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The role of the acidic domain of the Toc159 protein import receptor family in Toc complex assembly in *Arabidopsis thaliana*

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by

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I hereby declare that I am the only author of this thesis.

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Abstract

Most chloroplast proteins are encoded in the nucleus and translated in the cytosol with an N-terminal cleavable transit peptide, which can be recognized by the translocon at the outer envelope membrane of chloroplasts (Toc complex). The core Toc complex is composed of two GTPases, Toc159 and Toc34, and the Toc75 channel. atToc159, atToc132 and atToc120 are homologues of the Toc159 family in Arabidopsis, and have been shown to assemble into structurally and functionally distinct Toc complexes. Targeting of atToc159 to the chloroplast outer membrane is mediated by its GTPase (G-) domain. However, the role of the acidic (A-) domain in targeting of the receptor to chloroplasts is still unclear. The members of the Toc159 family are most variable in the A-domain suggesting that this domain might play a role in the functional specificity. The overall goal of this study is to clarify the role of the A-domain in the targeting of Toc159 to chloroplasts and in the assembly of distinct Toc complexes. In order to reach this goal, *in vitro* chloroplast targeting and solid-phase binding assays focusing on members of the Toc159 family were used. The data suggest that atToc132 interacts with both members of the Toc34 family in Arabidopsis (atToc33 and atToc34), but that the A-domain affects the targeting and assembly of atToc132 into specific Toc complexes by restricting the binding of this receptor parimarily to atToc34. *In vitro* chloroplast targeting assays show that competition with atToc159G also inhibits the targeting of atToc132, indicating that atToc132 may not exclusively bind with a specific member of the Toc34 family. The A-domain inhibits the targeting of atToc132GM (an A-domain deletion mutant of atToc132) to both wild-type and *ppi*3 (an atToc34 knockout mutant) chloroplasts

much more effectively than its targeting to *ppi*1 (an atToc33 knockout mutant) chloroplasts. In addition, *in vitro* solid-phase binding assays indicate that the A-domain deletion fragment of atToc132 increases its affinity for 33G. These results support the contention that the A-domain influences the targeting of atToc132 to chloroplasts and the assembly of atToc132-containing Toc complexes by inhibiting its interaction with atToc33, thereby promoting an interaction with atToc34.

Acknowledgement

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Table of contents

List of Figures

List of Abbreviations

Toc: translocon at the outer envelope membrane of chloroplasts

Tic: translocon at the inner envelope membrane of chloroplasts

132GM: truncated atToc132 construct, consisting of the G- and M-domains of atToc132

132G: truncated atToc132 construct, consisting of the G-domains of atToc132

132A: truncated atToc132 construct, consisting of the A-domains of atToc132

159G: truncated atToc159 construct, consisting of the G-domains of atToc159

33/34G: truncated atToc33/34 construct, consisting of the G-domains of atToc33/34

SPP: stromal processing peptidase

WT: wild-type

*ppi*1: plastid protein import mutant 1, which is atToc33 knockout mutant

*ppi*2: plastid protein import mutant 2, which is atToc159 knockout mutant

*ppi*3: plastid protein import mutant 3, which is atToc34 knockout mutant

IVT: *in vitro* tanscription/translation product

ATP: adenosine triphosphate

GTP: guanosine triphosphate

GTPase: guanosine triphosphatase

THL: thermolysin

PCR: polymerase chain reaction

IMAC: immobilized metal affinity chromatography

IPTG: isopropyl triogalactoside

THP: tetrahydrophranyl

DTT: dithiothreitol

1. Introduction

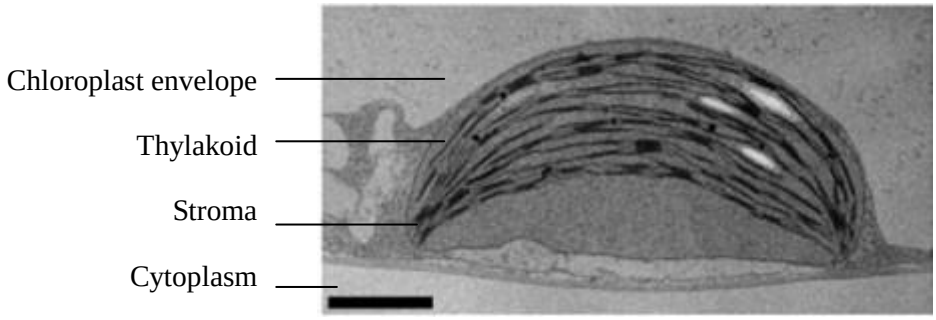
1.1 Plastids

Plastids are a diverse group of semiautonomous organelles found ubiquitously in plant and algal cells, and include chloroplasts, leucoplasts and chromoplasts (Pyke, 1999; Bauer et al., 2001; Wise, 2007). The progenitors of differentiated plastids are proplastids, which exist in the meristematic cells of plants. Depending on the gene expression program and resulting sets of proteins produced by the cell, plastids develop into one of the morphologically and functionally different types (Pyke, 1999; Bauer et al., 2001; Inoue et al., 2010; Schleiff & Becker, 2011). The members of the plastid family play crucial roles in numerous biochemical processes, such as photosynthesis, fruit, leaf and flower coloration, amino acid and lipid synthesis, and oil and starch storage (Bauer et al., 2001; Lopez-Juez & Pyke, 2005; Inoue et al., 2010). Chloroplasts, by far the best studied type of plastid, are responsible for photosynthesis, fatty acid and lipid synthesis, nitrogen assimilation and amino acid synthesis, sulfur metabolism, and oxygen metabolism (Smith, 2006; Wise, 2007).

Although all plastids are derived from proplastids, they develop different internal membrane systems depending on their specific function(s). Chloroplasts contain three distinct membranes: the double envelope membrane that separates the interior of the chloroplast from the cytoplasm, and the thylakoid membrane, which is the membrane where the protein complexes required for the biochemical reactions of photosynthesis are located (Waters & Langdale, 2009; Lemeille & Rochaix, 2010; Su et al., 2010). These three membranes separate chloroplasts into six subcompartments, which are the outer envelope membrane,

intermembrane space, inner envelope membrane, stroma, thylakoid membrane, and thylakoid lumen (Figure 1).

A.



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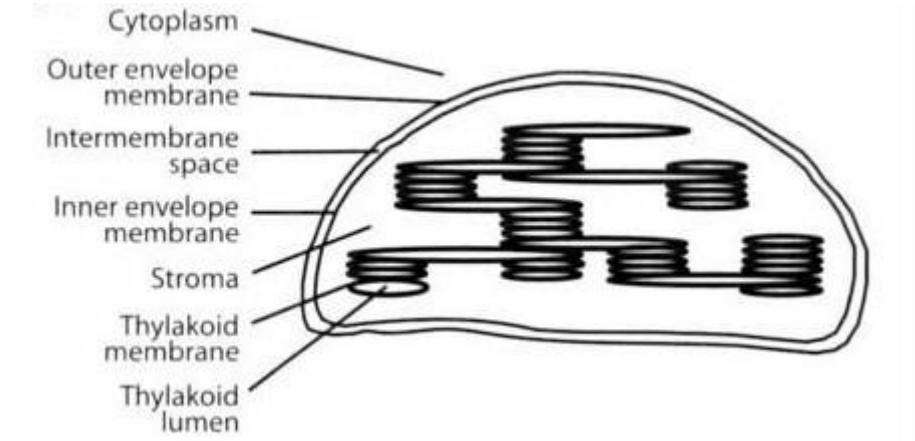


Figure 1. Chloroplast structure. A) a transmission electron micrograph of a wild-type chloroplast from *Arabidopsis thaliana* (Ivanova et al., 2004); B) a diagram of a chloroplast outlining structure and six subcompartments (Smith, 2006).

1.2 Protein import into organelles

As a control centre of eukaryotic cells, the nucleus contains most of the cell's genetic material. After the genes of the nuclear DNA are transcribed, the resulting mRNAs are translated into proteins on cytosolic ribosomes. Approximately 45-50% of these proteins ultimately reside in one of the many different organelles present in most plant cells (Dalbey & von Heijne, 2002), such as mitochondria and chloroplasts. This poses an interesting issue: that is, how are such proteins targeted and translocated into different organelles, especially mitochondria and chloroplasts, both of which are widely accepted to have originated from endosymbiotic events (Gray, 1992; Soll & Schleiff, 2004; Balsera et al., 2009; Schleiff & Becker, 2011). As a result of endosymbiosis, mitochondria and chloroplasts function as semiautonomous organelles, because the majority of their genetic information was transferred to the host nucleus, though both organelles still contain a small genome (Soll & Schleiff, 2004; Balsera et al., 2009).

