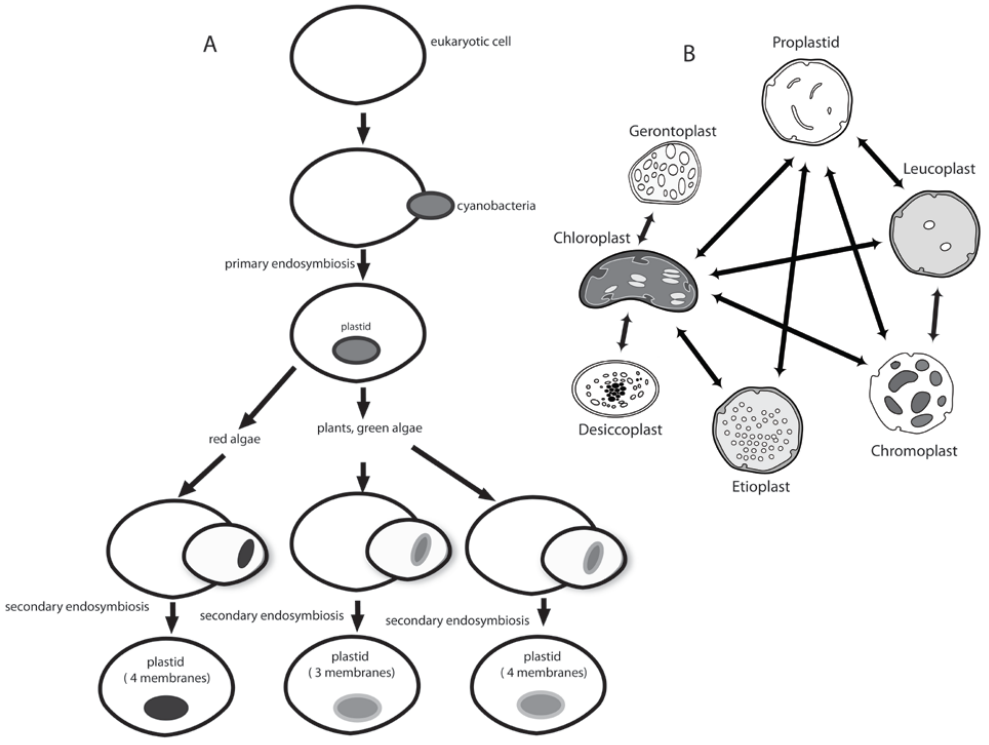

Figure 1. A. The origin and distribution of plastids through primary and secondary endosymbiosis. Primary endosymbiosis in which a photosynthetic cyanobacterial-like prokaryote was engulfed and retained by a eukaryotic phagotroph. These primary plastids are bounded by two envelope membranes. At least three secondary endosymbioses, in which a eukaryotic alga is engulfed and retained by eukaryotic phagotroph producing plastids with either three or four membranes. Modified from (McFadden 2002). **B.** A general scheme for interconversion of plastid types depending on function and origin in different plant tissues. Most of the plastid types are interconvertable under certain environmental and developmental conditions. Modified from (Solymosi and Keresztes 2012).

2.1. Chloroplasts

The plant chloroplast is known for photosynthesis, but it has other important functions such as generating ATP, synthesizing amino acids, fatty acids, sulfur and nitrogen metabolism. It contains a genome and replicates by division. The chloroplast is surrounded by two membranes called the inner and outer envelope membrane. There is a third membrane inside the chloroplast, the thylakoid membrane, which forms flattened discs called grana. The presence of three membranes of the chloroplast creates three soluble compartments; the intermembrane space between the two envelopes, the stroma between the inner envelope and the thylakoids, and the thylakoid interior lumen

(Cooper 2000). The chloroplast in Arabidopsis has 117 genes of which 87 encode proteins (Cui et al. 2006). The predicted total amount of different chloroplast proteins in Arabidopsis has gradually increased over the years and is currently estimated to be approximately 5000 chloroplast proteins (Lu et al. 2011). Thus, the majority of chloroplast localized proteins are nuclear encoded and translated in the cytosol prior to import into the chloroplast.

3. CHLOROPLAST LIPIDS

3.1. Lipid composition of chloroplast membranes

The membrane lipid composition of the individual subcellular compartments differs from one another to meet special requirements of organelle's function. The chloroplast has a cyanobacterial origin, thus its membrane lipid composition is similar to that found in cyanobacteria rather than that found in animals, fungi or non-photosynthetic bacteria. Chloroplast membranes contain a large proportion of galactoglycerolipids, i.e., 36 mol% monogalactosyldiacylglycerol (MGDG) and 29 mol% digalactosyldiacylglycerol (DGDG), and a small proportion of sulfoquinovosyldiacylglycerol (SQDG) and phosphoglycerolipids. Sterols and sphingolipids are present in the plant plasma membrane, Golgi apparatus and tonoplast but are absent from chloroplasts. Approximately 90 mol% of the membrane lipids in the chloroplast are present in the thylakoid membranes composed of 52 mol% MGDG , 32 mol% DGDG , 9.5 mol % phosphatidylglycerol (PG) and 6.5 mol% of SQDG (Andersson and Dörmann 2009; Benning 2009; Block et al. 1983).

In terms of lipid composition, the inner envelope membrane and the thylakoid membrane are similar, whereas the outer envelope membrane is more similar to extraplastidial membranes. In the outer envelope the amount of phospholipid, the ratio of DGDG to MGDG, and the lipid to protein ratio is higher than for the inner envelope membrane and the thylakoid (Andersson and Dörmann 2009; Block et al. 1983).

3.2. Biosynthesis and assembly of thylakoid membrane lipids

The chloroplast is the site of fatty acid synthesis in plants (Ohlrogge et al. 1979). Many land plants use two pathways for the assembly of thylakoid lipid precursors: (a) a prokaryotic pathway, where the glycerolipids are synthesized in the chloroplast and (b) a eukaryotic pathway, where the glycerolipids are synthesized in the ER and transported back to the chloroplast (Figure 2) (Roughan et al. 1980). Glycerolipids produced by the eukaryotic pathway have a different molecular composition (18-carbon fatty acids on the *sn*-2 position of the glycerol backbone) than those produced by the plastid pathway (16-carbon fatty acids on *sn*-2) (Heinz and Roughan 1983). On the bases of fatty acid composition plants are divided in two groups. Some plants are C16:3 plants, including *Spinacia oleracea* (Spinach), *Nicotiana sylvestris* (Woodland tobacco), *Tropaeolum majus* (Indian cress) and Arabidopsis, which contain hexadecatrienoic acid (16:3) on the *sn*-2 position of MGDG. However, most plants are C18:3 plants including

Pisum sativum (Pea) , *Avena sativa* (Oat), *Zea mays* (Maize) and *Vicia faba* (Braod bean) that exclusively contain linolenic acid (18:3) on the *sn*-2 position of MGDG and DGDG (Heinz and Roughan 1983; Mongrand et al. 1998).

The bulk of the thylakoid lipids are MGDG and DGDG. Their biosynthesis starts from fatty acid syntheses that take place with the help of an acyl carrier protein and NADPH. The major fatty acids synthesized in the stroma for both the chloroplast and the ER are 18:1 and 16:0 respectively (Ohlroggeav and Browseb 1995; Rawsthorne 2002). In the ER and the chloroplast a glycerol-3-phosphate is synthesized from dihydroxyacetonephosphate. Glycerol-3-phosphate is sequentially converted to lyso-phosphatidic acid and phosphatidic acid (PA). In the prokaryotic pathway the PA contains C18 on *sn*-1 and C16 on *sn*-2 and converted to diacylglycerol (DAG) and then to MGDG in the intermembrane space and DGDG on the outer envelope surface. Whereas in the eukaryotic pathway the PA contains C16 or C18 at *sn*-1 and C18 at *sn*-2. PA is further converted to phosphatidylcholine (PC). PA or PC-derived lipids, i.e., 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 transported to the thylakoid (Andersson and Dörmann 2009; Benning 2009).

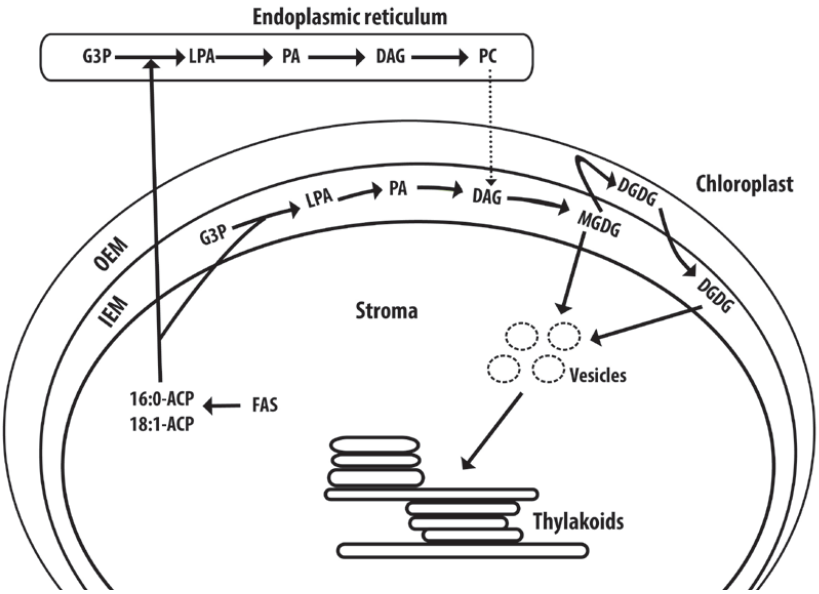

Figure 2. A model for galactolipid synthesis and trafficking. FAS; Fatty acid synthesis, ACP; acyl carrier protein, G3P; glycerol-3-phosphate, LPA; lyco phosphatidic acid, PA; phosphatidic acid, PC; phosphatidylcholine, DAG; diacylglycerol, MGDG; monogalactosyldiacylglycerol, DGDG; digalactosyldiacylglycerol, OEM; outer envelope membrane, IEM; inner envelope membrane.

3.3. Lipid transport to thylakoids

Thylakoid lipids are synthesized in the chloroplast envelope (Kelly and Dörmann 2004) and are transported to the thylakoid (Andersson et al. 2001; Rawyler et al. 1992). Different theories have been developed regarding lipid transport from the envelope to the thylakoid. The transfer of lipids could be mediated by physical contact sites between the envelope and the thylakoid and has been proposed a transient fusion between thylakoid and envelope (Rawyler et al. 1995) or lipids can be transported through diffusion with the help of soluble proteins. Thus far, these kind of transfer has not been demonstrated by ultra-structural or biochemical studies. However, there are both ultra-structural and biochemical evidence regarding vesicle transport for lipid transfer to the thylakoid membrane (Figure 2). One early piece of evidence regarding vesicle existence in the chloroplast came from the observation of vesicle structures inside the chloroplast stroma at low temperature (Morré et al. 1991b). At low temperatures, vesicles accumulate in the stroma, and the lipid transfer to the thylakoid significantly decreases (Andersson et al., 2001), because at low temperatures vesicle fusion does not occur fast enough at the thylakoid membrane, thus “freezing” the picture. These results show similarity to the ER-Golgi transport vesicles at low temperature, for which it has been shown that vesicle fusion, but not budding, is inhibited (Moreau et al. 1992).

