1 Introduction

Chloroplasts are organelles found in plant cells and eukaryotic algae that conduct photosynthesis. It has been estimated that about 3,500 proteins are required to build up a functional chloroplast (The Arabidopsis Genome Initiative, 2000; Emanuelsson et al., 2000). Among these 3,500 proteins, only about 100 proteins are encoded by the plastid genome while all the others are encoded by nuclear DNA and synthesized in the cytosol. Thus, to perform their function, all these nuclear-encoded proteins must be transported from outside into the chloroplast (Keegstra and Cline, 1999; Jarvis and Robinson, 2004).

However, transport of these proteins is complicated due to the existence of biological membranes which compartmentalize the chloroplast and maintain the characteristic differences between the contents of the chloroplast and the cytosol. Thus, for transporting of these nuclear-encoded proteins, elaborate protein transport systems have been developed in the membranes of chloroplast.

1.1 The structure of chloroplasts

The chloroplast of higher plants is made up of three types of membranes (Figure 1.1):

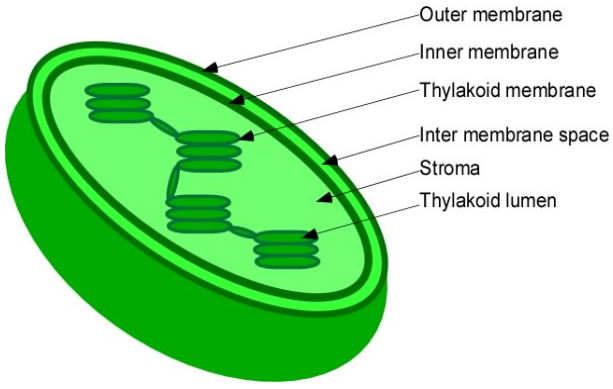

Fig 1.1: The structure of chloroplast.

(1) Outer membrane which is freely permeable to *small* molecules.
(2) Inner membrane which contains many transporters and is highly specialized with transport proteins.

(3) Thylakoid membranes which form a network of flattened discs called thylakoids. In the thylakoid membranes, the proteins responsible for photosynthesis and electron transport are embedded forming at least five multisubunit oligomeric complexes for photosynthesis, including the photosystems I and II and their light harvesting antenna (LHC, light harvesting complex), the cytochrome complex and the ATP synthase (Andersson and Barber, 1994; Herrmann, 1996). Some of these complexes work together to carry out the so-called "light-reactions" of photosynthesis.

Accordingly, separated by these three membranes, the chloroplast is divided into three distinct internal compartments:
(1) The intermembrane space between the two membranes of the chloroplast envelope;
(2) The stroma which lies inside the envelope but outside the thylakoid membrane. The stroma contains for example: (a) the enzymes, like RuBisCO, required to carry out the "dark-reactions" of photosynthesis; that is, the conversion of CO_2 into organic molecules like glucose; (b) a number of DNA molecules, each of which carries the complete chloroplast genome that encode around 100 proteins.
(3) The thylakoid lumen which contains many proteins that are important for photosynthesis processes like water splitting, electron transport etc.

1.2 Protein transport in chloroplasts

To allow protein passage through these three different membranes, chloroplast has developed different molecular machines in each membrane (Figure 1.2): for the outer and inner envelope membranes, the translocons referred to as Toc (Translocon at the outer envelope membrane of chloroplasts) and Tic (Translocon at the inner envelope membrane of chloroplasts), respectively. However, for transport into or across the thylakoid membrane, at least four transport mechanisms, called SRP (Signal Recognition Particle), Spontaneous, Sec (Secretory) and Tat (Twin arginine translocation)-dependent pathway, have been identified (Keegstra and Cline, 1999; Jarvis and Robinson, 2004; Gutensohn et al., 2006).

1.2.1 Passing through the envelope membrane (Toc and Tic)

The Toc translocon is composed of the receptor components, including Toc159 and Toc34, as well as Toc64 (Kessler et al., 2004; Qbadoua et al., 2007) for precursor recognition, and the translocation channel component (Toc75) (Schnell et al., 1994). Another component of the Toc complex is Toc12, which recruits the Hsp70 (Heat shock protein 70) of outer envelope membrane to the intermembrane space and facilitates the interaction of Hsp70 with the precursors (Becker et al., 2004). One recent model