There are two broad mechanisms of protein import into organelles: co-translational import, in which protein translocation is tightly coupled with translation, and post-translational import, in which protein translocation occurs after translation. To date, all nuclear-encoded mitochondrial and chloroplast proteins are known to take a post-translational import pathway to their target organelle. Although there are distinct protein targeting and import pathways for mitochondrial and chloroplast proteins, there are some general principles that are shared among these pathways. For example, precursor proteins are translated on cytosolic ribosomes with organelle-specific targeting signals, which can be recognized by

receptor components of translocons located on the surface of organelles (Schatz & Dobberstein, 1996; Schnell & Hebert, 2003; Jarvis, 2008). The proteins are then tranlocated across membranes through a protein channel, the targeting sequence is cleaved, and if the soluble compartment is the final destination, the mature proteins can be folded (Smith, 2006). In cases where the protein is destined for another sub-organellar compartment, the protein will contain additional targeting information to direct it to its final destination.

1.3 Chloroplast protein import

The majority of the genetic material from the cyanobacterial ancestor that represented the original endosymbiont was transferred to the nucleus of the host cells during the evolutionary transition from cyanobacteria to plastid (Schleiff & Becker, 2011). Approximately 95% of chloroplast proteins, representing 3000~4000 distinct proteins, are now encoded in the nucleus, translated on cytosolic ribosomes, and then transported through the chloroplast double envelope membrane in order to reach their correct destination (Smith, 2006; Jarvis, 2008; Kessler & Schnell, 2009). Most of these nucleus-encoded proteins are translated with N-terminal cleavable targeting sequences, known as transit peptides, to form preproteins. The preproteins are imported into chloroplasts via the Toc (translocon at the outer envelope membrane of the chloroplast) -Tic (translocon at the inner envelope membrane of the chloroplast) complex pathway (Agne & Kessler, 2009; Bauer et al., 2001; Schleiff and Soll, 2003; Smith, 2006; Jarvis, 2008). There are some proteins, however, that contain intrinsic targeting information instead of a cleavable N-terminal transit peptide to direct them

to the interior of the chloroplast (Jarvis, 2008). Other proteins, such as OEP14 (Outer Envelope Protein, 14 kDa) and Toc64/OEP64, are targeted and inserted into the chloroplast outer membrane with intrinsic targeting information (Jarvis, 2008). Moreover, other pathways exist for targeting proteins to distinct subcompartments. For example, thylakoid lumenal proteins are targeted via the Sec (secretory) and Tat (Twin-arginine translocase) pathway (Jarvis, 2008), while other thylakoid proteins are targeted via an SRP (signal recognition particle-dependent) pathway.

In the Toc-Tic protein import pathway, transit peptides play an essential role in targeting proteins to chloroplasts. Transit peptides vary in length from 20 to 100 amino acid residues and do not possess much sequence conservation (Smith, 2006; Jarvis, 2008). However, they are generally positively charged due to their enrichment in the hydroxylated amino acid residues, serine and threonine (Von Heijne et al., 1989; Jarvis, 2008). Transit peptide recognition is extremely faithful when multiple organelles are included in *in vitro* studies, in which mitochondrial or chloroplast proteins are directed to their destinated organelles without any mis-targeting (Vothknecht & Soll, 2007). In addition to ensuring the fidelity of protein import into chloroplasts, transit peptides can also interact with some cytosolic components, such as Hsp70 (heat shock protein of 70kDa) and 14-3-3 proteins, whose functions are to prevent the preproteins from folding prematurely as preproteins are translocated in an unfolded state (Vothknecht & Soll, 2007; Jarvis, 2008). It has also been reported that Hsp70 and 14-3-3 proteins may be involved in the targeting of preproteins to the chloroplast surface (May & Soll, 2000; Zhang & Glaser, 2002; Rial et al., 2003).

Import into chloroplasts via the Toc-Tic protein import pathway requires energy in the form of ATP and GTP at distinct steps. At first, the Toc complex recognizes and makes reversible contact with the transit peptide of a preprotein, which is an energy-independent step (Perry & Keegstra, 1994; Ma et al., 1996). At the second step, the preprotein is transported across the outer envelope membrane by inserting deeply into the Toc complex. At the same time, it makes contact with the Tic complex to initiate translocation across the inner envelope membrane. Here, a low concentration of ATP (< 100 μM) is required, as well as GTP (Ma et al., 1996; Young et al., 1999; Jarvis, 2008; Balsera et al., 2009). Finally, at the last step, when the preprotein emerges in the stroma, the transit peptide is cleaved by stromal processing peptidase (SPP); progression to this stage requires a high ATP concentration environment (Pain & Blobel, 1987; Theg et al., 1989; Balsera et al., 2009), so that the mature protein can be folded or subsequently targeted to one of the internal chloroplast sub-compartments (Figure 2).

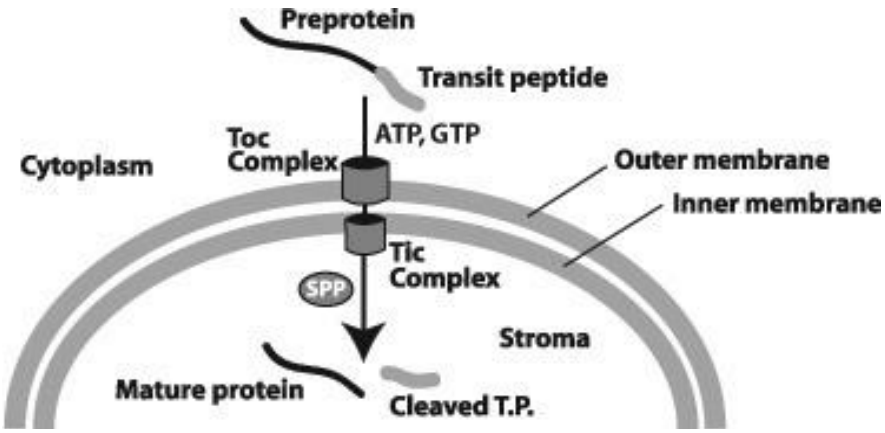


Figure 2. Toc-Tic complex protein import pathway. Schematic representation shows that most of nuclear-encoded chloroplast proteins are modified with N-terminal transit peptide, and transported across the double membrane envelope by the Toc-Tic complex pathway. This process requires energy in the form of ATP and GTP (adapted from Smith & Schnell, 2004).

1.4 The Toc-Tic complexes

The core Toc complex is comprised of a ß-barrel membrane channel Toc75 and two membrane-bound GTPases Toc34 and Toc159, all of which are named based on their molecular weight in kilodaltons (Schnell et al., 1994) (Figure 3). Together they form a functional Toc complex. The exact stoichiometry has not yet been conclusively determined, but a ratio of one Toc159:four Toc75:four Toc34 has been reported (Schleiff et al., 2003) and isolated Toc complexes have been reported to have an apparent mass of 500 kDa (Schleiff et al., 2003; Vothknecht & Soll, 2007; Jarvis, 2008). In addition to the core components, Toc64 and Toc12 have also been found in the Toc complex (Sohrt & Soll, 2000; Becker et al., 2004), however, the exact conditions under which they are associated with the Toc complex and precise roles of these components in import have not yet been conclusively determined. Most Toc and Tic components were originally identified in pea; however, due to its completely sequenced genome, *Arabidopsis thaliana* is now used broadly as the model organism for chloroplast targeting research, and Toc and Tic components from Arabidopsis are denoted by the inclusion of a two-letter prefix "at" as per the nomenclature agreed upon in 1997 (Schnell et al., 1997).

Toc75 is one of the most prominent proteins in the outer envelope membrane of chloroplasts. There are two domains in this protein: the N-terminal cytosolic domain that plays a role in recognizing and associating with the other components of the Toc complex, and the C-terminal membrane domain which forms the ß-barrel channel (Sveshnikova et al., 2000). Although four Toc75 isoforms (atToc75-I, atToc75-III, atToc75-IV and atToc75-V) have been found in *Arabidopsis*, the knockout of atToc75-III is embryo lethal, and is therefore thought to

be essential for the viability of plants from the embryonic stage (Baldwin et al., 2005; Smith, 2006; Balsera et al., 2009). atToc75-IV and atToc75-V have a role in growth in the dark and in the biogenesis of a small subset of proteins, respectively, while atToc75-I is not found to be expressed during development (Balsera et al., 2009).