The transfer between the envelope and the thylakoid is stimulated by ATP, GTP and stromal proteins, and the vesicles are similar to those formed on the ER (Morré et al. 1991a; Räntfors et al. 2000). Vesicle transport in the secretory pathway also requires nucleotides and soluble proteins (Bonifacino and Glick 2004), which indicates that the same kind of transport occurs in chloroplasts as well. Moreover, it has been shown that inhibitors of vesicle formation and fusion in the chloroplast also work in a similar way in the secretory pathway (Westphal et al. 2001). Chloroplasts are of prokaryotic origin, but vesicles were for a long time not observed other than in land plants, suggesting that there might be a late evolutionary development to cope with environmental challenges (Westphal et al. 2003). Nevertheless, there is now emerging evidence of vesicles inside photosynthetic organisms such as cyanobacteria (Nevo et al. 2007; Schneider et al. 2007), which shows that vesicle transport do exist in photosynthetic prokaryotes. In fact, in the chloroplast there are proteins found which are involved in vesicle transport having a cyanobacterial origin, indicating that these kind of proteins could retain the same function in cyanobacteria.

4. CHLOROPLAST PROTEINS

4.1. Protein import into the chloroplast

After the endosymbiotic event most genes have been transferred from the plastome to the nucleus. As many of the cyanobacterial functions are retained by the chloroplast, many nuclear encoded proteins must be transported back to the chloroplast. Most of these proteins are in the cytosol and are directed to the chloroplast by having N-terminal

sequences called transit peptides. In Arabidopsis there are at least 20 different protein components in the double layer envelope membrane for the translocation of the nuclear encoded chloroplast proteins (Aronsson and Jarvis 2009; Jackson-Constan and Keegstra 2001). These proteins are termed Toc or Tic components being part of the the TOC or TIC (Translocation of the outer/inner envelope membrane of chloroplasts) translocon complex (Schnell et al. 1997).

Upon arrival of preproteins (having transit peptides) the import can be divided into three different stages based on the energy requirement (Aronsson and Jarvis 2009). In the first stage the transit peptide makes reversible contact with the receptors of the TOC complex without consuming any energy (Kouranov and Schnell 1997; Perry and Keegstra 1994). In the second stage the preprotein becomes deeply inserted into the TOC complex and makes contact with the TIC complex, called docking. This stage is irreversible and requires a low amount of ATP and GTP (Kessler and Schnell 2006; Olsen and Keegstra 1992; Young et al. 1999). Finally, in the last step of import, the preprotein is completely translocated into the stroma, and the transit peptide is removed by a stromal processing peptidase.

The TOC machinery is responsible for the recognition of the preproteins. The TOC core complex is composed of three main proteins; Toc34, Toc75 and Toc159, according to their predicted molecular weight (Schnell et al. 1997). It has been proposed that for every Toc159 protein there are three or four Toc75 and Toc34 proteins (Kikuchi et al. 2006; Schleiff et al. 2003). Both Toc159 and Toc34 are responsible for protein recognition and are called receptors. Toc75 is deeply embedded in the outer membrane and works as a channel for preproteins. Two models exist regarding how the TOC receptor works. In the first model Toc159 is the primary receptor subsequently associated with Toc34 and transferring the preprotein to the Toc75 channel (Keegstra and Froehlich 1999). In the second model, Toc34 acts as the primary receptor for the preprotein and transfers it to Toc159, which, through GTP hydrolysis, acts as a motor and transfers the preprotein to Toc75 (Becker et al. 2004b; Soll and Schleiff 2004). In Arabidopsis different isoforms of these TOC receptors exist, e.g., Toc33 and Toc34 and Toc120, Toc132 and Toc159, and two different pathways have been proposed: (1) photosynthetic proteins use Toc159 and Toc33 (Bauer et al. 2000; Jarvis 2008) and (2) non- photosynthetic proteins use Toc132/Toc120 and Toc34 (Ivanova et al. 2004; Kubis et al. 2004; Yu and Li 2001). However, cross-talk is suggested to occur between these different pathways.

As for the TOC complex proteins, several homologues of the TIC complex are found. In Arabidopsis two homologues each exist for Tic22, Tic32 and HSP93, four each for Tic20, but only one each for Tic21, Tic40, Tic55, Tic62 and Tic110. Tic22, being located in the intermembrane space, might help the connection between the TOC and TIC complex (Becker et al. 2004a; Schnell et al. 1994).

In the TIC complex three proteins are considered to have a channel-like function, i.e., Tic20, Tic21 and Tic110. Tic40, Tic110 and HSP93 are suggested to have a close association with the each other (Kovacheva et al. 2004), operating as a motor complex. First, the preprotein's transit peptide is recognized by Tic110, and Tic40 works as a co-chaperone to coordinate association with Tic110 and HSP93, which helps the preprotein to transfer to HSP93 on the stomal side. Finally, the transit peptide of the preprotein is cleaved off by the stromal processing peptide (Richter and Lamppa 1999).

Other TIC components, such as Tic32, Tic55 and Tic62, are suggested to work as sensors for the chloroplast redox state and may help to increase import efficiency (Aronsson and Jarvis 2009).

4.2. Targeting of envelope membrane proteins

Most proteins in the intermembrane space and the inner envelope membrane possess transit peptides that are cleaved by stromal processing peptidase. However, Tic22 has a transit peptide but it is cleaved by a protease in the inner envelope membrane. Thus, two different pathways for protein targeting to the intermembrane space and inner envelope membrane may exist: firstly, the proteins using the normal TOC/TIC pathway into the stroma and then transported back to envelope membrane (Li and Schnell 2006; Lübeck et al. 1997; Tripp et al. 2007). In the second pathway the protein passes through only the TOC complex and thereafter enters the envelope membrane without passing the TIC complex (Brink et al. 1995; Knight and Gray 1995; Tripp et al. 2007).

There are some evidences from proteomics data that proteins can end up in the chloroplast envelope without having transit peptides (Kleffmann et al. 2004), such as Tic32 and the chloroplast envelope Quinone Oxidoreductase Homologue (ceQORH), that both end up in the inner envelope membrane without transit peptides (Aronsson and Jarvis 2009). The plant glutamate receptor (AtGLR3.4), which has no transit peptide (Paper **III**), is localized to the chloroplast inner envelop membrane of Arabidopsis and *Nicotiana tabacum* (Tobacco) (Teardo et al. 2010).

4.3. Targeting of proteins to the thylakoid

Thylakoids contain trans-membrane proteins and soluble proteins. The thylakoid membrane contains proteins necessary for the photosynthesis machinery and consists of four major complexes (PSI, PSII, the cytochrome *b6/f* complex and ATP synthase).

Proteins residing in the thylakoid lumen mostly contain two targeting sequences, one for targeting across the envelope membrane to the stroma and another one for luminal targeting (Figure 3) (Hageman et al. 1986). Luminal targeting signals have a characteristic three domain structure that comprises a positively charged amino-terminal region (N-domain), hydrophobic core region (H-domain) and more polar carboxy-terminal region (C-domain) ending with an A-X-A consensus sequence recognized by the thylakoid processing peptidase (Brink et al. 1997; Dalbey and von Heijne 1992). The secretory (Sec) pathway and the twin-arginine translocation (Tat) pathway have been proposed to be responsible for lumen protein targeting (Figure 3). Tat-directed proteins possess two arginine residues, whereas proteins taking the route of the Sec pathway have a lysine residue close to the H-domain. According to proteomic studies approximately 100 proteins reside in the thylakoid lumen, all nuclear encoded, with about half expected to be transported through the Tat pathway and the other half through the Sec or other pathways, based on the arginine and lysine residues (Peltier et al. 2002; Schubert et al. 2002). The signal recognition particle (SRP) and Spontaneous pathways target trans-membrane proteins to the thylakoid (Figure 3). These proteins have no target signal for the thylakoid; the targeting signal usually lies within the mature

part of the protein (Aldridge et al. 2009; Celedon and Cline 2012; Jarvis and Robinson 2004).

4.3.1. The Secretory (Sec) Pathway

A subset of luminal proteins, which includes plastocyanin, the 33 kDa oxygen-evolving protein (OE33) and PSII subunit F (PsaF), is transported by a Sec-type system (Figure 3) that resembles the Sec system in bacterial membranes (Mant et al. 1994; Robinson et al. 1994; Schuenemann et al. 1999; Yuan et al. 1994a). The bacterial Sec system contains SecA, an ATP-driven translocation motor, and the membrane-bound SecYEG translocation channel. It also contains an additional complex SecDFyajC and a chaperone called SecB (Driessen et al. 2001). SecA (cpSecA), SecY (cpSecY) and SecE (cpSecE) homologues have been identified in Arabidopsis and shown to be involved in thylakoid protein processing (Laidler et al. 1995; Schuenemann et al. 1999; Yuan and Cline 1994; Yuan et al. 1994a). The chloroplast lacks SecG, SecB and SecDFYajC, perhaps because they are not essential (Du Plessis et al. 2011). The chloroplast Sec pathway evolved to suit its environment, i.e., it required a different lipid composition and the ATPase activity is stimulated by thylakoid signal peptides rather than the *Escherichia coli* signal peptides (Sun et al. 2007).

The Sec pathway in the chloroplast is a slimmed-down version of the bacterial one that lacks many of the non-essential components, however there is evidence that both operate by a similar mechanism. The dependence on ATP, sensitivity to azide, that the antibody against cpSecY inhibits cpSecA-dependent protein translocation and complementation of cpSecE of *E. coli* all suggest similarity to the bacterial Sec system. Additionally, the insertion of Sec translocase into the thylakoid via the SRP pathway is similar to the bacterial plasma membrane (Fröderberg et al. 2001; Hulford et al. 1994; Mori et al. 1999; Yuan et al. 1994b). It has also been shown that cpSecA is essential for photosynthetic development in *A. thaliana* (Liu et al. 2010). The inability of the Sec pathway to transport folded proteins is similar to bacteria which required proteins to be in an unfolded state (Hynds et al. 1998; Marques et al. 2004).