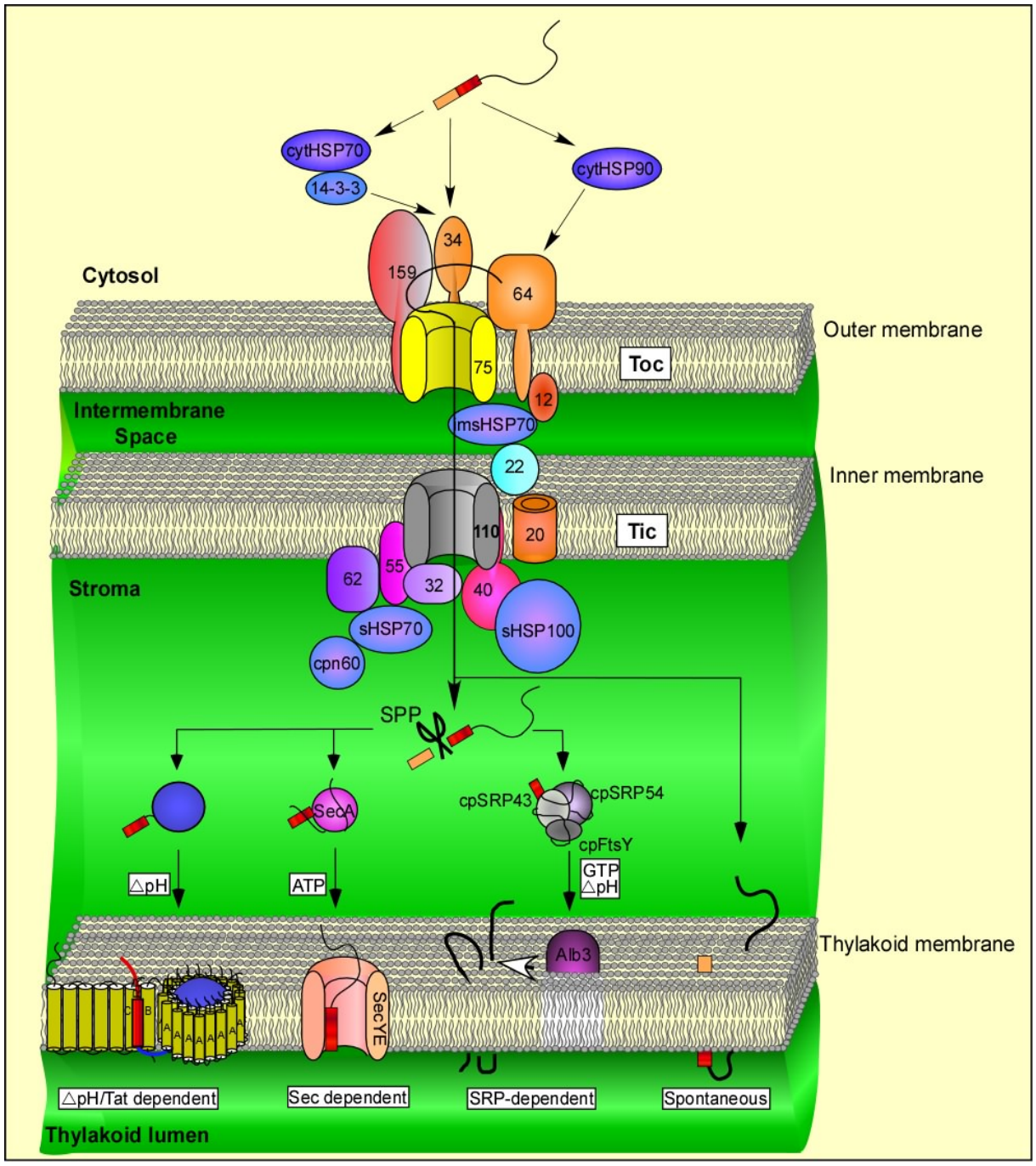

Fig 1.2: **Overview of the protein transport pathways in chloroplast.** The components of the Toc and Tic complexes are designated according to their molecular weight. For each of the four protein transport pathways operating at the thylakoid membrane, the stromal and thylakoidal factors involved are shown. The signal peptide of the stroma targeting domain (STD) is depicted as orange rectangle, thylakoid lumen targeting domain (LTD) as red rectangle. Likewise, the stromal processing peptidase (SPP) is shown as black scissors but thylakoid processing peptidase is not shown. For each pathway, the respective energy sources or driving forces are indicated. All further details are explained in the text. (14-3-3: 14-3-3 protein, cpFtsY: chloroplast SRP receptor FtsY, cpn60: chaperonin 60, cpSRP: chloroplast signal recognition particle, cyt-/ims-/s-HSP70: cytosolic-/intermembrane space-/stromal-heatshock protein 70 kDa, SPP: stromal processing peptidase.).

suggests that Toc translocon is formed by a single central Toc159 molecule that is surrounded by four copies of Toc75/Toc34 (Stengel et al., 2007). During the transport process, one set of precursors is directly recognized by Toc34 receptor, while another set of the precursors will be handled through either a cytosolic guidance complex with a 14-3-3 protein as the central component or cytosolic Hsp90 (May and Soll, 2000; Qbadou et al., 2006) and is then recognized by Toc34.