Toc34 is a major preprotein receptor. It inserts into the outer envelope membrane by a C-terminal transmembrane α-helix and exposes to the cytosol GTPase domain at the N-terminal. There are two homologues of Toc34 in *Arabidopsis*: atToc33 and atToc34, which are 60% identical in amino acid sequence (Jarvis et al., 1998). atToc33 is primarily expressed in photosynthetic tissues, and atToc34 is expressed more highly in non-photosynthetic tissues such as roots (Jarvis et al., 1998; Gutensohn et al., 2000). However, it has been shown that these two isoforms can substitute for each other *in vivo* (Jarvis et al., 1998; Wang et al., 2008; Balsera et al., 2009). As a GTPase preprotein receptor, the recognition of the preproteins by Toc34 depends on the binding of GTP to its N-terminal GTPase domain (Kouranov & Schnell, 1997; Sveshnikova et al., 2000; Jelic et al., 2002; Vothknecht & Soll, 2007). Furthermore, phosphorylation of preprotein transit peptides and specific sites on atToc33 may affect the binding of preproteins to atToc33, while atToc34 cannot be phosphorylated (Vothknecht & Soll, 2007).

The other GTPase at the outer envelope membrane of chloroplasts is Toc159. There are four homologues of the Toc159 family in Arabidopsis: atToc159 atToc132, atToc120 and atToc90 (Bauer et al., 2000). All members of this family have a tripartite architecture with a N-terminal acidic (A-) domain, a GTPase (G-) domain in the middle, and a membrane anchor

(M-) domain at the C-terminus (Figure 4b). The sequences of the GM-domains of the four isoforms share 65% identity whereas the A-domains are only 20% identical in amino acid sequence, and the length of these domains is variable among the family members (Figure 4a) (Bauer et al., 2000). It has been suggested that the A-domain may differentiate the functions of members in the Toc159 family, and this has been demonstrated recently by Inoue et al. (2010), and in our laboratory (Dutta and Smith, unpublished data). To date, atToc159 and atToc90 are proposed to play a role in recognizing photosynthetic proteins, while atToc132 and atToc120 may associate preferentially with non-photosynthetic proteins (Hiltbrunner et al., 2001a; Ivanova et al., 2004; Balsera et al., 2009). In addition, atToc132 and atToc120 were shown to be functionally redundant since the deletion of either of them individually did not result in a phenotype, however, a double mutant lacking both atToc132 and atToc120 was lethal (Ivanova et al., 2004). It has also been suggested that homologues of the Toc159 family specifically associate with different members in the Toc34 family functioning in the import of different classes of preproteins: atToc33 is reported to interact preferentially with atToc159 to transport photosynthetic preproteins, and atToc34 primarily associates with atToc132/120 to form Toc complexes for non-photosynthetic preprotein import (Ivanova et al., 2004; Wang et al., 2008; Balsera et al., 2009; Lee et al., 2009) (Figure 5).

There are two more components, Toc64 and Toc12, which have been reported as components of the Toc complex. Although it may not be required for import, Toc64 might function in transferring preproteins to the core Toc complex (Schleiff & Becker, 2011). Toc12 is anchored to the membrane and associates with Toc64 and Tic22 at the inner envelope

membrane of chloroplasts (Balsera et al., 2009).

The exact composition and functions of the translocon at the inner envelope membrane of chloroplast (Tic) are less well known than that of the Toc complex. Eight proteins are proposed to comprise the Tic complex: Tic110, Tic20, Tic22, Tic40, Tic62, Tic55, Tic32 and Tic21 (Figure 3). Tic110, as a central component of the inter membrane translocon, is essential for preprotein import into chloroplasts. It contains two membrane domains at the N-terminus and a C-terminal hydrophilic domain on the stromal side, which may function in recruiting chaperones (Kessler & Blobel, 1996; Balsera et al., 2009; Lee et al., 2009). In addition, Tic110 has been proposed to form the preprotein import channel in the inner membrane (Heins et al., 2002), although this is not universally accepted (Teng et al., 2006; Kikuchi et al., 2009). Another inner membrane translocon component proposed to form at least part of the channel is Tic20 (Chen et al., 2002). Tic20 is also reported to be distantly related to Tim17, a mitochondrial inner membrane translocon (Reumann & Keegstra, 1999). Tic22 is a soluble protein in the intermembrane space (IMS), which interacts with Tic20, the Toc complex and preproteins, and therefore it may play a role in passing on preproteins from the Toc complex to the Tic complex (Kouranov & Schnell, 1997; Vothknecht & Soll, 2007). Tic40 is anchored to the inner membrane by a single N-terminal transmembrane domain. It works on coordinating the association of chaperones with preproteins during late stages of import (Chou et al., 2003). Furthermore, its stromal domain forms a potential Hsp70 binding site (Chou et al., 2003). There are three other components, Tic62, Tic55 and Tic32, believed to be regulatory members of the Tic complex. All of them are proposed to interact with Tic110

(Caliebe et al, 1997; Kuchler et al., 2002; Hormann et al., 2004). Finally, the function of Tic21, the most recently added Tic complex component (Sun et al., 2001), remains to be conclusively determined, but is likely a component of the pore through the inner envelope membrane (Teng et al., 2006; Kikuchi et al., 2009).

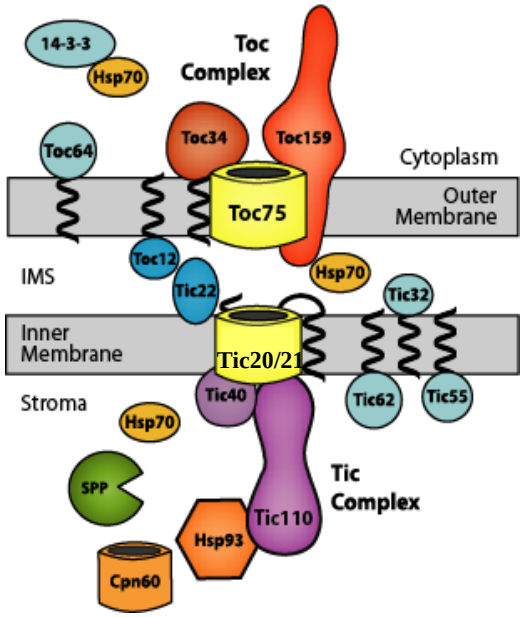
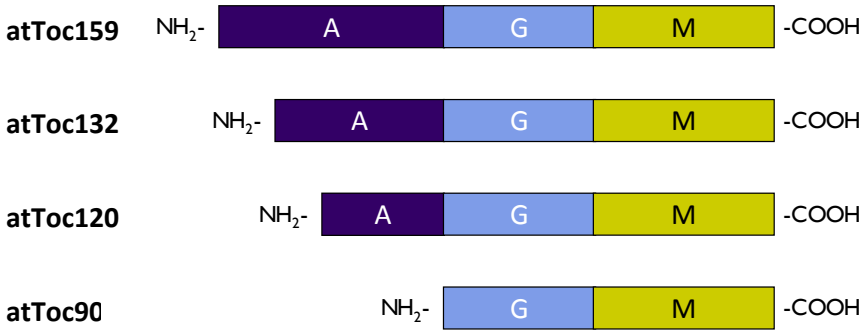


Figure 3. The chloroplast protein import apparatus. Diagrammatic representation illustrating the Toc-Tic complex is responsible for the recognition and import of preproteins from the cytoplasm into the stroma. Components in red are the core translocons responsible for recognizing and binding preproteins; components in yellow function in membrane translocation; components in purple are involved in translocation and maturation of preproteins; the functions of components in blue are still unknown (Smith 2006).

A.



B.

Figure 4. Schematic representation showing the domain structure of the four homologues of the Toc159 family in *Arabidopsis thaliana* (B), and a general structure among the Toc159 family (A).