4.3.2 The twin-arginine translocation (Tat) pathway

The Tat pathway is proposed to transport luminal proteins, e.g., the 16 and 23 kDa oxygen-evolving proteins (Figure 3) (Gutensohn et al. 2006). As the pathway name suggests, the signal peptide of proteins transported by this pathway contains an amino terminal twin-arginine motif upstream of the hydrophobic region (Chaddock et al. 1995). Translocation is dependent on thylakoid ΔpH and does not require ATP, unlike the Sec pathway (Cline et al. 1992; Klösgen et al. 1992; Mould and Robinson 1991), and transports proteins in a folded state (Clark and Theg 1997). Three proteins (Tha4, Hcf106 and cpTatC) are important for Tat pathway translocation (Motohashi et al. 2001; Walker et al. 1999; Voelker and Barkan 1995): the homologues of TatA, TatB and TatC, respectively. The receptor complex is formed by the cpTatC and Hcf106 proteins, and the Tha4 oligomer forms a separate complex, which forms a channel or ring like structure. The receptor and oligomer complex is associated with the presence of precursor and proton gradients (Cline and Mori 2001; Dabney-Smith et al. 2006; Mori and Cline 2002). First, the precursor protein binds to the receptor complex. This binding

stimulates the assembly of the oligomer with the receptor complex, and the precursor is transported to the lumen by crossing the thylakoid membrane in the presence of a proton gradient (Aldridge et al. 2009).

4.3.3 The signal recognition particle (SRP) pathway

It has been proposed that the SRP pathway translocates thylakoid trans-membrane proteins (Figure 3). This system is also found in prokaryotes. It has been shown that light harvesting complex proteins (LHCPs) are translocated to thylakoid membranes through the SRP pathway. Thylakoid targeting of LHCPs depends on three stromal factors, cpSRP54 (Franklin and Hoffman 1993; Li et al. 1995), cpSRP43 (Schuenemann et al. 1998) and cpFtsY (Kogata et al. 1999). CpSRP54 has a GTPase domain, which might be responsible for the insertion of protein into the thylakoid. It has another domain called methionine-rich domain (M-domain) (Franklin and Hoffman 1993). CpSRP43 has two structural domains. The first are chromodomains, where one chromodomain (CD1) is located in the N-terminal region (Eichacker and Henry 2001) and two others (CD2 and CD3) are located at the C-terminal (Klimyuk et al. 1999). The second domain structure is four ankyrin repeats (ANK1 - ANK4) that are located between CD1 and CD2/CD3 and are responsible for protein-protein interaction (Klimyuk et al. 1999). LHCP has an 18 amino acid span between transmembrane two and three called the L18 domain, which interacts with SRP43 at ANK3 (Tu et al. 2000). SRP54 also binds directly to the LHCP at the third transmembrane region (High et al. 1997; Li et al. 1995). Similarly, interaction between SRP54 and SRP43 occur via the M-domain and the CD2 domain (Goforth et al. 2004; Jonas-Straube et al. 2001; Sivaraja et al. 2005). The third stromal factor, cpFtsY, is required to target the transient complex to the thylakoid. CpFtsY has the NG domain, which has three motifs for GTP binding and contain the target sequence for the thylakoid (Kogata et al. 1999; Stengel et al. 2007). Finally, LHCP is inserted into the membrane with the help of the integral membrane protein Alb3 (Moore et al. 2000).

4.3.4. Spontaneous pathway

It has been suggested that the insertion of bitopic membrane proteins, such as CFo-II, PsbW, PsbX and PsbY (Kim et al. 1998; Lorković et al. 1995; Michl et al. 1994; Thompson et al. 1999), does not depend on stromal factors, nucleoside triphosphates, transthylakoidal proton gradients, (Michl et al. 1994) stromal exposed receptors or translocases in the thylakoid membrane (Kim et al. 1998; Robinson et al. 2003). This would then indicate a spontaneous pathway (Figure 3). Proteins suggested to insert spontaneously have a bipartite transit peptide for stromal and thylakoid targeting. In this pathway, the bipartite sequence possesses two hydrophobic regions, one close to the N-terminal on the target peptide and another on the C-terminal. These proteins do have cleavage signals to be recognized by a luminal protease but no signal for the stromal processing protease, which shows that these proteins could be targeted to the thylakoid without the removal of the transit peptide (Gutensohn et al. 2006).

4.3.5. Vesicle transport pathway

Evidence of vesicle transport inside chloroplasts suggested that it could be similar to the secretory system in the cytosol. Vesicle transport in the secretory system transports both lipids and proteins, but so far evidence has only been shown for lipid transport through vesicles inside chloroplasts. If indeed chloroplast vesicle transport is similar to that in the cytosol, then it should also be responsible for the transport of proteins as cargos (Figure 3). As it has been suggested that vesicles in the chloroplast transport lipids from the envelope to the thylakoid membrane (Andersson et al. 2001), it could also be possible that proteins are transported from the envelope to the thylakoid with the help of vesicles, in addition to the above mentioned thylakoid targeting pathways. This hypothesis is supported by a recent study that suggested that LHCB normally transported to the thylakoid via SRP pathway could also be transported through vesicles with the help of a *snowy cotyledon 2 protein (SCO2)* (Tanz et al. 2012). In addition, we found conserved signals on many thylakoid proteins. These signals are important for incorporation in COPII vesicles in the cytosol (Paper **III**). In our search we found these signals on LHCP proteins, which have been shown to be targeted to the thylakoid by the SRP pathway, and the PSII protein, which is targeted by the spontaneous pathway, suggesting it could take an alternative route as well with the help of vesicle transport. It has been proposed that the NADPH:protochlorphyllide oxidoreductase (POR) enzyme, which requires NADPH and ATP for association with the membrane (thylakoid), could be transported in yet another pathway apart from those four already proposed (Aronsson 2001). In fact, we found that POR co-immunoprecipitated with CPSAR1 (unpublished result, Khan NZ, Karim S, Aronsson H), suggesting that this additional pathway could be vesicle transport. We also found transmembrane components of the SRP and Sec pathways that could be transported with the help of vesicles as cargos to the thylakoid membrane (Paper **III**), suggesting that membrane bound components are provided by vesicles transport. In the case of luminal proteins cargos, we didn't identify any arginine or lysine residues on most of the proteins (Paper **III**), which are important for transport in the Sec and Tat pathways, respectively. These results open up the possibility that proteins could be transported through vesicles, as only a subset of the proteins identified are transported with the help of the other four thylakoid targeting pathways.

4.4. Dual targeting of proteins destined for the chloroplast

There are cases where the same proteins are found in more than one compartment. e.g., the cytosol, ER, mitochondrion and chloroplast. Distribution to two compartments follows different routes: 1) two genes transcribed into their mRNAs, but only one carrying the targeting signal; 2) two mRNAs transcribed from one gene but having two different start sites and only one encoding the targeting signal; 3) one mRNA is spliced and one is not spliced, and the spliced one loses the targeting signal for distribution to an organelle and the rest of the cell; 4) because of two initiation codons, two messages are translated with one containing the target signal; or 5) a single protein having the targeting signal is distributed to the organelle and the rest of the cell (Danpure 1995; Karniely and Pines 2005; Small et al. 1998). If two different signals exist for targeting to different compartments on the same protein, there will be a competition between these

two compartments. If these signals are accessible then the distribution may be dictated by the relative affinities to their receptors, but if the signal is ambiguous than it can be recognized by two different organelles, e.g., chloroplasts and mitochondria. Different distribution is achieved if the targeting signal is inaccessible to its destination receptor due to improper folding, post-translational modification or incomplete importation. Proteins can also be retrieved through retrograde transport to the cytosol from organelles through translocons, leakage or by active transport (Karniely and Pines 2005).

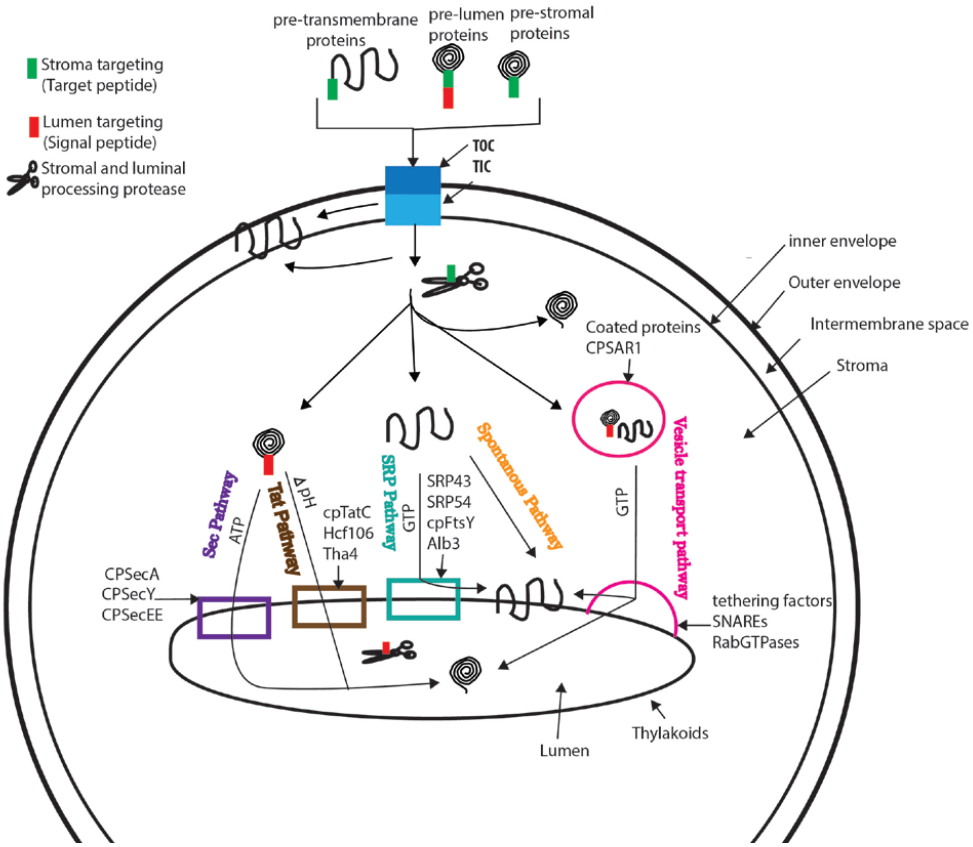

Figure 3. Transport pathways of thylakoid proteins after import into the chloroplast. Most chloroplast proteins with target peptides are imported from the cytosol with the help of the TOC/TIC complex. After import the proteins are destined for the envelope membrane, stroma and thylakoid. Five pathways are proposed for transport of proteins towards the thylakoid. The Sec and Tat pathways transport lumen proteins having lumen targeting signals. The SRP and spontaneous pathways transport trans-membrane proteins towards the thylakoid. The vesicle transport pathway proposed here is capable of both lumen and trans-membrane proteins.