Tic translocon is probably composed of at least Tic110, Tic62, Tic55, Tic40, Tic32, Tic22, and Tic20 (Fig. 1.2; Soll and Schleiff, 2004; Gutensohn et al., 2006). Among them, the presumed basic components of Tic machinery include: (1) Tic22, a soluble protein that is peripherally associated with the inner envelope membrane from the inter membrane space and is assumed to be the first Tic component interacting with the incoming precursor protein (Kouranov and Schnell, 1997; Kouranov et al., 1998); (2) two integral membrane proteins, Tic20 and Tic110, both are assumed to be the translocation pore components (Kessler and Blobel, 1996; Kouranov et al., 1998; Heins et al., 2002); (3) several molecular chaperones including Hsp100 and Chaperonin-60 (Cpn60), which have been reported to interact from the stromal side with the Tic complex (Kessler and Blobel, 1996; Akita et al., 1997; Nielsen et al., 1997). At present, more works are required for understanding the Tic complex and its transport mechanism.

1.2.2 Passing through the thylakoid membrane

After passing through the Toc and Tic complexes, the proteins arrive in the stroma (Fig 1.1 and 1.2), where the N-terminal transit peptide, the so-called stroma-targeting-domain (STD), is proteolytically removed by a large monomeric enzyme called stromal processing peptidase (SPP) (Van der Vere et al., 1995). Depending on their final destination, further sorting of these proteins into or across the thylakoid membrane is handled by at least four protein transport pathways: the SRP-dependent and 'spontaneous' pathways mainly for insertion of proteins into the thylakoid membrane, and the Sec-dependent and ΔpH/Tat-dependent pathways mainly for transport of proteins into the thylakoid lumen (Fig 1.2 and Jarvis and Robinson, 2004; Gutensohn et al., 2006).

The SRP dependent pathway

The well known substrate of this pathway (Fig 1.2) is light-harvesting chlorophyII a/b-binding protein (LHCP). The analysis of this protein has shown that, after the signal peptide being removed by SPP inside the stroma, the substrate will be recognized and bound by a soluble 54 kDa GTPase (cpSRP54) and a plant specific stromal 43-kDa protein (cpSRP43). Together, they form a "transit complex"(Schünemann, 2004). The transit complex interacts further with cpFtsY – a protein that possibly functions as

a receptor (Kogata et al., 1999). Then the complex targets to a not yet identified translocase for membrane integration. Alb3, one of such an integral membrane protein, has been shown to be involved in this integration process (Sundberg et al., 1997; Moore et al., 2000). However, it is still not known, how many other membrane components are involved in LHCP integration and how the LHCP inserts into the thylakoid membrane. GTP hydrolysis provides the power for LHCP integration and ATP stimulates this process if it is present in combination with GTP (Hoffman and Franklin 1994; Yuan et al., 2002). Possibly, GTP is required to regulate the interaction of the GTPases cpSRP54 and cpFtsY during delivery of LHCP to the translocon (Schünemann, 2004). However, the role of ATP in LHCP insertion is completely unresolved since no ATP-binding proteins involved have yet been identified (Schünemann, 2004). Additionally, plastome encoded thylakoid membrane proteins, like D2, CP43, PSI-A and CFoIII, possibly also use SRP-dependent pathway to integrate into the thylakoid membrane but in a co-translational manner (Pasch et al., 2005).

The spontaneous pathway

Another set of thylakoid integral membrane proteins seem to require neither any known protein transport machinery or essential targeting factors nor energy for insertion into the thylakoid membrane. This feature leads to the designation of "spontaneous insertion mechanism" which constitutes a mainstream pathway for bitopic membrane proteins (Schleiff and Klösgen, 2001). Example substrates of this pathway are CFoII, the photosystem II subunits PsbW, PsbX, and PsbY as well as PsaK from Photosystem I and the SecE subunit (cpSecE) (Michl et al., 1994; Lorkovic et al., 1995; Kim et al., 1998; Thompson et al., 1998). The key feature requirements for the substrates of this pathway are: (i) they must have two hydrophobic domains provided one by the membrane anchor of the mature protein and the other by the signal peptide; (ii) the hydrophilic domain between the two hydrophobic domains is negatively charged while the extreme termini of the two hydrophobic domains, i.e. the N-terminus of the first hydrophobic domain and the C-terminus of the second hydrophobic domain, must be positively charged (Michl et al., 1994, 1999).

The Sec pathway

Beside the SRP dependent and the spontaneous pathway, which integrate most of the thylakoid membrane proteins, some other membrane proteins, like PSI-F, Cytochrome f, plastid fusion/protein translocation factor (Pftf), and Rieske protein, can also be integrated into the thylakoid membrane but by the other two transport pathways: Sec- (Cytochrome f, PSI-F) and Tat-dependent pathway (Pftf, Rieske) (Karnauchov et al., 1994; Nohara et al., 1996; Summer et al., 2000; Molik et al., 2001). However, these

latter two pathways are mainly responsible for the transport of proteins that function inside the thylakoid lumen. These two pathways have been characterized based on their transport requirements.

One subgroup of precursors is absolutely dependent on ATP as well as stromal extract and is stimulated by the thylakoidal ΔpH. This is the Sec pathway, which resembles the well-characterized Sec systems in bacterial inner membranes.