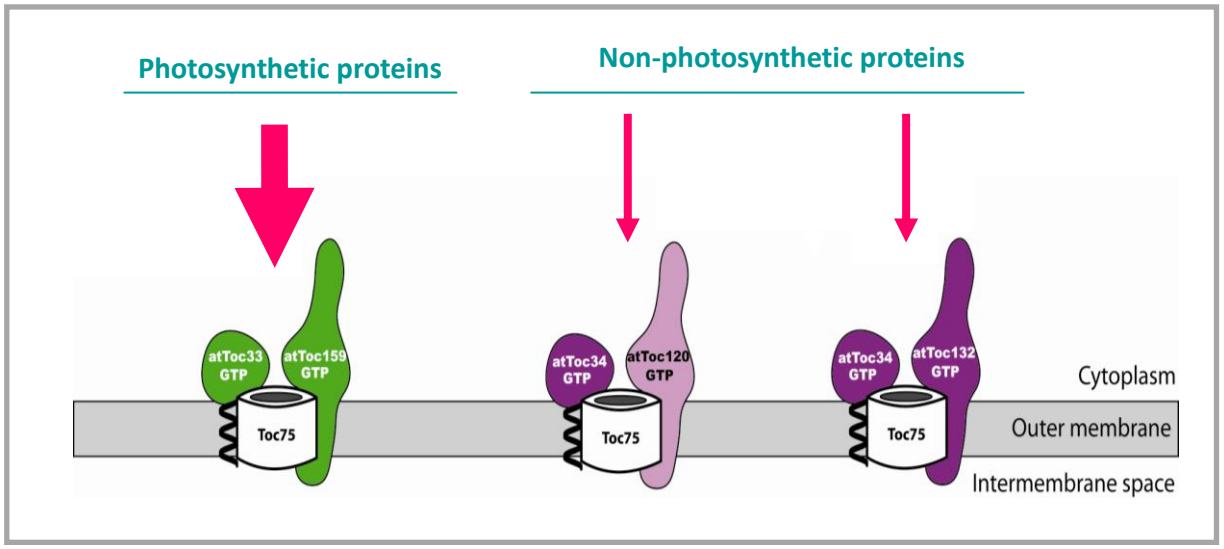


Figure 5. Distinct Toc complexes in Arabidopsis that demonstrate functional specificity. atToc159 interacts with atToc33 and functions in recognizing photosynthetic proteins; atToc120 and atToc132 associate with atToc34 to recognize non-photosynthetic proteins.

1.5 Targeting of Toc159 to chloroplast

The majority of outer membrane resident proteins are targeted by intrinsic targeting information instead of a cleavable N-terminal transit peptide (Jarvis, 2008). Toc75 is the only known outer membrane protein that uses a cleavable transit peptide for targeting to the chloroplast outer membrane (Schnell et al., 1994; Tranel et al., 1995; Inoue et al., 2005; Andres et al., 2010). There are two portions of the Toc75 targeting signal. One is at the N-terminus, which is the same as a standard transit peptide in the Toc-Tic protein import pathway. It is cleaved by the stromal processing peptidase (SPP). The other component of the targeting signal resides to the C-terminal side of the transit peptide, and is necessary to mediate Toc75 targeting to the outer membrane. This targeting signal is cleaved by a type I signal peptidase (Inoue et al., 2005; Jarvis, 2008). The exact mechanism used by Toc75 for targeting to the outer envelope membrane of chloroplast remains undefined.

Toc34 is targeted to the outer membrane of chloroplasts by an intrinsic signal located at the C-terminus. Indistinguished from Toc-Tic pathway, this process requires both membrane translocon and an energy source (Tsai et al., 1999; Jarvis, 2008; Dhanoa et al., 2010). It has been shown that the targeting signal of both atToc33 and atToc34 consists of almost the entire protein, including their GTPase domain and the C-terminal transmembrane domain (Dhanoa et al., 2010). In addition, Dhanoa et al.'s study (2010) also indicates that atToc33 and atToc34 themselves serve as a protein receptor during their targeting to the outer membrane.

The discovery that Toc34 from pea can form homodimers through an interaction between individual GTPase domains, combined with the high degree of identity between the

GTPase domains of Toc159 and Toc33 gives rise to the possibility that the binding of Toc159 to chloroplasts involves a homotypic G-domain interaction (Smith et al., 2002b; Jarvis, 2008). While the exact mechanism remains enigmatic, targeting of Toc159 to chloroplasts can be divided into two steps based on different requirements of GTP during the process. In the first step, Toc159 binds to the chloroplast surface: this "binding" step requires the presence of a nucleotide, but is supported equally well by GTP or GDP (Smith et al., 2002b; Wallas et al., 2003). The second step is the insertion of the protein into the outer membrane, during which the interaction between two GTPases may happen and insertion efficiency increases in the presence of GDP (Smith et al., 2002). When studied *in vitro* using chloroplast targeting assays, Toc159 that has been inserted into the membrane (i.e. reached the second step) can be detected by treating the chloroplasts with thermolysin. Thermolysin is a non-specific protease, which is able to degrade all exposed regions of a protein. In this case, the protease degrades all parts of Toc159 that are not protected by chloroplast membranes, meaning only the membrane anchor domain is protected for properly targeted and inserted proteins.

In Smith et al.'s (2002) paper, another experiment was done to test the adequacy of each domain of atToc159 in targeting itself to chloroplasts. Their analysis showed that both atToc159GM (an A-domain deletion fragment of atToc159) and atToc159G (an A- and M-domain deletion fragment of atToc159) bind to the chloroplast surface with the same efficiency as full-length atToc159. The M-domain alone (atToc159M) has lower binding to chloroplasts, but no insertion was detected in the absence of the G-domain. The A-domain (atToc159A) itself was not able to bind to chloroplasts at all. Intriguingly, the deletion of the

A-domain does not affect the targeting of atToc159. However, another set of experiments done by Richardson (2007) indicates that a deletion of the A-domain of atToc132 (a homologue of the Toc159 family) stimulates its targeting to chloroplasts.

1.6 The A-domain of Toc159

Although its function in targeting members of the Toc159 family to chloroplasts is unclear, it is reported that the A-domain is an intrinsically disordered protein (IDP) domain (Richardson et al., 2009). IDPs are defined as entire proteins or large segments of proteins which lack a well-structured three-dimensional fold (Dyson & Wright, 2005). Interestingly, this characteristic of protein structure is common in functional proteins and plays a role in many crucial areas such as transcriptional regulation, translation and cellular signal transduction (Dyson & Wright, 2005). Around 30% of all proteins in higher eukaryotes are IDPs (Dyson & Wright, 2005; Fink, 2005). One of the probable reasons for the disorder of proteins is that there is a low content of bulky hydrophobic amino acids, such as Val, Leu, Ile, Met, Phe, Trp and Tyr, to form the core of a well-folded protein. Moreover, there is a higher percentage of some polar and charged amino acids like Gln, Ser, Pro, Glu and Lys present in this group of proteins (Dyson & Wright, 2005).

Earlier studies found that the A-domain contains high ratio of charged acidic amino acid residues, repetitive amino acid sequences, and phosphorylation sites (Hirsch et al., 1994; Kessler et al., 1994; Bolter et al., 1998; Chen et al., 2000; Tompa, 2003; Receveur-Brechot et al., 2006; Agne et al., 2010). It has also been observed that full-length atToc132 and atToc159

(i.e. including the A-domain), and the A-domains themselves migrate aberrantly during SDS-PAGE (Richardson et al., 2009; Agne et al., 2010). In Richardson's experiment, they also show that the random coil secondary structure content in atToc132 and atToc159 is 76% and 63%, respectively. In total, these data confirm that the A-domain, which constitutes a large portion of the Toc159 proteins, is an intrinsically disordered protein. To date, the data also suggest that the A-domain may work as a functional regulator in Toc159 as a preprotein receptor at the outer membrane of chloroplast.

1.7 Overall objectives

Toc159 has been shown to be a major chloroplast protein receptor at the outer envelope membrane of chloroplasts in Arabidopsis. Moreover, the targeting of Toc159 to chloroplasts and its assembly into the Toc complexes are primarily mediated by the interactions between the G-domains of the Toc159 and the Toc34 families. Although the paper by Smith et al. (2002b) shows that the deletion of the A-domain does not affect binding or insertion efficiency of atToc159 to chloroplast membranes, the exact mechanism of how the A-domain affects the targeting of Toc159 and the Toc complex assembly remains undetermined. The overall objective of this study is to determine the role of the A-domain in targeting of atToc132 to the outer envelope membrane of chloroplasts and in the assembly of atToc132-containing Toc complexes. This thesis is presented in three portions, and the specific objectives of these sections are: 1) to determine the effect of the A-domain on targeting atToc132 to wild-type chloroplasts; 2) to investigate the specific interactions

between the members of the Toc159 family and those of the Toc34 family as they relate to Toc132/159 targeting; 3) to determine the role of the A-domain in the assembly of distinct Toc complexes.