In the chloroplast there are several examples of dual targeting. FtsZ, a filament forming protein involves in plastid division, has been found in the cytosol and the chloroplast in the moss *Physcomitrella patens.* Another example are aminoacyl-tRNA synthetases which are found in chloroplasts and mitochondria in Arabidopsis (Duchêne et al. 2005). Plant glutamate receptors (AtGLR3) have been shown to be localized in the chloroplast and plasma membrane in Arabidopsis and tobacco (Teardo et al. 2011), but according to different subcellular prediction tools all of the members of this family are not localized in the chloroplast, instead they are strongly predicted to be in the secretory system (Paper **III**). Putative dynamin like large GTPase (AtDRP1a/AtADL1a) is localized in the thylakoid membrane and is responsible for thylakoid biogenesis (Park et al. 1998) without having any signals for localization to the chloroplast, mitochondrion or secretory system (Paper **III**). However, it also has a role in clathrin-coated vesicle formation in the endocytosis (Fujimoto et al. 2010), suggesting a dual localization in the chloroplast and the secretory system. On the other hand, dynamin like protein AtADL2a has a role in both the secretory system (Zhang and Hu 2008) and the chloroplast (Kim et al. 2001), and has a targeting signal for the chloroplast (Paper **III**) . The subunit of potassium channel AtTPK3 is localized in the thylakoid membrane (Zanetti et al. 2010) and vacuoles (Voelker et al. 2010) with no chloroplast targeting signal predicted. Another potassium channel, AtTPK5, has also been found in the vacuole (Dunkel et al. 2008; Voelker et al. 2006), but is strongly predicted to be localized in the chloroplast (Paper **III**). These examples suggest that chloroplast targeting signals for localization are important, but proteins can be targeted to the chloroplast without the presence of targeting signals to the envelope and even to the thylakoid. Furthermore, the dual localization of dynamin proteins, important factors in vesicle transport and potassium channels, which are transported in COPII vesicles as a cargo (Mikosch et al. 2006; Sieben et al. 2008), show the possibility that vesicle components in both the secretory system and chloroplasts can be dual localized. In fact, when we used bioinformatics to search for the vesicle components in secretory systems, we found that these components can be localized in the chloroplast as well (Paper **III**).

4.5. Bioinformatics tools used to predict protein localization, structure and function in chloroplasts

4.5.1. Prediction of chloroplast localized proteins and their topology

There are different *in silico* methods available to predict protein localization in different organelles. As chloroplasts are semiautonomous organelles, most proteins are imported into the chloroplast. The proteins possess an N-terminal chloroplast transit peptide for targeting. The structure of transit peptides is very variable, which makes prediction tools not fully reliable, but they are usually considered as a first step prior to proteomics localization and assigning functions to a particular protein. Usually the transit peptide contains multiple domains responsible for interaction with envelope lipids, chloroplast receptors and stromal processing peptidase (Bruce 2000). It also contains a low content of acidic residues and an over-representation of hydroxylated residues compared to the mature parts of chloroplast proteins (von Heijne 1990). Despite this, several tools have been developed to find chloroplast targeted proteins with a reasonable accuracy.

Similarly, to predict whether the protein is located in the stroma, lumen, envelope or thylakoid membrane *in silico* methods have been developed to predict possible transmembrane regions.

4.5.2. Integrated databases for protein localization

It is preferable to use experimental data if available together with a bioinformatics approach to confirm predictions. There have been several databases developed over the years that hold experimental data.

ARAMEMNON is a database for plant membrane proteins for dicotyledonous plants i.e. Arabidopsis*,* grape vine (*Vitis vinifera*) and poplar (*Populus trichocarpa)* and monocotyledonous plants i.e. rice (*Oryza sativa*)*,* maize (*Zea mays*) and purple false brome (*Brachypodium distachyon*) plants. It covers all the proteins for Arabidopsis in particular (http://aramemnon.uni-koeln.de/index.ep) (Schwacke et al. 2003). In recent years there has been lots of progress in developing the tools for proteins localization and structure but still variation exists between these tools. One of the advantages of using ARAMEMNON is not only that it predicts the topology by using 18 and 6 different prediction tools for transmembrane alpha helix and beta barrels, respectively, but two built in consensus methods (TmConsens and ConPred_v2) are used on the bases of individual prediction of transmembrane helix. A third extended consensus method (TmMutliCon) is also used, which combines the consensus of several homologous proteins. Similarly, ARAMEMNON uses 17 different prediction tools for localization of proteins in chloroplasts, mitochondria and the secretory pathway, and combines the individual predictions to develop a built-in consensus prediction method (Schwacke et al. 2003) (http://aramemnon.uni-koeln.de/).

The subcellular localization database for Arabidopsis proteins (SUBAIII) gives information about protein localization with 22 different prediction programs with a built in consensus similar to ARAMEMNON, as well as a protein-protein interaction prediction tool. It stores 3788 entries based on green florescent protein (GFP) localization and 22191 based entries based on subcellular proteomic studies (mass spectrometry), both of which are increasing rapidly. It covers the proteins localized in 13 different locations including the cell plate, cytoskeleton, cytosol, endosome, ER, extracellular, Golgi, mitochondria, nucleus, peroxisome, plasma membrane, plastid and vacuole. In short, it is a very useful tool for exploring protein function, protein redundancy and of the biological inter-relationship among proteins (http://suba.plantenergy.uwa.edu.au/) (Heazlewood et al. 2005; Heazlewood et al. 2007).

The plant proteome database (PPDB) is useful to search within the Arabidopsis and maize proteome. The PPDB stores experimental data from in-house proteome and mass spectrometry analyses, curated information about protein function, properties and subcellular localization. In addition, the proteins are curated for suborganellar plastid location and function; this involves integrated, peripheral and soluble proteins in the envelope membrane, stroma, thylakoid, plastoglobuli, nucleoid and ribosomes (http://ppdb.tc.cornell.edu/default.aspx) (Sun et al. 2009).

4.5.3. Predicting the function of proteins

The primary structure of a protein is composed of a linear sequence of amino acids, the more similar these sequences between proteins, the more identical or homologous they should be. One of the common ways to predict the homology of an unknown protein is to use basic local alignment search tool (BLAST), which compares primary sequences with the available datasets and finds the conserved region above the threshold selected. There are different algorithms developed for BLAST search and one of them is called PSI-BLAST (position specific iterated BLAST). PSI-BLAST is useful to find distant relatives or evolutionary relationships between proteins. The first step is similar to normal blast by searching the query sequence against the datasets by creating a profile based on the significant features present in the sequences of available datasets and a group of protein is found. On the bases of this group another profile is developed and the process is repeated. The procedure can be iterated as often as desired or until there is no significantly sequence detected (Altschul et al. 1997).

The domains are structural and functional parts of a protein that can evolve independently and form the three dimensional structure. So by identifying a particular structure, one can predict the function of that protein. There are various web-based tools where domains can be identified in a given proteins sequence.

Prosite (Release 20.2) is a database containing patterns and profiles for more than a thousand protein families and domains (Hulo et al. 2006). Similar to other tools for domain searching, e.g., Pfam (http://pfam.sanger.ac.uk/), Prosite can be used to identify domains by searching protein sequences. In addition, it has the advantage of creating manual patterns on the basis of ProRules and also allows the creation of conserved patterns in a set of protein sequences using the PRATT web tool. In the Prosite web based tool, in addition to pattern searching, it allows searching the domain entries not only against the Swiss-Prot protein database but also by uploading manually created datasets in FASTA format (http://prosite.expasy.org/).

5. CYTOSOLIC VESICLE TRANSPORT

The cytosolic pathway consists of several functionally and structurally different compartments including the ER, the Golgi apparatus, various post-Golgi intermediate compartments, the vacuoles/lysosomes and the plasma membrane. The majority of proteins and lipids are transported between these compartments via vesicles. Three types of vesicular pathway are identified by the recruitment of their coatomers, i.e., coat protein complexes (COPI, COPII) or clathrin coated vesicles (CCV) and their partners (Figure 3). Generally, all these coated vesicles start from a GTPase activation of a GTPase, e.g., Sar1 or Arf1, at a 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 vesicle continues to move towards the acceptor membrane where it is tethered with the help of tethering factors. Finally, fusion of the vesicle occurs with the help of SNAREs and delivery of the cargo to the

acceptor membrane occurs (Bonifacino and Glick 2004). Recently it has been shown that uncoating does not start immediately after budding, but somehow interaction with the fusing machinery (such as tethering factors) helps to pair up the vesicle with the target membrane prior to uncoating (Trahey and Hay 2010). Homologues of almost all the COPI, COPII and clathrin components needed for these three types of vesicle transport have been identified in Arabidopsis, though the mechanisms have not yet been studied in detail, unlike in yeast or mammals (Bassham et al. 2008). Moreover, multiple homologues have been found for most of the components in Arabidopsis.

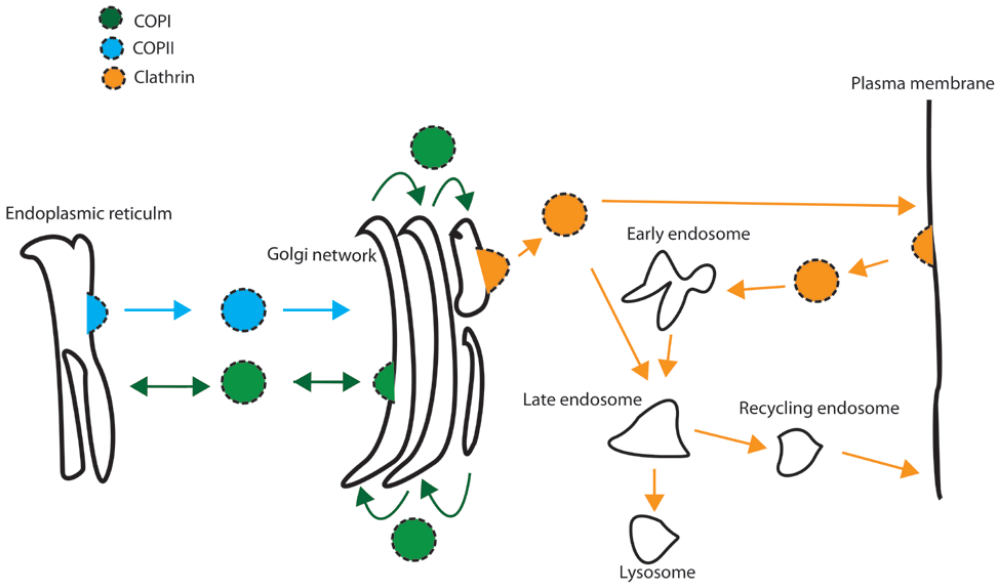

Figure 4. Cytosolic vesicle transport in the secretory pathway. COPII vesicle transport from endoplasmic reticulum (ER) to the Golgi. COPI operates from Golgi to ER, from ER to Golgi and in between Golgi cisternae Clathrin coated vesicle transport occur between Golgi, Plasma membrane and in endocytosis pathway.