***The Sec signal peptides*:** The Sec signal peptides comprise three domains: a short, positively charged amino-terminal domain (N-domian); a central hydrophobic domain (H-domain); and a more polar carboxy-terminal domain (C-domain) containing the signal peptidase cleavage site (von Heijne, 1998). The length of N-domain is variable for different precursors while the H-domain is ∼15 residues long, on average. The end of the N-domain (to the H-domain terminus) is often occupied by charged residues. In the C-domain, basic residues are always lacking in contrast to the Tat signal peptides which frequently contain basic residues (Mori and Cline, 2001).

***The Sec translocon*:** In comparison with the Sec system in bacteria, thylakoid Sec components, SecA, SecY and SecE (Fig. 1.2) have been cloned (Berghöfer et al., 1995; Berghöfer and Klösgen, 1996) and shown to be involved in thylakoid protein transport process using *in vitro* assays. Presumably, SecY and SecE form the translocation pore in the thylakoid membrane (Mori and Cline, 2001; Jarvis and Robinson, 2004; Gutensohn et al., 2006). In bacteria, additional components like SecB, SecG, SecD, SecF or YajC, are also involved in the Sec transport. However, no chloroplastic homologous subunits have been identified in the *Arabidopsis* genome. Thus, it remains to be seen whether additional components are involved and how the thylakoidal Sec translocon is organized.

***The chaperones involved in Sec transport*:** Like its bacterial counterpart, proteins transported by thylakoid Sec pathway are also in an unfolded state which has been demonstrated experimentally using dihydrofolate reductase (DHFR) as a transport substrate (Endo et al., 1994; Hynds et al., 1998). In bacteria, this unfolded state is maintained by the action of chaperones like SecB (Manting and Driessen, 2000). Several chaperones like Hsp70, Hsp60, Rubisco activase were found in the stroma of chloroplasts (Jackson-Constan et al., 2001). However, no such chaperones affecting the Sec pathway have been identified.

SecA functions as an ATPase that powers the translocation of the polypeptides across the Sec translocase (Lill et al., 1989). Chloroplast SecA (cpSecA) has a dual localization in both the stromal and thylakoid fractions (Nakai et al., 1994; Yuan et al., 1994). The

presence of galactolipid and only a small fraction of anionic lipid optimally stimulate the SecA activity (Sun et al., 2007). Furthermore, SecA activity could only be stimulated by thylakoidal Sec-dependent signal peptides but not *E.coli* Sec signal peptides indicating that cpSecA probably has been evolved to be specifically well suited for the environment of the chloroplast thylakoid and to recognize thylakoidal Sec-dependent proteins thus ensures the pathway specificity (Sun et al., 2007). In this regard, chloroplast SecA could be considered as a chaperone.

***The energetics of Sec transport*:** When stroma extract was preincubated with anti-cpSecA antibodies prior to *in vitro* import assays, the transport of Sec precursors like OEC33 kDa protein and Plastocyanin (PC) into thylakoids was completely blocked (Nakai et al., 1994). Chloroplast SecA can be azide insensitive (spinach) or azide sensitive (pea) (Berghöfer et al, 1995). Furthermore, depletion of ATP by apyrase (Hulford et al., 1994) or by use of the ATP-analog AMP-PNP all result in a Sec-transport abolishment (Berghöfer, 1998) suggesting that ATP hydrolysis is absolutely required for thylakoid Sec-dependent translocation. A trans-membrane potential is not essential for Sec-dependent transport, however, translocation of some precursor proteins could be stimulated by the presence of ΔpH (Yuan and Cline, 1994; Mant et al., 1995).

Even though only limited number of experiments have fosused on this aspect, it seems that thylakoidal Sec transport and its bacterial counterpart are highly similar in mechanism. For example, spinach plastocyanin can be transported by the Sec pathway of bacteria (Haehnel et al., 1994). Briefly, when thylakoidal Sec precursors arrive in the stroma of chloroplast, cpSecA binds (Sun et al., 2007) and directs these precursors to the thylakoid membrane (Keegstra and Cline, 1999). Then, they form a stable complex within the membrane that also contains chloroplast SecY (Mori and Cline, 2001). Functioning as a translocation motor, cpSecA partially inserts into the lipid bilayer and pushes the precursors through the Sec translocon (Fig 1.2).

The ΔpH/Tat pathway

In sharp contrast to Sec pathway, Tat pathway is very unique in several features (Mori and Cline, 2001; Robinson and Bolhuis, 2004; Müller and Klösgen, 2005; Gutensohn et al., 2006). First, *in vitro* thylakoid experiments have shown that the transport solely and strictly depends on the transmembrane proton gradient while no requirements of soluble factors or nucleoside triphosphates were found to be involved (Mould and Robinson, 1991; Cline et al., 1992; Klösgen et al., 1992). Second, a twin pair of arginine residues is located at the boundary of N- and H-domain of the Tat signal peptides which give rise to the name of Tat (Twin-arginine translocation) (Chaddock et al., 1995). Third, probably the most remarkable feature is, however, that this pathway is

able to transport folded polypeptide chains across the membranes. This pathway was evolutionary conserved both in thylakoid membranes of chloroplast and in bacteria and thus the known features of this pathway will be summarized together here.