2. Materials and Methods

2.1 *in vitro* chloroplast protein import assays

2.1.1 Model organism

Although pea was the original model system of chloroplast protein import research, *Arabidopsis thaliana* is now applied as a model organism due to its completely sequenced genome and advanced genetic and molecular tools. The reasons that *Arabidopsis thaliana* is utilized broadly are that: 1) small genome of around 125Mb; 2) rapid life cycle, which is about six weeks from germination to mature seed; 3) easy growth conditions; and 4) a large number of mutant lines are available. All these advantages make *Arabidopsis thaliana* a good model organism for genetic and molecular research on chloroplasts and many other plant systems.

2.1.2 Plant growth conditions

Seeds of wild-type (ecotype Columbia), *ppi1* (atToc33 deletion mutant) (Jarvis et al., 1998) and *ppi3* (atToc34 deletion mutant) (Constan et al., 2004) *Arabidopsis thaliana* were sterilized by washing in 95% ethanol for 5 min, 30% bleach with 0.02% (v/v) Triton-X 100 for 20 min at 4℃, and subsequently washed 5 times with sterile water in a sterile flowhood. Approximately 30 mg of seeds were sown on 150mm x 15mm growth medium plates composed of 4.4 g/L Murashige and Skoog media, 10 g/L sucrose and 8 g phytoblend agar with final pH of 5.7. Seeds were then stratified to break dormancy at 4℃ for 48 h. Plants were grown at 22℃ under a 16:8 hour light:dark cycle in growth chamber (Encornair, Bigfoot

Series) for 14-21 days on plates.

2.1.3 Isolation of intact chloroplasts from *Arabidopsis thaliana*

Chloroplasts from wild-type, *ppi*1 and *ppi*3 plants were isolated as described previously (Brock et al., 1993; Schulz et al., 2004). All centrifuge tubes and buffers were pre-chilled on ice. Approximately 40-100 g of green tissue from 14-21 days old Arabidopsis seedlings were harvested using a razor blade to separate the tissue from the agar, and homogenized in pre-chilled grinding buffer (50 mM Hepes-KOH, pH 7.5, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 330 mM sorbitol, 100 mM ascorbic acid, 0.25% (w/v) BSA, 0.05% (v/v) Sigma Protease Inhibitor Cocktail (P9599)) using a PowerGen Homogenizer (Fisher Scientific) at setting 6 for approximately 15 seconds. The homogenate was filtered through 2-layer of Mirachloth (Calbiochem) into an ice-cold 500 ml centrifuge tube and spun at 1000×g for 8 min at 4℃ (Beckman Coulter Avanti J-30I centrifuge, JLA 10.5 rotor). The chloroplast pellets were resuspended in 4-8 ml of cold grinding buffer by shaking gently on ice. Vigorously shaking and pipetting the chloroplast suspension was avoided as this would lead to breakage of intact chloroplasts. The suspension was equally layered onto two Percoll gradients consisting of a lower (7 ml) 85% Percoll layer (85% (v/v) Percoll, 50 mM Hepes-KOH, pH 7.5, 330 mM sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 50 mM ascorbic acid, 0.2% (w/v) BSA) and an 8 ml upper (8 ml) 40% or 35% Percoll layer (40% or 35% Percoll, 50 mM Hepes-KOH, pH 7.5, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 330 mM sorbitol, 50 mM ascorbic acid, 0.05% (v/v) Sigma Protease Inhibitor Cocktail). Gradients were then

centrifuged at 7,700×g for 15 min at 4℃ in a swinging-bucket rotor (Beckman Coulter JS13.1) with slow acceleration and deceleration to avoid the mixing of two Percoll gradients. Intact chloroplasts at the interface between the two Percoll layers were collected using a 1 ml micropipet with a tip that had been cut to produce a wider opening at the end, and transferred into another pre-chilled 50 ml centrifuge tube containing HS buffer (50 mM Hepes-KOH, pH 7.5, 330 mM sorbitol). Chloroplasts were collected by centrifugation at 1000×g for 6 min at 4℃. 600-1000 µl cold HS buffer was added to resuspend the final intact chloroplast pellets by shaking it on ice slowly by hand.

The chlorophyll concentration of isolated intact chloroplasts was measured at 652 nm in a spectrophotometer by diluting it 100 times into 80% acetone (Arnon, 1949). The final chlorophyll concentration was calculated using the formula: A_{652}×dilution factor/36 (dilution factor = total volume/volume of sample added = 100) in mg/ml (Richardson, 2007). Chilled HS buffer was added to the chloroplasts to dilute the chlorophyll concentration to 1 mg/ml for *in vitro* targeting assays.

2.1.4 *in vitro* transcription/translation of radiolabeled Toc159 homologues

Constructs used as templates for *in vitro* transcription/translation were pET21a:atToc132$_{NoHis}$ (Bauer at al., 2000; Ivanova et al., 2004) , pET21a:132GM$_{NoHis}$ (Richardson, 2007), pET21a:132GM$_{His}$ (described in section 2.3) and pET21d:atToc159 (Smith et al., 2002b). Translation was achieved using the T_NT Coupled Reticulocyte Lysate System or T_NT Coupled Wheat Germ System (Promega) according to the manufacturer's

instructions. Briefly, 1 μg of plasmid DNA was used for a 50 ul reaction that also included [^{35}S]Methionine (EXPRES S^{35} Protein Labeling Mix, PerkinElmer) instead of unlabelled methionine at 30℃ for 2 h. Consequently, all methionines of the protein produced during the reaction were radiolabeled and could be detected in dried SDS-PAGE gels using a phosphorimager (Bio Rad Personal Molecular Imager FX).

2.1.5 *in vitro* chloroplast protein import assays

in vitro chloroplast protein import assays were done according to the protocol described in Smith et al. (2002a). Each reaction contained chloroplasts equivalent to 30-50 μg chlorophyll, 1 mM dithiothreitol (DTT), 5 mM ATP, 1mM GTP, 10 mM methionine, 50 mM Hepes-KOH, pH 7.5, 3-4 μl radiolabeled protein, and in some cases different concentrations of a protein competitor in a final reaction volume of 100 μl. Before adding a radiolabeled version of Toc159 or its homologues, the other components of the reaction were equibrated at 26℃ for 5 min. The import reaction took 30 min in a 26℃ waterbath. Chloroplasts were reisolated by diluting the reaction with 400 μl HS buffer, transfering it to the top of an 800 μl cushion of 40% or 35% Percoll, and centrifuging at 1000×g for 6 min to collect intact chloroplasts and to remove unbound radiolabeled proteins. Chloroplast pellets (~100 μl) were resuspended with 100 μl HS buffer and the thermolysin treatment was done on ice for 30 min after adding $CaCl_2$ to a final concentration of 1 mM, and 0.2 mg/ml thermolysin. 10 mM EDTA was added to stop the proteolysis. The final chloroplasts were pelleted down at 1000g for 5 min and resuspended completely with 20 μl 2×SDS-PAGE sample buffer for analysis on

SDS-PAGE. This analysis included a lane on the gel containing 10% of the *in vitro* translated radiolabeled protein that was added to each reaction. Each import reaction (with or without the thermolysin treatment) was done in duplicate every time, and was repeated at least two times.