5.1. COPII

The COPII vesicle pathway operates from the ER to the Golgi (Figure 4) and has been extensively studied in yeast. COPII vesicle formation starts by the activation of Sar1 by a SEC12 protein that is a guanosine nucleotide exchange factor (GEF) (Barlowe et al. 1993; Barlowe and Schekman 1993). This activation causes the recruitment of coated proteins. First the coat proteins Sec23 and Sec24 start the budding process, and cargo proteins are enriched by binding to the Sec24 (Bi et al. 2002). Later on two other coat Proteins (Sec13 and Sec31) form the outer layer of the ongoing budding vesicle and help in the invagination of the donor membrane (Lederkremer et al. 2001).

Arabidopsis encodes five homologues of Sec23, four of Sec24, two each of Sec13 and Sec31 (Table 1) (Sanderfoot and Raikhel 2003). Similarly five homologues of SAR1 GTPases (Table 2) (Bassham et al. 2008; Robinson et al. 2007) and two SEC12 proteins of which one each was isolated by complementation of yeast mutants and shown to associate with the ER (Bar-Peled and Raikhel 1997; d'Enfert et al. 1992).

Table 1. Main coated components involved in the formation of COPII, COPI and CCV. (Bassham et al. 2008; Robinson et al. 2007). Green color indicates putative localization of the components in chloroplasts (Andersson and Sandelius 2004, Paper **III)**.

COPII		COPI		CCV	
Sec13	At3g01340	**B-COP**		**Triskelion**	
	At2g30050	α-COP	At1g62020	Heavy chain	At3g11130
	At3g49660		At2g21390		At3g08530
	At2g43770	β'-COP	At1g52360	Light chain	At2g40060
Sec31	At1g18830		At3g15980		At3g51890
	At3g63460		At1g79990	**AP1**	
Sec23	At3g23660	ε-COP	At2g34840	γ	At1g60070
	At1g05520		At1g30630		At1g23900
	At5g43670	**F-COP**		β1/2	At4g11380
	At4g14160	β-COP	At4g31480		At4g23460
	At2g21630		At4g31490	μ	At1g60780
	At4g01810	γ-COP	At4g34450		At1g10730
Sec24	At3g07100	∂-COP	At5g05010	σ	At2g17380
	At4g32640	ζ-COP	At1g60970		At4g35410
	At2g27460		At3g09800	**AP2**	
	At3g44340		At1g08520	α	At5g22770
	At4g32640				At5g22780
				β1/2	At4g11380
					At4g23460
				μ2	At5g46630
				σ2	At1g47830
				AP3	
				δ	At1g47830
				β3	At3g55480
				μ3	At1g56590
				σ3	At3g50860
				AP4	
				ε	At1g31730
				β4	At5g11490
				μ4	At4g24550
				σ4	At4g24550

5.2. COPI

COPI type vesicle transport occurs between the ER and the Golgi in both directions (Figure 4). Activation of Arf1 is essential for the recruitment of the heptomeric COPI complex from the cytosol (Orci et al. 1993). This heptomer consists of two main sub-complexes, the F-COPI subcomplex (*β*, *γ, ∂*, *ζ)* and the B-COPI sub complex (*α*, *β* ', *ε*) (Fiedler et al. 1996). Arf1 activation is stimulated by the Sec7 family of GEFs (D'Souza-Schorey and Chavrier 2006). GBF1, the only known GEF localized to the cis-Golgi, plays an important role in mediating protein trafficking between the ER and the cis-Golgi (Claude et al. 1999; Kawamoto et al. 2002). Unlike the COPII coat, where the Sar1 GAP is an integral part of the coat, stimulation of GTP hydrolysis on Arf1 to promote coat disassembly is not mediated by a subunit of the coat *per se* but by a separate ARF GAP. In yeast, the Golgi-localized GAPs Glo3 and Gcs1 have functions in COPI coat disassembly (Dogic et al. 1999; Poon et al. 1999).

Arabidopsis encodes between nine and twelve ARF GTPases (Table 2) (Jürgens and Geldner 2002; Vernoud et al. 2003). In Arabidopsis eight homologues of ARF-GEFs (Anders and Jürgens 2008) and 15 GTPase-activating ARF-GAPs have been identified (Vernoud et al. 2003). Except for δ-COP and γ-COP, plants have multiple genes encoding for COPI proteins (Table 1). Thus, there are two isoforms for α-COP, β-COP, *ε* -COP and three for β′-COP and *ζ*-COP (Bassham et al. 2008; Robinson et al. 2007). The multiplicity of COPI isoforms might reflect different classes of COPI-coated vesicles. In Arabidopsis, two different sized COPI-vesicle populations exist: COPIa derived from *cis*-cisternae, and COPIb from medial and *trans*-cisternae, which suggests that the transport from *cis*-cisternae to the ER is conducting via COPIa and from *trans* to *medial* and finally to *cis*-cisternae is via COPIb (Donohoe et al. 2007)

Table 2. Arabidopsis thaliana Coat GTPases . (Bassham et al. 2008; Robinson et al. 2007). Green color shows localization in the chloroplasts (Andersson and Sandeļius. 2004, Garcia et al., 2010).

Sar1 GTPases		Arf1 GTPases	
SARA1a	At1g09180	ArfA1a	At1g23490
SARA1b	At1g56330	ArfA1b	At5g14670
SARA1c	At4g02080	ArfA1c	At2g47170
SARA1d	At3g62560	ArfA1d	At1g70490
SARA1e	At1g02620	ArfA1e	At3g62290
CPSAR1	At5g18570	ArfA1f	At1g10630
		ArfB1a	At2g15310
		ArfB1b	At5g17060
		ArfB1c	At3g03120
		ArfD1A	At1g02440
		ArfD1b	At1g02430

5.3. Clathrin coated vesicles (CCV)

Transport occurs between the Golgi and the plasma membrane via the CCV pathway (Figure 4). There are two kinds of adapter proteins (AP): AP1, which is found on the trans-Golgi network (TGN) and endosomes, and AP2, which is found on the plasma membrane (Keen 1990). Clathrin coated components are called light and heavy chain proteins and collectively called triskelions (Fotin et al. 2004). AP complexes are

components of clathrin coated vesicles associated with the TGN and the plasma membrane, respectively. They attach the clathrin to the membrane, select the vesicle cargo and recruit accessory proteins that regulate the vesicle formation. Two additional adaptor complexes, AP-3 and AP-4, have also been identified. Like AP-1, AP-3 and AP-4 are found on TGN/endosomal membranes, with AP-3 localized more to endosomes and AP-4 more to the TGN (Robinson and Bonifacino 2001).

The Arabidopsis genome encodes homologues of all of the adaptins of these APs and triskelion (cage) found in mammals and yeast (Table 1) (Bassham et al. 2008).

5.4. Rab (Ras-related in brain) GTPases

Rab GTPases form the largest family of the Ras superfamily of small GTPases. The Arabidopsis genome encodes 57 Rab proteins, divided into eight subfamilies (RabA to RabH) based on sequence similarities (Table 3) (Rutherford and Moore 2002; Vernoud et al. 2003).

Like other regulatory GTPases, the Rab proteins switch between an active GTP bound form and an inactive GDP bound form. Rab GTPases are reversibly associated with membranes by hydrophobic geranylgeranyl groups that are attached to one or two carboxy-terminal Cys residues and regulate membrane traffic. Rab GTPase plays a central role by regulating vesicle trafficking in all eukaryotic cells from vesicle budding, uncoating to fusion (Stenmark 2009).

5.5. Tethering Factors

Tethering factors ensure the correct docking before fusion of newly formed vesicles from the donor membrane to the acceptor membrane. Tethering factors have been divided into three functional classes: 1) oligomeric complexes that bind to SNAREs and typically act as Rab effectors, i.e., the DCGE group that includes Dsl1 complex, Conserved Oligomeric Golgi (COG) complex, Golgi-associated retrograde protein (GARP) complex, and Exocyst; 2) oligomeric complexes that function as GEFs for Rab proteins, i.e., Transport Protein Particle (TRAPP I and TRAPP II) and Heterohexameric homotypic fusion and vacuole protein sorting complex (HOPS), which works as a GEF and an effector; and 3) coiled-coil tethers (Sztul and Lupashin 2009). In Arabidopsis most of the tethering factor homologues have been identified (Table 4) (Koumandou et al. 2007; Latijnhouwers et al. 2005).

5.6. Soluble N-ethylmaleimide-sensitive factor activating protein receptors (SNAREs)

SNAREs help the vesicles fuse with the acceptor membrane. SNAREs found on the vesicle are termed v-SNAREs (vesicle membrane SNAREs), whereas those on the target membrane are called t-SNAREs (target membrane SNAREs) (Söllner et al. 1993). Q-SNAREs contain glutamine conserved residues, whereas R-SNAREs contain arginine conserved residues All v-SNAREs belongs to the R-SNARE group, whereas t-SNAREs belongs to the Q-SNARE group. Q-SNAREs are further classified as Qa, Qb and Qc SNAREs on the basis of amino acid composition (Bock and Scheller 1999; Fasshauer et al. 1998; Jahn et al. 2003). Functional SNARE complexes that drive

membrane fusion form parallel four-helix bundles, requiring one each of the Qa, Qb, Qc and R-SNAREs (Jahn and Scheller 2006).
In Arabidopsis a total of 64 SNAREs have been identified and classified in five different subfamilies, namely Qa, Qb, Qc, R and SNAP25 (Table 5) (Sanderfoot 2007).

Table 3. Rab GTPases in Arabidopsis thaliana. RabA class could work during TGN-post golgi vesicles , RabB on the Golgi apparatus, RabC during cell polarization, RabD between ER-Golgi, RabE during polarized secretion, RabF on the endososmes, RabG on lysosomes/vacuoles and RabH could be involved during golgi-ER retrograde transport (Vernoud et al. 2003). Green color indicates putative localization of the components in chloroplasts (Paper **III** & **IV**).