***Tat signal peptides*:** Beside the common principles of signal peptides like tripartite structure (Schatz and Dobberstein, 1996; von Heijne, 1998), Tat signal peptides have several specific features particularly when compared to the Sec signal peptides:
(i) In the N-domain, the most notable one is the presence of a characteristic -RR- motif just prior to (i.e. on the N-terminal side of) the H-domain. Even at this point two highly atypical Tat substrates (Pftf and the Rieske FeS protein) could be considered as exceptions (Summer et al., 2000; Molik et al., 2001). Mutagenesis studies have shown that replacement of both arginine residues, even by lysine, lead to a complete block in Tat dependent translocation while the conservative substitution of a single Arg by Lys usually affects the translocation rate only (Chaddock et al., 1995; Stanley et al., 2000; DeLisa et al., 2002; Ize et al., 2002). The RR-motif is, however, not diagnostic for Tat-specific export. In *Bacillus subtilis*, only two of a large number of RR containing signal peptides have so far been proven to direct their passenger proteins to a Tat translocase (Jongbloed et al., 2000; van Dijl et al., 2002). Thus an RR-consensus motif even if predicted by improved algorithms (Dilks et al., 2003) is not compelling for a Tat-dependent export.
(ii) The H-domain of Tat signal peptides is less hydrophobic and relatively long when compared to that of Sec signal peptides. These features have been considered to be one of the so-called "Sec-avoidance" determinants (Cristobal et al., 1999). Further studies have also shown that, beside the important -RR- motif, the presence of a highly hydrophobic residue at the + 2 or + 3 positions, relative to the second arginine residue, was almost equally important for Tat transport (Brink et al., 1998).
(iii) The C-domain of Tat signal peptides is characterised by a high proportion of basic amino acids and is often positively charged when compared to the Sec signal peptides. This feature has been suggested as another determinant of the "Sec-avoidence" (Bogsch et al., 1997; Ize et al., 2002; Blaudeck et al., 2003). However, since only a subset of Tat substrates possesses this "Sec-avoidance" signature, other potential determinants for escaping the Sec pathway have to be identified or characterized (Müller and Klösgen, 2005). Additionally, it has been proven that the polarity or the charge of this domain has an effect on the signal peptidase cleavage (Frielingsdorf and Klösgen, 2007).

The 3D structure of Tat signal peptides is not very well characterized to date, except for two examples from bacteria. By use of Nuclear Magnetic Resonance (NMR) and H/D exchange mass spectrometry, it has been shown that the N- and H-regions of the signal sequence, including the twin-arginine motif, form an unfolded conformation regardless

of a completely and correctly folded mature domain following it (Kipping et al., 2003). In another study, analysis of SufI signal peptide by Circular Dichroism (CD) which is the simplest indication of protein and peptide secondary structure, it was concluded that this Tat signal peptide has two distinct states depending on the surrounding environment: In aqueous solution, the signal is unstructured while in membrane-mimetic environments such as SDS micelles or water/trifluoroethanol, the signal peptide contains α-helical structure which located in the center of the peptide, starting either just before or at the twin-arginine motif (Miguel et al., 2003). Further works are required at this point.

***The Tat translocon*:** Owing to the remarkable feature of Tat pathway to transport folded proteins (Clark and Theg, 1997; Hynds et al., 1998; Marques et al., 2003, 2004) and the fact that transported Tat substrates are highly variable in size (from 10-100 kDa), shape and surface features (Berks et al., 2000; Müller and Klösgen, 2005), the Tat translocase must be able to either change its pore size or to form series of different size pores to accommodate its divergent transport substrates, meanwhile avoiding the leakage of ions across the membrane. This special character indicates that possibly the Tat translocase is a dynamic or an active translocation pore. Indeed, recently it has been suggested that a functional Tat translocase is assembled only on demand, i.e. in the presence of transport substrate and a trans-membrane proton gradient (Mori and Cline, 2002).

The current model identifies TatA as the pore-forming component. It has been reported that TatA monomers oligomerize to form a transient pore to translocate the Tat substrates (Mori and Cline, 2002; Alami et al., 2003; Dabney-Smith et al., 2006). After transport, the translocon will be disassembled (Mori and Cline, 2002) for the next round of transport. In line with this, the *E. coli* TatA protein assembles when overexpressed in the membrane as series of oligomers, at least homotrimers or homotetramers (de Leeuw et al., 2002). This oligomerization is a intrinsic property of the transmembrane helix as its removal results in the recovery of TatA monomers (Porcelli et al., 2002). Using single particle electron microscopy, the low-resolution 3D structures show that these TatA oligomers of different sizes are similar in shape, i.e. a ring-shaped structure. Each thick-walled ring has an asymmetric lid at one end. The ring is approximately 50Å deep (enough to span the bilayer) and ∼30Å wide (Gohlke et al., 2005). Most importantly, their internal cavities of these rings increased with the number increase of the TatA monomer. Even though these results possibly only stand for a resting stage of Tat system, they strongly suggest that it is possible to change the pore size by adding or removing the TatA monomers. However, how TatA oligomerize together to form a functional translocase, whether an appropriately sized TatA channel is selected or if

the channel is formed by active recruitment of TatA monomers remains to be an open question (Sargent et al., 2006).