2.2 *in vitro* solid-phase binding assays

in vitro solid-phase binding assays were performed following the protocol of Smith et al. (2002b) and Ivanova et al. (2004). Briefly, a His-tagged protein (the bait) that had been purified from *E. coli* was immobilized on a 8 μl of Ni-charged resin, and incubated with a radiolabled protein (the prey). The binding efficiency of the prey with the bait could be quantitated in an SDS-PAGE gel using a phosphorimager after washing and eluting proteins from the Ni-charged resin. The reactions started with the preparation of a total amount of resin to be used at one time by equilibrating three times with two resin volumes of water followed by three washes with two resin volumes of HMK buffer (50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 40 mM KOAc). The resin was then resuspended with 4 resin volumes of HMK and aliquots containing 8 μl of resin were distributed into 1.5 ml microcentrifuge tubes. The bait (atToc33G-His or atToc34G-His) at a final concentration of less than 20 mM imidazole was added and rotated with the resin at room temperature for 30 min to bind completely to the resin. Increasing amounts of bait were immobilized on the resin (see figure legends for amounts). The resin with immobilized bait was washed once with 250 μl of HMKIT (50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 40 mM KOAc, 10 mM imidazole, 0.1% Triton X-100, 0.1

mM GTP). 2 µl of radiolabeled protein was diluted 50 times with HMKIT and incubated with the resin for 30 min while rotating at room temperature. All steps from this point on were done in a 4℃ cold room in order to limit the rate of dissociation of bait and prey. The resin was washed with 350 µl HMKIT three times and resuspended with ~20 µl 1×SDS-PAGE sample buffer (350 mM Tris, 5% (w/v) SDS, 80 mM DTT, 7.5% (v/v) glycerol, 1.5% (v/v) saturated bromophenol blue, 775 mM imidazole) to elute resin-bound proteins. The eluted samples plus 10% loading control of the *in vitro* translated radiolabeled protein were resolved on SDS-PAGE and visualized in dried gels using a phosphorimager.

2.3 Generation of the different atToc132 constructs

2.3.1 Generation of atToc132GM construct

The cDNA encoding the GM-domain of atToc132 (132GM, corresponding to basepair 1366-3618) was amplified by PCR using GoTaq Hot Start Polymerase (Promega). Gene specific primers (Appendix 1, primer set 1) that did not include the stop codon in the antisense primer were used, so that the target cDNA could ultimately be expressed as a fusion protein with a C-terminal His-tag when inserted into pET21 expression vector. The template used was a full-length atToc132 cDNA cloned into pET21a as described in Bauer et al. (2000). The PCR-amplified atToc132GM fragment was then ligated into the pTZ57R/T PCR cloning vector using the InsTAclone PCR Cloning Kit (Fermentas Life Science) following the manufacturer's instructions. Restriction enzymes NdeI and XhoI were used to release the 132GM construct from the pTZ57R/T vector by incubating at 37℃ for 3 h. The resulting

132GM cDNA fragment with sticky endings was then ligated with pET21a, which was digested by the same restriction enzymes, by incubating at 16℃ overnight in the presence of T4 DNA Ligase (NewEngland BioLabs). The resulting plasmid including the pET21a-132GM$_{His}$ insert, was then transformed into *E. coli* strain DH5α and was sequenced from the T7/T7 Terminator primers, and using gene specific primers (Appendix 1, primer set 2) different from those used in PCR (ATCG Sequencing Facility, Sick Kids Hospital, Toronto).

pET21a:132GM$_{His}$, was transformed into *E. coli* strain BL21 Codon Plus (Novagen) for the purpose of over-expressing the corresponding recombinant protein. A number of different expression conditions were attempted, but none were successful: 1) 0.4 mM IPTG or 1 mM IPTG, 37℃, 3 h; 2) 0.4 mM IPTG, 30℃, 5 h; 3) 0.1 mM, 0.5 mM, or 0.7 mM IPTG, room temperature, overnight; and 4) 1.5 mg/ml or 2 mg/ml The Induccer (Molecula Research Laboratories), room temperature, overnight. While over-expression was not successful and therefore recombinant atToc132GM$_{His}$ was not used in the thesis, I have included the information here for the benefit of future students who might be interested in using this reagent.

2.3.2 Generation of atToc132G construct

Both the prey and competitor used in solid-phase binding assays must not have a His-tag in order to avoid an unwanted association with the His-Bind resin. Consequently, an atToc132G construct, which contains a cleavable His-tag, was required as a competitor for

this group of assays. A TEV protease cleavage site was chosen to sit between the target protein and His-tag, so that it could be cleaved by the TEV protease to release the target protein without a His-tag.

The cDNA encoding the G-domain of atToc132 (corresponding to basepairs 1366-2382), was amplified from pET21a:atToc132 (Bauer et al., 2000; Ivanova et al., 2004) by PCR with HotStar HiFidelity DNA Polymerase (Qiagen). Sequence specific sense and antisense primers (Appendix 1, primer set 3) were designed to include NdeI and SacI restriction enzyme sites, respectively. The PCR-amplified fragment was then directly digested sequentially by NdeI and SacI at 37℃ for 6-18 h, and inserted into the corresponding sites of the pET21a:TEV vector, by incubating at 16℃ overnight with T4 DNA Ligase (New England BioLabs). The ligation product was transformed into *E. coli* strain DH5α by heat-shock at 42℃ for exactly 90 sec and incubated at 37℃ overnight. 50 out of ~500 colonies were screened by colony PCR (Taq DNA Polymerase, New England Biolabs) using sequencing primers T7 and T7 Terminator. Positive colonies were grown up in small overnight cultures, and the plasmids were isolated using a commercial minipreps kit (Promega). The presence of an insert of the correct size (1 kb) was confirmed by double digestion of the plasmid with NdeI and SacI (analyzed by agarose gel electrophoresis). 0.8% agarose gels were used at each step to check the DNA. The positive constructs were sequenced to definitively confirm their identity (ATCG Sequencing Facility, Sick Kids Hospital, Toronto). While over-expression was not done and therefore recombinant atToc132G_{His} was not used in the thesis, I have included the information here for the benefit of future students who might be interested in using this

reagent.

2.4 Expression and purification of recombinant proteins

Recombinant versions $132G_{His}$ (A- and M-domain deletion of atToc132) and $132A_{His}$ (a GM-domain deletion mutant of atToc132) were expressed in *E. coli* and purified following standard procedures. Specifically, the A-domain was purified according to Richardson (2007), and the purification of $132G_{His}$ was similar to that described by Smith et al. (2004). Expression and purification conditions are described in sections 2.4.1 and 2.4.2. The purified proteins were used as competitors in *in vitro* chloroplast import assays (refer to section 2.1.5).

2.4.1 Expression and purification of $132G_{His}$

Another protein produced for the purpose of using it as a competitor in place of $132GM_{His}$, was $132G_{His}$ (the G-domain fragment of atToc132 with a C-terminal 6×His tag), which was cloned with a C-terminal His-tag. BL21 Codon Plus *E. coli* containing the pET21a-atToc132G-His plasmid was inoculated into 10 ml LB broth with 100 μg/ml ampicillin (Amp), 25 μg/ml chloramphenicol (Chl), and 50 μg/ml streptomysin (Strep), and was incubated at 37℃ overnight while shaking at 240 rpm. The overnight culture was used to inoculate into 1 L of fresh LB (Amp, Chl and Strep) and incubated with shaking at 37℃. When the culture reached an OD_{600} of 0.6-0.8, expression of 132G-His was induced with the addition of 2 mg/ml Inducer at room temperature overnight.

Cell pellets were collected by centrifugation of the culture at 6000×g for 15 min at 4℃

(Beckman Coulter JLA 10.500 rotor). 30 ml of binding buffer (BB; 25 mM Tris-HCl, pH 6.8, 50 mM NaCl, 20 mM imidazole) containing 0.8 mg/ml lysozyme were used to resuspend and lyse the cell pellet by rotating at 4℃ for 1 h and sonicating with a microtip for 15 sec for 8 times (setting 6 on a Branso Sonifier 150 sonicator). The cell lysate was centrifuged at 50000×g for 30 min (Beckman Coulter JS13.1 rotor) to collect the supernatant containing soluble proteins, which were stored at -20℃ for later purification. Immobilized metal affinity chromatography (IMAC) was used to purify the target proteins from other proteins of the lysate. 2 ml His-Bind Ni-charged Resin (Novagen) was allowed to settle in a small chromatography column by gravity, and was washed with 6 column volumes of sterile water and twice with 4 ml of BB. The soluble proteins of the lysate were applied twice to the resin by gravity flow (200 µl of the flowthrough was collected for SDS-PAGE). Non-specifically bound proteins were removed by washing the resin with 6 column volumes of washing buffer (WB; 25 mM Tris-HCl, pH 6.8, 50 mM NaCl, 40 mM imidazole), and $132G_{His}$ was eluted in 6 1-ml fractions of elution buffer (EB; 25 mM Tris-HCl, pH 6.8, 50 mM NaCl, 250 mM imidazole). 50% glycerol was added into the elution fractions to a final concentration of 10% and proteins were stored at -80℃.