RabA		RabB		RabG	
RABA1a	At1g06400	RABB1a	At4g17160	RABG1	At5g39620
RABA1b	At1g16920	RABB1b	At4g35860		
RABA1c	At5g45750	RABB1c	At4g17170	RABG2	At2g21880
RABA1d	At4g18800				
RABA1e	At4g18430	**RabC**		RABG3a	At4g09720
RABA1f	At5g60860	RABC1	At1g43890	RABG3b	At1g22740
RABA1g	At3g15060			RABG3c	At3g16100
RABA1h	At2g33870	RABC2a	At5g03530	RABG3d	At1g52280
RABA1i	At1g28550	RABC2b	At3g09910	RABG3e	At1g49300
				RABG3f	At3g18820
RABA2a	At1g09630			**RabH**	
RABA2b	At1g07410	**RabD**		RABH1a	At5g64990
RABA2c	At3g46830	RABD1	At3g11730	RABH1b	At2g44610
RABA2d	At5g59150			RABH1c	At4g39890
		RABD2a	At1g02130	RABH1d	At2g22290
RABA3	At1g01200	RABD2b	At5g47200	RABH1e	At5g10260
		RABD2c	At4g17530		
RABA4a	At5g65270				
RABA4b	At4g39990	**RabE**			
RABA4c	At5g47960	RABE1a	At3g53610		
RABA4d	At3g12160	RABE1b	At5g59840		
RABA4e	At2g22390	RABE1c	At3g46060		
		RABE1d	At5g03520		
RABA5a	At5g47520	RABE1e	At3g09900		
RABA5b	At3g07410				
RABA5c	At2g43130	**RabF**			
RABA5d	At2g31680	RABF1	At3g54840		
RABA5e	At1g05810				
		RABF2a	At5g45130		
RABA6a	At1g73640	RABF2b	At4g19640		
RABA6b	At1g18200				

Table 4. Tethering factors in Arabidopsis thaliana. Based on its homologues in yeast and human, Golgin are located on the golgi apparatus on both *cis* and *trans* golgi network. The conserved oligomeric Golgi (COG) and transport protein particles (TRAPP I and TRAPPII), facilitate ER–Golgi and intra-Golgi transport. DSL1 between Golgi–ER retrograde transport and the Golgi-associated retrograde protein (GARP) complex work in retrograde transport from endosomes to the Golgi/TGN. The Exocyst and HOPS are involved in post-Golgi trafficking events (Koumandou et al. 2007; Latijnhouwers et al. 2005). Green color indicates putative localization of the components in chloroplasts (Paper **III**).

Golgin	
AtGC1	At2g19950
AtGC2	At1g18190
AtGC3	At3g61570
AtGC4	At2g46180
AtGC5	At1g79830
AtGC6	At3g27530
AtGRIP	At5g66030
AtCASP	At3g18480
Dsl1Complex	
Dsl1like/ZW10	AT2G32900
Sec20-like	At3g24315
Tip20-like	At1g08400
	At3g47700
COG Complex	
COG1-like	At5g16300
COG2-like	At4g24840
COG3-like	At1g73430
COG4-like	At4g01400
COG5-like	At1g67930
COG6-like	At1g31780
COG7-like	At5g51430
COG8-like	At5g11980
Exocyst Complex	
Exo70	
Exo70A1	At5g03540
Exo70A2	At5g52340
Exo70A3	At5g52350
Exo70B1	At5g58430
Exo70B2	At1g07000
Exo70C1	At5g13150
Exo70C2	At5g13990
Exo70D1	At1g72470
Exo70D2	At1g54090
Exo70D3	At3g14090
Exo70E1	At3g29400
Exo70E2	At5g61010
Exo70G1	At4g31540
Exo70G2	At1g51640
Exo70F1	At5g50380
Exo70H1	At3g55150
Exo70H2	At2g39380
Exo70H3	At3g09530
Exo70H4	At3g09520
Exo70H5	At2g28640
Exo70H6	At1g07725
Exo70H7	At5g59730
Exo70H8	At2g28650
Sec3-like	At1g47550
	At1g47560
Sec5-like	At1g21170
	At1g76850
Sec6-like	At1g71820
Sec8-like	At3g10380
Sec10-like	At5g12370
Sec15-like	At3g56640
	At4g02350
Exo84	
Exo84a	At1g10385
Exo84b	At5g49830
Exo84c	At1g10180
TRAPPI Complex	
Bet3-like	At5g54750
Bet5-like	At1g51160
Trs85-like	At5g16280
Trs20-like	At1g80500
Trs23-like	At5g02280
Trs31-like	At5g58030
Trs33-like	At3g05000
TRAPPII Complex	
Trs120-like	At5g11040
Trs130-like	At5g54440
GARP Complex	
Vps52-like	At1g71300
Vps53-like	At1 g50500
Vps54-like	At4g19490
HOPS	
Vps11-like	At2g05170
Vps16-like	At2g38020
Vps18-like	At1g12470
Vps33-like	At3g54860
Vps39-like	At4g36630
	At1g22860
Vps41-like	At1g08190

Table 5. Arabidopsis thaliana SNARE proteins (Bassham et al. 2008; Sanderfoot 2007). Green color indicates putative localization of the components in chloroplasts (Paper **III**). ER: endoplasmic reticulum, LE: late endosome, PM: plasma membrane, PVC: pre vacuolar compartment, TGN: trans Golgi network.

Qa-SNAREs		Localization
SYP81	At1g51740	ER
SYP31	At5g05760	Golgi
SYP32	At3g24350	Golgi
SYP41	At5g26980	TGN
SYP42	At4g02195	TGN
SYP43	At3g05710	TGN
SYP21	At5g16830	Vacuole, LE
SYP22	At5g46860	Vacuole, LE
SYP23	At4g17730	Vacuole, LE
SYP24	At1g32270	Vacuole, LE
SYP111	At1g08560	PM, CP
SYP112	At2g18260	PM, CP
SYP121	At3g11820	PM
SYP122	At3g52400	PM
SYP123	At4g03330	PM
SYP124	At1g61290	PM
SYP125	At1g11250	PM
SYP131	At3g03800	PM
SYP132	At5g08080	PM
Qb-SNAREs		
SEC20	At3g24315	ER
MEMB11	At2g36900	ER/Golgi
MEMB12	At5g50440	ER/Golgi
GOS11	At1g15880	Golgi
GOS12	At2g45200	Golgi
VTI11	At5g39510	Vacuole, TGN
VTI12	At1g26670	Vacuole, TGN
VTI13	At3g29100	Vacuole, TGN
VTI14	At5g39630	Vacuole, TGN
NPSN11	At2g35190	PM, TGN
NPSN12	At1g48240	PM, TGN
NPSN13	At3g17440	PM, TGN
Qc-SNAREs		
USE11	At1g54110	ER
USE12	At3g55600	ER
BET11	At3g58170	ER/Golgi
BET12	At4g14455	ER/Golgi
SFT11	At4g14600	Golgi
SFT12	At1g29060	Golgi
SYP51	At1g16240	Vacuole, LE/PVC, Golgi
SYP52	At1g79590	Vacuole, LE/PVC, Golgi
SYP61	At1g28490	TGN
SYP71	At3g09740	PM, ER
SYP72	At3g45280	PM, ER
SYP73	At3g61450	PM, ER
Qa+b-SNAREs		
SNAP33	At5g61210	PM
SNAP29	At5g07880	PM
SNAP30	At1g13890	PM
R-SNAREs		
SEC221	At1g11890	ER,Golgi
SEC222	At5g52270	
YKT61	At5g58060	Golgi, Vacuole
YKT62	At5g58180	Golgi, Vacuole
VAMP711	At4g32150	TGN, Vacuole
VAMP712	At2g25340	TGN, Vacuole
VAMP713	At5g11150	TGN, Vacuole
VAMP714	At5g22360	TGN, Vacuole
VAMP721	At1g04750	TGN, PM
VAMP722	At2g33120	TGN,PM
VAMP723	At2g33110	TGN, ER
VAMP725	At4g15780	TGN, PM
VAMP726	At2g32670	TGN, PM
VAMP728	At3g24890	TGN, PM
VAMP724	At1g04760	TGN, PM
VAMP727	At3g54300	TGN, PM, EE
TYN11	At5g05570	PM
TYN12	At4g35560	PM

6. VESICLE TRANSPORT INSIDE THE CHLOROPLAST

The observations of vesicles in leaves exposed to low temperature (Morré et al. 1991b) and in isolated chloroplasts treated with specific vesicle fusion inhibitors (Westphal et al. 2001) indicate similarities to vesicle transport in the cytosol. Several proteins have been identified and predicted to play a role in vesicle formation, scission and fusion being the most important for thylakoid biogenesis.

6.1. Vesicle-inducing protein in plastids 1 (VIPP1)

VIPP1, a nuclear encoded protein, has been shown to be involved in vesicle budding at the inner envelope of the chloroplast. It is required for thylakoid membrane maintenance, however it has been suggested that VIPP1 is not required for lipid accumulation (Kroll et al. 2001). This opens up the possibility that VIPP1 could be involved with the transport of proteins as a cargo from the inner envelope membrane to thylakoids. It has been shown that VIPP1 could be involved in the reorganization of the thylakoid structure to facilitate protein transport in the cpTat pathway (Lo and Theg 2012). The VIPP1 proteins form a complex or ring-like structure on the inner envelope membrane; this oligomerization is important for binding to the envelope (Aseeva et al. 2004; Otters et al. 2012), and for VIPP1 function in thylakoid membrane formation, but is not important for the assembly of thylakoid protein complexes (Aseeva et al. 2007). Interestingly, the VIPP1 ring forms a rod-like structure similar to the microtubule structure, which is regulated by a HSP70 chaperone in *Chlamydomonas* (Liu et al. 2007). This cytoskeleton type of structure is needed for vesicle transport in the secretory system, thus VIPP1 could function as a motor for vesicles in the chloroplast.

In addition, many of the components of the TOC-TIC protein import apparatus and VIPP1 were identified by mass spectroscopy in material co-immunoprecipitated with antibodies to actin, suggesting that an actin-TOC-TIC-VIPP1 complex may provide a means of channeling cytosolic preproteins to the thylakoid membrane (Jouhet and Gray 2009a; b). Thus, VIPP1 could be involved in several functions during vesicle transport: it could function in a similar way to the cytosolic vesicle coated proteins by being involved in membrane bending and protein sorting, and the VIPP1 ring-like structure could function in vesicle fission similar to a dynamin GTPase (Vothknecht et al. 2012).

6.2. Thylakoid formation1 (THF1)

In Arabidopsis it has been shown that THF1 is important for leaf and chloroplast development by maintaining thylakoid stacks. It has been shown to be localized in the envelope, thylakoid and the stroma of the chloroplast (Wang et al. 2004). Interestingly, it has been shown that in *thf1* the non-green areas in the leaves show vesicle accumulation when lacking thylakoids, suggesting that vesicles fusion does not occur due to the absence of an organized thylakoid structure (Wang et al. 2004). THF1 could perform more than one function as it has been shown that it is also localized to the outer envelope membrane as well in the stroma in root plastids, and it interacts with the

plasma membrane G-protein GPA1, which works together in sugar signaling mechanisms (Huang et al. 2006).