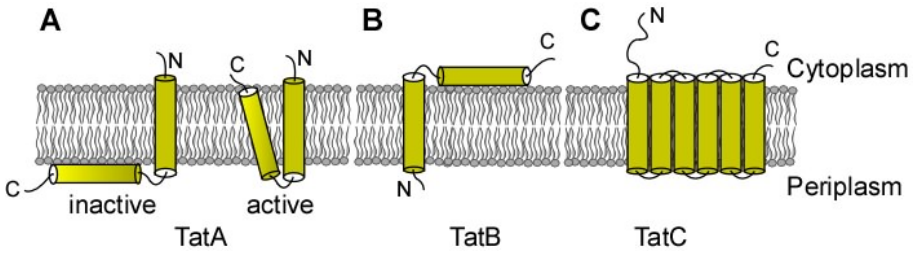

Fig 1.3: **The predicted topology of Tat proteins in *E.coli*. A**, TatA has a dual topology: (a) The inactive state with single transmembrane orientation in which N-terminus located on the cytoplasm side while C-terminus on the periplasm side; (b) The active state with double transmembrane orientation in which both N- and C-terminus located on the cytoplasm side. While it has to be pointed out that this topology is not yet fully settled. **B**, TatB is with its C-terminus on the cytoplasmic side and with its N-terminus on the periplasmic side while both N- and C-terminus of TatC are on the cytoplasmic side **(C)**. In thylakoids, cytoplasm corresponds to the stroma side and periplasm corresponds to the lumenal side. For TatA from plants, no such dual topology shift was predicted or shown so far. According to Berks et al. (2000) and Chan et al. (2007).

TatA is an integral membrane protein anchored in the membrane by one N-terminal transmembrane helix. Secondary structure predictions and circular dichroism spectroscopy suggest that TatA consists of two α-helices at its N-terminus, one hydrophobic and one amphipathic, followed by a larger, unstructured C-terminus (Porcelli et al., 2002). The transmembrane helix has been postulated to play an important role during the oligomerization process. Particularly, a conserved glutamate residue in the transmembrane helix is essential for the activity of TatA as conservative substitutions by aspartate and the structurally conserved glutamine impair Tat transport (Dabney-Smith et al., 2003). Another conserved residue, glycine, that is located in the amphipathic helix and seems to be part of a flexibility-conveying hinge region (Barrett et al., 2003; Hicks et al., 2003), probably plays a role for the interactions of TatA with its neighboring monomers as well (Chan et al., 2007). The N-terminus of TatA has been implied to be located in the periplasm based on predictions using the "positive-inside rule" and protease sensitivity experiments (Porcelli et al., 2002). However, recent experimental data have shown an opposite result, i.e. the N-terminus of TatA located in the cytoplasm in bacteria (Chan et al., 2007). Thus, it is not yet fully settled for the topology of TatA. In contrast, the C-terminus of TatA has a dual topology conformation depending on the presence of an intact membrane potential (Fig 1.3; Gouffi et al., 2004; Chan et al., 2007). This might also be true for plant TatA but needs experimental confirmation.

TatB and TatC are the other two components that have been identified to be required for a functional Tat transport. Like TatA, both proteins are also integral membrane

proteins (Fig 1.3; Settles et al., 1997; Walker et al., 1999; Motohashi et al., 2001). TatB, in several aspects, shares limited, but significant, sequence similarities with TatA (Sargent et al., 2006). For example, their amino acid residues are 25% identical in *E. coli*; both possess a transmembrane α-helix at their extreme N-terminus, followed by an amphipathic α-helix. In the case of TatB, however, the amphipathic helix is longer than that of the TatA protein and is probably not exposed to the trans-membrane side under any circumstances (Bolhuis et al., 2001). Finally, like TatA, the extreme C-terminal region of TatB is predicted to be unstructured and is not essential for a successful Tat translocation (Lee et al., 2002). Despite of these similarities, however, the two proteins have a different topology and fulfil different functions during the transport process (Müller and Klösgen, 2005; Lee et al., 2002).

TatC is the largest and most highly conserved component of the Tat machinery (Sargent et al., 2006). TatC has six transmembrane domains as predicted and experimentally confirmed by analysis of TatC reporter fusions (Gouffi et al., 2002; Behrendt et al., 2004; Ki et al., 2004). TatC protein also contains a number of conserved residues and some of them, when mutated, indeed interfere with the activity of TatC (Allen et al., 2002; Buchanan et al., 2002). However, our current knowledge on TatC structure and function is surprisingly rudimentary (Müller and Klösgen, 2005).