2.4.2 Expression and purification of atToc132A$_{His}$

Expression and purification of atToc132A$_{His}$ (132A; the A-domain fragment of atToc132 with a C-terminal 6×His tag) was achieved following the protocol of Richardson (2007). In detail, pET21b:132A$_{His}$ in BL21 Codon Plus was grown up in the same way as the strain

containing pET21a:132G_{His} (section 2.4.1) in 1 L LB with Amp, Chl and Strep until the culture reached an OD_{600}=0.6-0.8. Expression of 132A_{His} was induced with the addition of 1 mM IPTG at 37℃ for 3 h by shaking at 240 rpm. The supernatant of the cell lysate was collected by centrifugation and 132A was purified by IMAC, following the same protocol for the purification of 132G_{His}, except that the buffers were slightly different. Buffers used for IMAC purification of 132A_{His} were BB (10 mM Tris-HCl, pH 8, 50 mM NaCl, 20 mM imidazole), WB (10 mM Tris-HCl, pH 8, 50 mM NaCl, 30 mM imidazole), and EB (10 mM Tris-HCl, pH 8, 50 mM NaCl, 250 mM imidazole).

132A_{His} was further purified by Q-sepharose Fast Flow ion exchange media (GE Health Sciences) according to Richardson et al. (2009). All the elution fractions containing relatively large amounts of proteins (showing thicker bands on SDS-PAGE gels) were combined and diluted 1:1 with ion exchange binding buffer (IEBB; 20 mM piperazine, pH 4.5, 0.2 M NaCl). 1.5 ml ion exchange resin was washed with IEBB in a glass vial 4 times before proteins were added and incubated for 10 min at room temperature while rotating. The resin was then allowed to settle by gravity and the supernatant (containing unbound proteins) was removed. The resin was washed by mixing it completely 3 times with 5 ml of IEBB each time. The resin was transferred into a clean tube and pelleted using a pulse spin to remove as much IEBB as possible. Pure 132A_{His} was eluted by incubating the resin with 4 ml of exchange elution buffer (20 mM piperazine, pH 4.5, 0.55 M NaCl) at room temperature while rotating.

To obtain more concentrated proteins, and to exchange the protein into HS buffer, a centrifugal filter device (Amicon Ultra-15 Ultracel 10k, Millipore) was used. After the

centrifuge filter was equilibrated in HS buffer, purified $132A_{His}$ was concentrated and exchanged into HS buffer by first diluting up to 13 ml with HS buffer and centrifuging at 4000×g for 25 min at 20℃ 3 times. The flowthrough was discarded and the volume of the retentate was recorded each time. Glycerol was added to a final concentration of 10% to the concentrated $132A_{His}$ for storage at -80℃ for further usage.

2.4.3 Expression and purification of $34G_{His}$

$34G_{His}$ (the G-domain fragment of atToc34 with a C-terminal 6×His tag) was needed as a bait for *in vitro* solid-phase binding assays. Similar to the expression of $132G_{His}$ and $132A_{His}$, pET3d:atToc$34G_{His}$ (Weibel et al., 2003) in BL21 Codon Plus strain was grown up until an OD_{600}=0.6-0.8 was reached. Expression of $34G_{His}$ was induced using 0.1 mM IPTG while shaking at room temperature overnight. Cell lysate was obtained following the protocol for $132G_{His}$ and $132A_{His}$ and soluble proteins were separated by spinning at 50,000×g for 30 min at 4℃. The buffers used in the IMAC purification of $34G_{His}$ were BB (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM THP (tetrahydrophranyl), 10 mM imidazole), WB (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM THP, 40 mM imidazole) and EB (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM THP, 250 mM imidazole). Purification was performed in a 4℃ cold room to protect the protein from degradation.

2.5 SDS-PAGE and phosphorimager analysis

Recombinant proteins and chloroplast proteins from the *in vitro* chloroplast protein

import assays and proteins eluted in the *in vitro* solid-phase binding assays at the final steps were resolved using SDS-PAGE (4% stacking gel, 10% or 12% resolving gel depending on the size of the proteins) run at constant current (15 mA through stacking and 25 mA through resolving gel). Gels were stained with Coomassie blue (0.25% (w/v) Coomassie Blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid). For the gels that didn't contain radiolabeled proteins, gel drying films (Promega) was used to air dry the gels. Gels containing radiolabeled proteins (products from *in vitro* transcription/translation, *in vitro* chloroplast targeting assays and *in vitro* solid-phase binding assays) were dried using a heated gel-drying bed (Bio Rad Model 224 Gel Slab Dryer) connected to a vacuum pump. Dried gels were exposed to a phosphor screen (Bio-Rad Laboratories Ltd.) for 4 to 14 days. The screen was then scanned using a phosphorimager (Personal Molecular Imager FX, Bio-Rad Laboratories Ltd.), and the radioactivity was quantitated using Quantity One 1-D Analysis software v4.6 (Bio-Rad Laboratories Ltd.).

3. The role of the A-domain in targeting of atToc132 to isolated chloroplasts and in Toc complex assembly

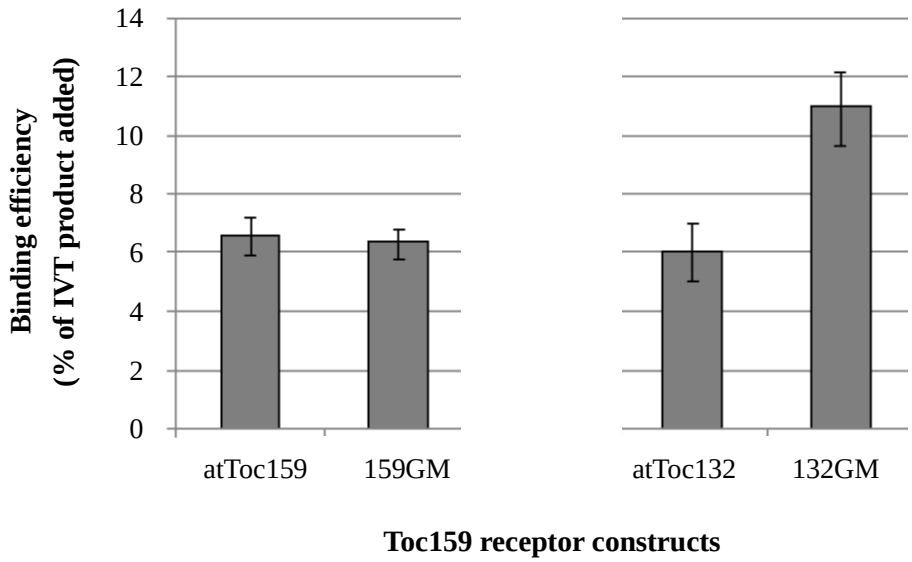
3.1 Background

It has been demonstrated that targeting of Toc159 to the outer membrane of Arabidopsis chloroplasts is initiated by the interaction between the G-domains of atToc33 and atToc159 (Smith et al., 2002b), which gives rise to the notion that the targeting of Toc159 to chloroplast happens concurrently with its integration into the Toc complex.

Members of the Toc159 receptor family are distinguished from each other by the size and sequences of their A-domains (Bauer et al., 2000; Ivanova et al., 2004), which have also been shown to be intrinsically disordered protein domains (Richardson et al., 2009). Together, these properties lead to the possibility that the A-domain may function in managing the specific association between Toc159 family members and other members of the Toc complex. Richardson (2007) showed that the A-domain does not affect the binding (Figure 6A, atToc159 and 159GM) of atToc159 to wild-type chloroplasts. However, the insertion efficiency declines when the A-domain of atToc159 is deleted (Figure 6B, atToc159 and 159GM). On the contrary, there is a clear increase in the binding efficiency (Figure 6A, atToc132 and 132GM) of atToc132 when the A-domain is cleaved, while no dramatic differences are observed in its insertion efficiency (Figure 6B, atToc132 and 132GM). These data give rise to the hypothesis that the A-domain might influence the insertion of atToc159 and the binding of atToc132 to the chloroplast outer envelope membranes. Both observations suggest that the A-domains do play a role in the assembly of distinct Toc complexes.

In the current study, *in vitro* chloroplast targeting assays were used to compare the targeting efficiency of atToc132 and 132GM to wild-type chloroplasts. In addition, increasing concentrations of a competitor, 132A, were added to determine its effect on targeting of 132GM. For these assays, radiolabeled proteins (full-length atToc132 and 132GM) generated using an *in vitro* transcription/translation system were targeted to isolated wild-type chloroplasts. For the competition experiments, increasing concentrations of competitor were included. The amount of radiolabeled protein that co-purifies with chloroplasts following the protein import assays can be quantified using phosphorimaging after resolving the proteins by SDS-PAGE.