6.3. Dynamin GTPases and fusion proteins

Dynamin is a large GTPase group that plays a critical role in vesicles scission, organelle fission, fusion and cytokinesis (Praefcke and McMahon 2004). In the chloroplast there are indications of localization of dynamin-like proteins. In Arabidopsis FZL (Fuzzy-onions Like), an FZO_like dynamin-like protein, is found in both the inner envelope membrane and the thylakoid and is important for thylakoid and chloroplast morphology. In accordance with FZO, it is suggested that FZL could also function in membrane fusion (Gao et al. 2006). Although its role in membrane fusion is unclear, the existing evidence for vesicle transport from the envelope that FZL could be a potential candidate for vesicle fusion at the thylakoid membrane. The ADL1a is dynamin-like protein in Arabidopsisand has been shown to be localized in the thylakoid and involved in thylakoid biogenesis (Park et al. 1998). Another dynamin-like protein (ADL2a) in Arabidopsis has been shown to be involved in vesicle formation, particularly the vesicle scission at the chloroplast inner envelope membrane (Kim et al. 2001).

NSF (N-ethylmaleimide-sensitive factor) functions as a SNARE chaperone and utilizes the energy of ATP hydrolysis to disassemble SNAREs, thus facilitating SNARE recycling (Zhao et al. 2007). It has been shown that a plastid fusion and/or translation factor (Pftf) has been involved in the vesicle fusion process. This factor shows high sequence similarity to yeast and animal NSF, and FtsH of bacteria (Hugueney et al. 1995). Indeed, the yeast and animal NSF also shows similarities to the chloroplastic FtsH chaperone. Interestingly, FtsH1 and FtsH5 are suggested to be involved in thylakoid formation (Chen et al. 2001; Sakamoto et al. 2002; Takechi et al. 2000). As there are SNAREs predicted to be localized in the chloroplast, this suggests that SNARE disassembly takes place in thylakoids with the help of FtsH (Paper **III**).

7. COPII RELATED TRANSPORT IN CHLOROPLAST

Transport from ER and Golgi is mediated by COPII vesicles and is well characterized in yeast and animals, and to some extent in plants. Vesicle transport in the chloroplast shows a similarity to the cytosolic secretory system (Morré et al. 1991b; Westphal et al. 2001), which indicates that there could be similar factors involved in both systems. In fact, it has been shown using bioinformatics that homologues of the COPII i.e. Sec23/Sec24, Sec13/Sec31 and Sar1 GTPase and COPI GTPase Arf1 could exist (Andersson and Sandelius 2004). To expand whether there are more components involved in chloroplast COPII-like transport we also did a bioinfomatic search and found that there could be possible candidates for vesicle initiation, budding, tethering and fusion (Paper **III**). We predicted a Sec12-like GEF that may activate CPSAR1 on the inner envelope. There could be tethering factors in the chloroplast to help in docking, and SNAREs to fuse the vesicles possibly on the thylakoids (Figure 5). We found that the components required for vesicle initiation and budding are more related to COPII, but the SNAREs and tethering factors are similar to the one helping COPI and CCV

components during docking and fusion. Interestingly, preliminary evidence shows that there are no COPI and CCV components in the chloroplast (unpublished results, Alezzawi M, Khan NZ, Aronsson H), suggesting that the chloroplast is only using COPII-like vesicle transport during budding, but for fusion it needs components which are more related to post-Golgi trafficking. Furthermore, we also predict the possibility of cargo proteins to be transported from the envelope to the thylakoid (Figure 5). In COPII transport the cargo that is selected in these vesicles possess signals like di-hydrophobic, di-acidic, di-basic for transmembrane proteins and ILV for soluble proteins (Sato and Nakano 2007). These signals have been confirmed on the thylakoid proteins (Paper **III**). Altogether the existence of vesicles, prediction of COPII components and cargos suggest the possibility that in addition to lipids, proteins could be transported as a cargo from the envelop to the thylakoid.

The previous results raise the possibility that if COPII type vesicle transportation exists in the chloroplast it could be involved in the transport of lipids and proteins from the envelope to the thylakoid. According to the prediction tools and proteomic data the components could be localized in the chloroplast but there is evidence that most of the predicted components (Andersson and Sandelius 2004; Paper **III**) are localized in the secretory system, which could be explained by a dual targeting mechanism. So far, there is no direct evidence of the involvement of COPII mediated transport, so there is a need for biochemical and genetic characterization of these components to confirm whether COPII's role is similar to its cytosolic counterparts. If the localization confirms it to be in the chloroplast, it would mean that the same kind of system is utilized in both the cytosol and chloroplasts.

7.1. CPSAR1

In COPII vesicle transport the Sar1 GTPase is involved in the initiation of budding at the ER. A similar protein found in chloroplasts has been named CPSAR1 due to its presence in chloroplasts (Andersson and Sandelius 2004; Garcia et al. 2010). Its role has been debated due to its similarity with the Obg (Spo0B-associated GTP-binding protein) subfamily. It has been characterized under different names such as CPSAR1 (Paper **I**) , 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 subfamily function is still unknown but previous results suggest that it could be involved in stress response, sporulation and ribosome synthesis (Kobayashi et al. 2008). It has been proposed that CPSAR1 (ObgC) has a role in ribosome biogenesis (Bang et al. 2012; Bang et al. 2009). In line with this it has also been suggested that CPSAR1 affects the PEP (Plastid-Encoded RNA Polymerase) pathway for plastid encoded genes, (Bang et al. 2012). However, the phenotype of RNAi *obgc* mutant is more severe in the seedling stage than in mature stage, conflicting the fact that PEP enzyme levels are increasing as the plastid matures (Cahoon et al. 2004). At the mature level the severe effect in the RNAi *obgc* mutant is abolished (Bang et al. 2012) therefore not fully supporting an important role of ObgC/CPSAR1 for PEP. Furthermore, it has been shown that plastid genes are transcribed by NEP (Nucleus-Encoded RNA Polymerase) in PEP-deficient tobacco plastids (Krause et al. 2000), thus the absence of ObgC/CPSAR1 does not necessarily have to be linked to PEP. Moreover, the highest expression of CPSAR1

occur at the early stages (Paper **I**) where the PEP enzyme level is low, and in the absence of CPSAR1 the plants are lethal (Paper **I**), indicating an additional function such as the proposed vesicle transport.

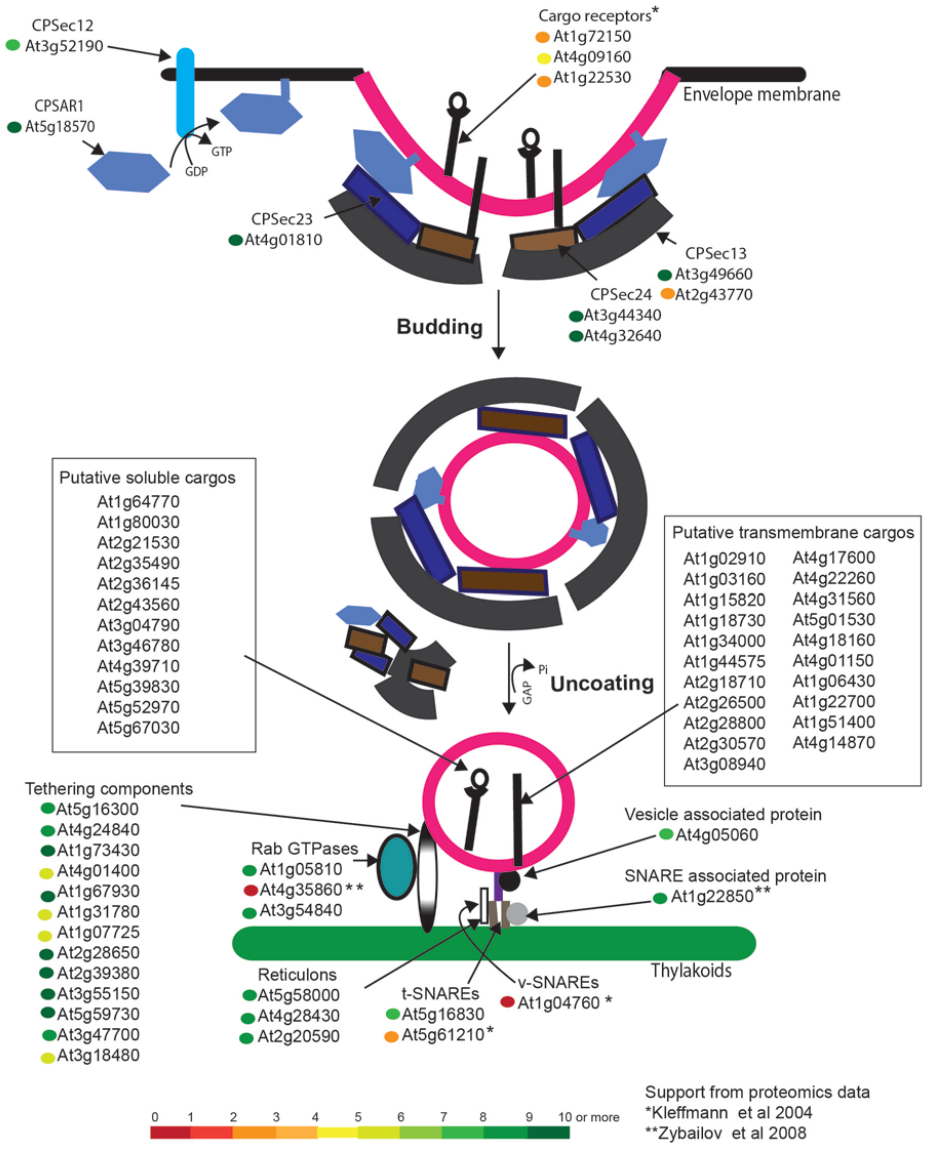

Figure 5. Putative model for vesicle transport inside the chloroplasts. The score given by ARAMEMNON is here represented by a color code, with a high score from left to right.

CPSAR1 has dual localization, being present both in soluble form in the stroma and membrane bound in the inner envelope, but not in the thylakoid (Paper **I**). This is similar to other GTPases such as Sar1, which is a soluble protein but contains a **S**ar1–NH_2-terminal activation recruitment (STAR) motif for membrane binding (Jones et al. 2003). However, CPSAR1 does not seem to have a STAR-like domain, but instead contains a long N-terminal stretch that is unique to CPSAR1. Given that CPSAR1 has been shown to be in the envelope, this N-terminal was suggested to help in binding to the envelope. Also, as it has been shown to be co-localized with vesicles in the stroma at low temperatures, this strengthens the proposal that CPSAR1 has a role in vesicle transport (Paper **I**). In addition, preliminary data show a slight increase of lipid when radiolabelled isolated envelopes from spinach were incubated with CPSAR1 (unpublished results, Khan NZ, Lindquist E and Aronsson H), which gives an indication of vesicle budding/transport that requires GTP hydrolysis (Räntfors et al. 2000). It is still unclear whether CPSAR1 plays a direct role in vesicle initiation and budding, though at this point it is evident that it is involved in vesicle transport.