The stoichiometric ratio of TatA:TatB:TatC in the *E. coli* cytoplasmic membrane has been estimated to be approximately 20-30:1:0.4 (Berks et al., 2003). In plants, however, the amounts and ratios of Tat proteins varied depending on the species (like pea or Arabidopsis) and the developing stages of the plant (M. Jacob et al., submitted). On blue-native polyacrylamide gel electrophoresis (BN-PAGE), these components have been found to form series of high molecular weight complexes (Berghöfer and Klögen 1999; Bolhuis et al., 2001; Cline and Mori, 2001; Sargent et al., 2001; de Leeuw et al., 2002; Oates et al., 2003; Oates et al., 2005; Behrendt et al., 2007). TatA and TatB form complexes in a ladder-like pattern after solubilization of the cytoplasmic membrane of bacteria after overexpression. Depending on the number of monomers involved, the complexes ranged from about 100 kDa to over 880 kDa for TatB (Behrendt et al., 2007) and from 100 kDa to over 600 kDa with average differences of 34 kDa between the ladders of TatA complexes (Oates et al., 2005). Further, there are also cross-reactions between these three Tat components. In equimolar quantities, TatB and TatC form a complex with molecular weight of 560 and 620 kDa in thylakoids (Berghöfer and Klögen 1999; Cline and Mori, 2001) and of $\sim$600 kDa in bacteria (Bolhuis et al., 2001). It has been reported that TatC is highly unstable in the absence of TatB (Sargent et al., 1999), and that the TatBC complex is also unstable without TatA (Mangels et al., 2005), suggesting the important relevance of these three components.

***Chaperones involved in Tat transport*:** Tat system can transport folded proteins and some Tat substrates are cofactor containing proteins. However, it is still a mystery how the folding state is sensed and how the cofactor-containing proteins are held in a transport-waiting state before the cofactors become correctly incorporated (Palmer et al., 2005). It is likely that some chaperones or not yet identified accessory proteins contribute to these features (Müller and Klösgen, 2005).

Interestingly, specific proteins that bind to Tat signal sequences (Oresnik et al., 2001; Dubini and Sargent, 2003; Jack et al., 2004) and function as specific molecular chaperones in the targeted insertion of cofactors into Tat substrates have been described in bacteria (Driessen et al., 2001; Jack et al., 2004; Hatzixanthis et al., 2005; Graubner et al., 2007; Maillard et al., 2007; Perez-Rodriguez et al., 2007).

In the thylakoidal Tat system, such chaperones have not yet been identified. Generally, no stroma was added in the *in thylakoido* import assay which is widely used for Tat transport analysis. In other words, without any stroma factors, the Tat substrates could still be efficiently imported into the thylakoid lumen. This strongly suggests that probably chaperones are not involved in the thylakoid Tat transport system. However, the addition of stroma in such import assays sometimes increases the amount of imported substrates (S. Frielingsdorf, personal communication) indicating that probably for thylakoid Tat machinery, some yet-to-be-identified chaperones which probably increase the transport efficiency do exist. Interestingly, the analysis of one Tat substrate, Rieske protein, demonstrated that indeed stromal components, including cpn60 chaperonine, are involved in the targeting process (Molik et al., 2001).

***The driving force of Tat transport*:** In contrast to most of the protein transport powered by NTP hydrolysis (Alder and Theg, 2003b), Tat protein translocation is very unique as this transport solely depends on the transmembrane proton gradient (Cline et al., 1992; Klösgen et al., 1992; Santini et al., 1998; Yahr and Wickner, 2001; Alder and Theg, 2003a) which costs 3% of the total energy output of the chloroplast (Alder and Theg 2003a). This feature gave rise to the initial name of this pathway as ΔpH-dependent pathway. This character is further enhanced by the finding that overproduction of PspA, a protein involved in the maintenance of the H^+-motive force, favours Tat export in *E. coli* cells (DeLisa et al., 2004). It remains unknown, however, how the proton gradient was coupled to the Tat transport process.

However, the requirement of ΔpH in the Tat pathway has been challenged recently by *in vivo* analysis of *Chlamydomonas reinhardtii* (Finazzi et al., 2003) and transfected tobacco protoplasts (DiCola et al., 2005). The reason for the current discrepancy bet-

ween *in vitro* and *in vivo* data is not known, but two possibilities have been suggested (Theg et al., 2005): First, some factor(s) probably missing in the *in vitro* system, which the *in vivo* experiments might contain, alter the energetic requirements of the transport reaction; The second, the transmembrane $\Delta\psi$ and/or ΔpH contribute(s) to power the Tat pathway. It has been shown that the steady-state $\Delta\psi$ substantially decreased in isolated thylakoids, which have been used generally for *in vitro* assay for Tat transport analysis. If $\Delta\psi$ indeed was involved, this decrease could contribute to the discrepancy observed for the differences between *in vitro* and *in vivo* results. Interestingly, it has been reported in *E. coli* recently that ΔpH is not required, instead two kinds of $\Delta\psi$ with short and long duration are required, respectively. The short one is required for an early transport step while the long duration one is necessary to drive a later transport step (Bageshwar and Musser, 2007). In thylakoid system, it has also been reported recently that $\Delta\psi$ can replace ΔpH as a driving force for Tat transport (Braun et al., 2007).