To search for possible proteins co-expressed with CPSAR1 the public available database ATTED-II were used and proteins were found having functions for GTP binding, protein synthesis, and embryo lethality (Paper **II**). However, unlike our prediction, no coated proteins were identified (Andersson and Sandelius 2004; Paper **III**). As CPSAR1 (ObgC) has been shown to interact with ribosomal subunits (Bang et al. 2012) and some of the proteins predicted by ATTED-II database were involved in protein synthesis, it suggests that it plays a role in ribosome biogenesis. On the other hand, a co-immunoprecipitation using CPSAR1 as bait detected several thylakoid proteins, most of them having a role in photosynthesis (unpublished results, Khan NZ, Karim S, Aronsson H). One of the proteins detected was an LHC protein that has recently been suggested to be transported using vesicles (Tanz et al. 2012). Another protein found was THF1 which is important during vesicle transport (Wang et al. 2004). This data support the idea that CPSAR1 has a role in vesicle transport. At this stage it has to be suggested that CPSAR1 (ObgC) could be involved in more than one function: either it retains the bacterial Obg function involving ribosome biogenesis, or it could have evolved during endosymbiosis to acquire a new cellular function – in this case it would be vesicle transport.

7.2. CPRabA5e

Rab GTPases play a vital part in vesicle transport from budding to fusion by recruiting their effector proteins, such as tethering factors, adopters, kinases, phosphatases and motors (Stenmark 2009). In Arabidopsis 57 Rab GTPases have been classified (Rutherford and Moore 2002; Vernoud et al. 2003), of these three Rab GTPases (AtRabA5e, AtRabB1c and AtRabF1) could be localized to the chloroplast (Paper **III**).

One of the three putative chloroplast localized Rab GTPases, AtRabA5e, from a sub class of 5 Rab GTPases (AtRabA5a-AtRabA5e), has been shown to be localized in the chloroplast (Paper **IV**) and is therefore named hereafter CPRabA5e (CP = chloroplast localized) to follow the same nomenclature used for CPSAR1. Previous data predicted CPRabA5e to be an Arf1 protein (Andersson and Sandelius 2004), but due to

the presence of Rab characteristic Cysteine residues present at the C-terminal and prediction of a Rab domain in Prosite Scan the name CPRabA5e is retained (Paper **III** and **IV**) as proposed previously (Rutherford and Moore 2002; Vernoud et al. 2003). In support of this the CPRabA5e cannot complement the yeast Arf1 homologue, but it instead complements the yeast Rab GTPase Ypt31/Ypt32 (Paper **IV**), which confirms that it could be working as a Rab GTPase. Furthermore, Ypt31 and Ypt32 are involved in vesicle transport in yeast (Segev 2001) further strengthen the possibility that CPRabA5e can have a similar role.

CPRabA5e has been shown to be localized with thylakoids and in the stroma but not with the envelope (Paper **IV**). This result gives us the idea that, similar to other Rab GTPases, its soluble inactive form is in the stroma but its active GTP form attaches itself to the membrane (the thylakoid membrane in this case). The active Rab GTPases work by regulating their effector molecules (Grosshans et al. 2006). The best understood function of Rabs is suggested during tethering factors and SNAREs. Tethering factors can work as a Rab GEF or Rab effectors to regulate downstream reactions (Cai et al. 2007). Tethering factors have been predicted to exist in the chloroplast, e.g., the COG complex, Exo 70 and AtCASP (Paper **III**) could all work as Rab effectors (Grosshans et al. 2006). One of the tethering complex called TRAPP works as a GEF for Ypt31/Ypt32, the closest homologues in yeast of CPRabA5e (Jones et al. 2000). However, there is no TRAPP or GEF found for Rab in the chloroplast using a bioinformatics search (Paper **III**). Thus, how CPRabA5e or other Rab GTPases predicted to be in the chloroplast are activated remains to be investigated. However, the presence of putative tethering factors that work as effectors for Rab GTPases and could be recruited and help vesicles tether to the thylakoid do exist (Paper **III**).

Our recent characterization of CPRabA5e show that mutant chloroplasts preincubated at 4ºC contained more vesicles close to the envelope membranes than wild-type chloroplasts. In addition, it has also been shown that CPRabA5e has a role in seed germination and oxidative stress (Paper **IV**). Under oxidative stress, vesicle formation was enhanced compared to wild type. Furthermore, in *cprabA5e* the plastoglobuli (storing lipids of thylakoid membrane) size are increased, which suggests a function in the maintenance of the thylakoid membrane by regulating the plastoglobuli (Paper **IV**). Further study is needed to confirm its exact role in seed germination and stresses. Anyhow, CPRabA5e does seem to have a clear link to vesicle transport as shown by ultrastructure analysis of its chloroplasts. The closest homologues in yeast and mammals of CPRabA5e work on the post-Golgi trafficking by helping tethering factors and SNAREs during fusion, and interact with the cytoskeleton to help motility of vesicles. Whether the CPRabA5e conserves all these functions and works in conjunction with tethering factors and SNAREs in the chloroplast, or whether it has evolved to have a different role in the chloroplast is still to be resolved.

8. CONCLUSION AND FUTURE PERSPECTIVE

It has to be considered that in chloroplasts the proteins shown to be involved in vesicle transport (e.g. VIPP1, THF1, FZL and CPSAR1) all are important for thylakoid development. This gives us an indication that these proteins could work together during vesicle transport from the envelope to the thylakoid or could be involved in different kinds of vesicles. As discussed it has been proposed that VIPP1 is responsible for making a rod-like structure similar to tubules, and VIPP1 has been found in both the envelope and thylakoid membranes. Thus, it could help in vesicle motility to the thylakoid from the envelope or it could have a role in vesicle coating. CPSAR1 could help in vesicle formation, with the help of coat proteins, to select cargos and lipids. ADL2a and FZL may be involved in vesicle fission and fusion, respectively. THF1 could have role in the fusion of vesicles with the thylakoid, collaborating with SNAREs and tethering factors to deliver the cargo and lipids. Finally, FtsH, in accordance with its similarity to NSF, may help in the recycling of vesicle components, especially the SNAREs.

The chloroplast vesicle transport field is just above 20 years old by now. It initially mainly considered for lipid transport to the thylakoid. However, whether vesicles can transport protein to the thylakoid as well to maintain the thylakoid integrity and its photosynthesis activity are both still to be proven. My thesis is an attempt to expand the amount of knowledge about possible components involved in chloroplast vesicle transport, which seems more similar to the COPII transport system in the cytosol when predicting budding and tethering factors, SNAREs and Rab GTPases for chloroplast localization. Through my studies and results I speculate that cargo transport in chloroplast vesicles may include proteins and not only lipids. So far, CPSAR1and CPRabA5e have been characterized and shown to be in the chloroplast, and both counterparts do have roles in the cytosolic vesicle transport. However, in the chloroplast their roles are still ambiguous and further experimentation is needed in order to clarify their exact involvement in vesicle transport in chloroplasts. In the meantime, preliminary data have identified protein interactors of both CPSAR1 and CPRabA5e using Co-IP and yeast-2-hybrid; both results that will need further investigation. In parallel it is also important to confirm the localization of predicted proteins identified through the bioinformatics approach. If one can confirm the localization of the coated proteins, SNAREs and tethering factors, it will give us a more reliable indication of the existence of a vesicle transport system similar to the secretory system.

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11. Populärvetenskaplig sammanfattning på svenska

Fotosyntes kallas processen i växter där kolhydrater och syre bildas. Processen är av stor vikt för människan eftersom vi behöver båda dessa produkter, som mat och för att andas. För att fotosyntesen ska fungera behöver växten koldioxid och solljus. Med hjälp av detta produceras kolhydrater, med syrgas som en biprodukt. Fotosyntesen sker inuti växtceller i en avdelning som kallas kloroplast, närmare bestämt på ett ställe som kallas grana inuti kloroplasten.

Membran kallas det hölje som omger eller utgör olika strukturer i celler. Kloroplasten omges av ett membran som kallas envelop, och grana omges av ett membran som kallas tylakoid. Det är i tylakoidmembranet som apparaten för fotosyntes finns. Tylakoidmembranet består av lipider och proteiner, men de flesta av dessa tillverkas utanför kloroplasten och måste därför transporteras till tylakoidmembranet.

I kloroplasten, mellan envelop och tylakoiden, finns en vattenbaserad lösning som kallas stroma. Eftersom lipider inte är vattenlösliga behövs ett transportsystem för att de ska ta sig från envelop till tylakoiden. Lipider transporteras till tylakoiden genom att de bildar en bubbla, en så kallad vesikel, och på så vis kan de färdas i vattenlösningen.

Vi tror att dessa vesiklar också kan bära med sig proteiner. Ett liknande mer välstuderat system för transport av lipider och protein finns utanför kloroplasten i cellens cytosol, som är vätskan i cellen. I cytosolen finns tre typer av vesiklar, en av dessa kallas COPII. COPII vesiklar transporterar proteiner från olika ställen i cellen; från endoplasmatiska retikulumet till Golgi-apparaten. Systemet fungerar med hjälp av olika proteiner; Sar1 heter ett protein som startar vesikelbildningen, andra protein omger vesikeln och underlättar att krökning av membranet sker så vesikeln blir rund och lossnar.

Man har trott att proteiner som liknar Sar1 samt de omgivande proteinerna också kan finns i kloroplaster och i denna avhandling visar vi att det mycket riktigt finns ett protein som liknar Sar1 i kloroplasten. Detta protein visade sig vara viktigt för uppbyggnad av tylakoiden och är en del av vesikeltransporten. Detta gav stöd åt hypotesen att det finns ett vesikelsystem liknande COPII i kloroplasten.

Det behövs dock fler protein än de som nämnts ovan för en funktionell vesikeltransport. Det behövs till exempel proteiner som gör att vesikeln fångas in och fästs på tylakoiden. Även dessa proteiner har förutspåtts att finnas i kloroplasten genom att vi gjort jämförelser med de proteiner som jobbar i cytosolen.

Våra resultat stöder att det finns vesikeltransport i kloroplasten men mycket återstår att undersöka, t ex behöver fler medverkande protein karakteriseras.