***Current working model of the Tat transport process*:** Combining the results from cross-linking, immunoprecipitation and immunoblotting, as well as the analysis of chimeric proteins, our present understanding of the Tat protein transport process could be summarized as following: After most of the Tat precursors, if not all, first insert

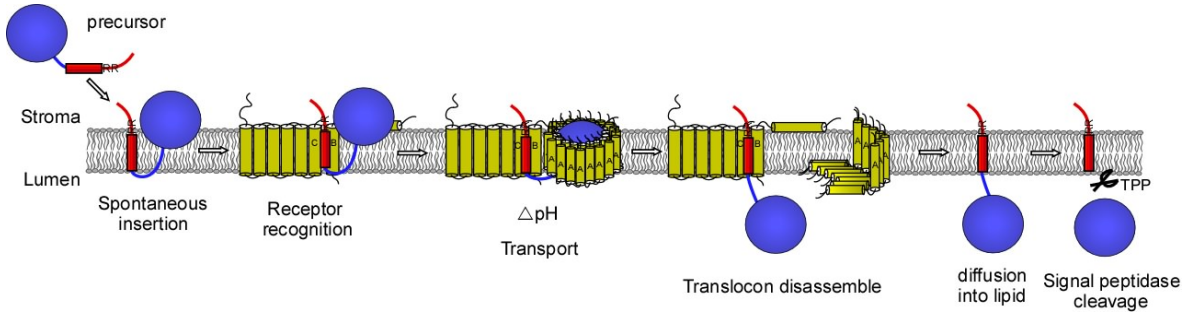

Fig 1.4: **Current working model of Tat protein transport process.** Based on the analysis of 16/23 chimera, Tat transport could be divided into the following steps: (1) Membrane insertion and receptor recognition (Transport intermediate Ti-1); (2) Translocation; (3) Translocon disassemble and precursor diffusion into the lipid (Transport intermediate Ti-2); (4) Maturation (TPP cleavage). For details see text.

into the thylakoid membrane or plasma membrane in an unassisted or spontaneous manner (Hou et al., 2006; Shanmugham et al., 2006) by use of their signal peptide forming a loop structure (Fincher et al., 1998), the precursors will be recognized by a receptor complex, which is 560-620 kDa in size (Berghöfer and Klösgen, 1999; Hou et al., 2006) and composed of TatB and TatC (Bolhuis et al., 2001; Cline and Mori, 2001; Alami et al., 2003). Then, in the presence of a trans-membrane proton gradient, the precursor is transported by the Tat translocase which is formed by recruitment of TatA

(Mori and Cline, 2002; Alami et al., 2003). After being successfully transported across the membrane, the precursors are probably released by lateral diffusion into the lipid membrane (Frielingsdorf and Klösgen, 2007). Then the signal peptidase will cleave off the Tat signal peptide and the mature proteins will be released into the lumenal side of the membrane. Meanwhile, as soon as the precursors being released into the lipid bilayer, the Tat translocon will be disassembled for next round transport (Müller and Klösgen, 2005). The oligomerization of TatA to form a transient translocon (assemble) (Dabney-Smith et al., 2006) and disassembled back into monomers could explain how the Tat system can accommodate folded proteins of varied size. It also explains in part how the system can exist in the membrane without compromising its ion and proton permeability barrier (Mori and Cline, 2002). For moving the substrate across the membrane, probably the dual topology of TatA has some functional indications: in the presence of proton motif force, the C-terminus of TatA will be oriented in the cytoplasm-side of the membrane probably in a flip-flopping manner, which probably could facilitate the formation of the translocon from a structure point of view (Chan et al., 2007).

1.2.3 The goal of the work

The goal of this work was to characterize the mechanism of thylakoid Tat protein transport. To this end, *in vitro* protein transport experiments were performed using isolated intact chloroplasts (*in organello*) or thylakoid vesicles (*in thylakoido*). Specifically, this thesis aimed to answer the following three questions: (I) In cryptophytes, the phycobiliproteins are located at the thylakoid lumenal side, but how these proteins are sorted is not known. To analyze the mechanism involved, one of the phycobiliproteins, notably phycoerythrin alpha, has been analyzed with the heterologous thylakoid system; (II) Tat transport machinery can transport folded proteins, but it is not known how the Tat system can accommodate the Tat substrates with different sizes. For this purpose, a "train-like" chimeric 16/23-EGFP protein has been constructed and analyzed. This allows for the analysis of the translocation steps and to get an idea about how Tat transport machinery can transport substrates with different sizes; (III) After Tat protein transport, Tat signal peptides are cleaved off by thylakoid processing peptidase, but it is not known what happens afterwards to these small peptides. To analyze the fate of Tat signal peptides, a "tandem-substrate", in which two precursors have been fused in a sequential order, has been constructed and used. This chimeric protein gives rise to an easier detection of the Tat signal peptide and one of its subfragment after a cleavage event.