A.



B.

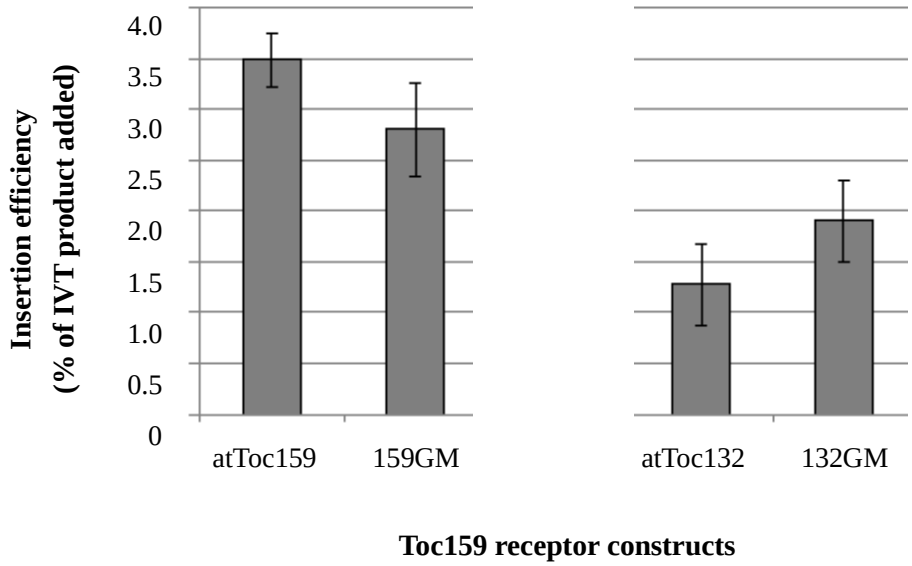


Figure 6. Targeting efficiency of Toc159 receptor family-related proteins to chloroplasts isolated from WT Arabidopsis. Binding efficiency (A) and insertion efficiency (B) are quantified (Richardson, 2007).

3.2 Objectives and hypothesis

The objective of the experiments presented in this chapter was to investigate the role of the A-domain in targeting of atToc132 to chloroplasts and in the assembly of atToc132-containing Toc complexes. I hypothesized that the A-domain restricts the targeting of atToc132 by inhibiting its binding to the surface of the outer membrane of chloroplasts.

3.3 Results

3.3.1 Expression and purification of atToc132A$_{His}$

atToc132A$_{His}$ (132A$_{His}$) was expressed and purified to be used as a competitor in *in vitro* chloroplast targeting assays following the protocol of Richardson et al. (2009). The A-domains of both atToc132 and atToc159 were originally predicted to be intrinsically disordered proteins due in part to a high percentage of acidic amino acid residues (Richardson et al., 2009). The preponderance of acidic proteins also causes an aberrant electrophoretic mobility on SDS-PAGE gels (Bolter et al., 1998; Dyson & Wright, 2005; Richardson et al., 2009). 132A$_{His}$ migrated aberrantly at an apparent molecular weight at around 100 kDa instead of according to its theoretical molecular weight of ~50 kDa on an SDS-PAGE protein gel (refer to section 1.6; Figure 7). Purification of 132A$_{His}$ was achieved using sequential IMAC and ion-exchange chromatography steps. However, sorbitol was added to a final concentration of 330 mM to all purified proteins that were used as a competitor in the chloroplast targeting assays to maintain isotonic conditions for the chloroplasts and therefore to avoid unwanted chloroplast lysis.

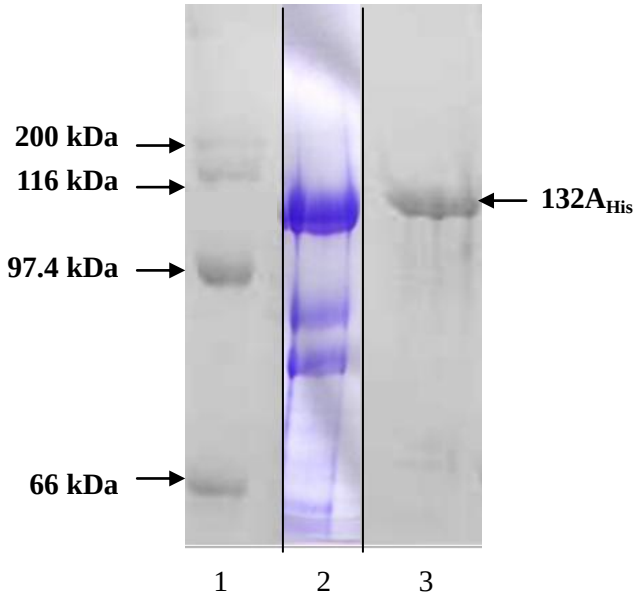


Figure 7. Expression and purification of atToc132A$_{His}$. Protein samples were resolved on a 10% SDS-PAGE gel and stained with Coomassie blue. Molecular weight markers (labeled to the left in kDa) are shown in lane 1; lane 2 is loaded with IMAC-purified 132A$_{His}$; lane 3 contains further ion-exchange-purified 132A$_{His}$. The black lines separate lanes from different SDS-PAGE gels.

3.3.2 Effect of the A-domain on targeting of atToc132 to wild-type chloroplasts

The targeting efficiency of atToc132 and 132GM to wild-type, *ppi*1 (atToc33 knockout mutant) and *ppi*3 (atToc34 knockout mutant) chloroplasts was confirmed using *in vitro* chloroplast targeting assays. A doublet appears in all *in vitro* transcription/translation products of full-length atToc132 (Figure 8, lanes 1, 2; Richardson et al., 2009). In addition, an extra band of approximately 86 kDa is also noticeable in some *in vitro* transcription/translation products (e.g. Figure 8, lane 2), which may be due to the non-specific proteolysis of the A-domain (Bolter et al., 1998). This phenomenon is supported by the finding that "Toc86", an early putative preprotein receptor at the outer membrane of chloroplasts, in fact represents only a proteolytic fragment of a larger polypeptide, now named Toc159 (Bolter et al., 1998). Indeed, the proteolytic sensitivity of the A-domain is well-documented (Chen et al., 2000). Following thermolysin treatment of chloroplasts re-isolated from the chloroplast targeting assays, bands can be visualized on SDS-PAGE gels at ~52 kDa. This band represents the M-domain of atToc132 (132M, Figure 8, lanes 3, 6), which anchors the protein in the outer membrane of chloroplasts and is therefore protected from proteolysis (Bauer et al., 2000; Richardson, 2007). The formation of this band after protease treatment is a characteristic feature of the Toc159 family and is commonly used to confirm insertion of the proteins into the outer envelope membrane, and the Toc complex.

By quantitating the density of each band on the phosphorimager-visualized SDS-PAGE gel, using the software QuantyOne, the binding and insertion efficiency can by calculated as the percentage of *in vitro* transcription/translation product added into each reaction. The data

shows that the binding efficiency of the A-domain deletion construct of atToc132 (132GM) is ~15%, which is approximately 4% higher than that of full-length atToc132 running at 11% (Figure 9). These data are consistent with that of a former student in the Smith's lab (Richardson, 2007; Figure 6), which suggests that the A-domain may play a role in inhibiting the binding of atToc132 to wild-type chloroplasts. However, the insertion efficiency of both atToc132 and 132GM are approximately equal (~3.7% of *in vitro* transcription/translation products added to import reaction; Figure 9), which indicates that the deletion of the A-domain may not affect the insertion of atToc132 into the outer membrane. Intriguingly, the results on the targeting to the mutant chloroplasts used in this study (i.e. *ppi*1 and *ppi*3) are not consistent with those of the previous study by Richardson (2007): 1) full-length atToc132 and 132GM are targeted to *ppi*1 chloroplasts with approximately equal binding and insertion efficiency of ~12% and ~1.9%, respectively, whereas Richardson reported that the binding and insertion efficiency of the A-domain deletion fragment increased by 6% and 0.6% compared with full-length atToc132; 2) in Richardson's study, the binding efficiency of 132GM to *ppi*3 chloroplasts is approximately equal with that of atToc132, while 132GM has a 3% higher binding efficiency than atToc132 in this work (Figure 9). These data illustrate that the A-domain may play an inhibitory role in targeting atToc132 to a specific member of the Toc34 family.

A.

B.

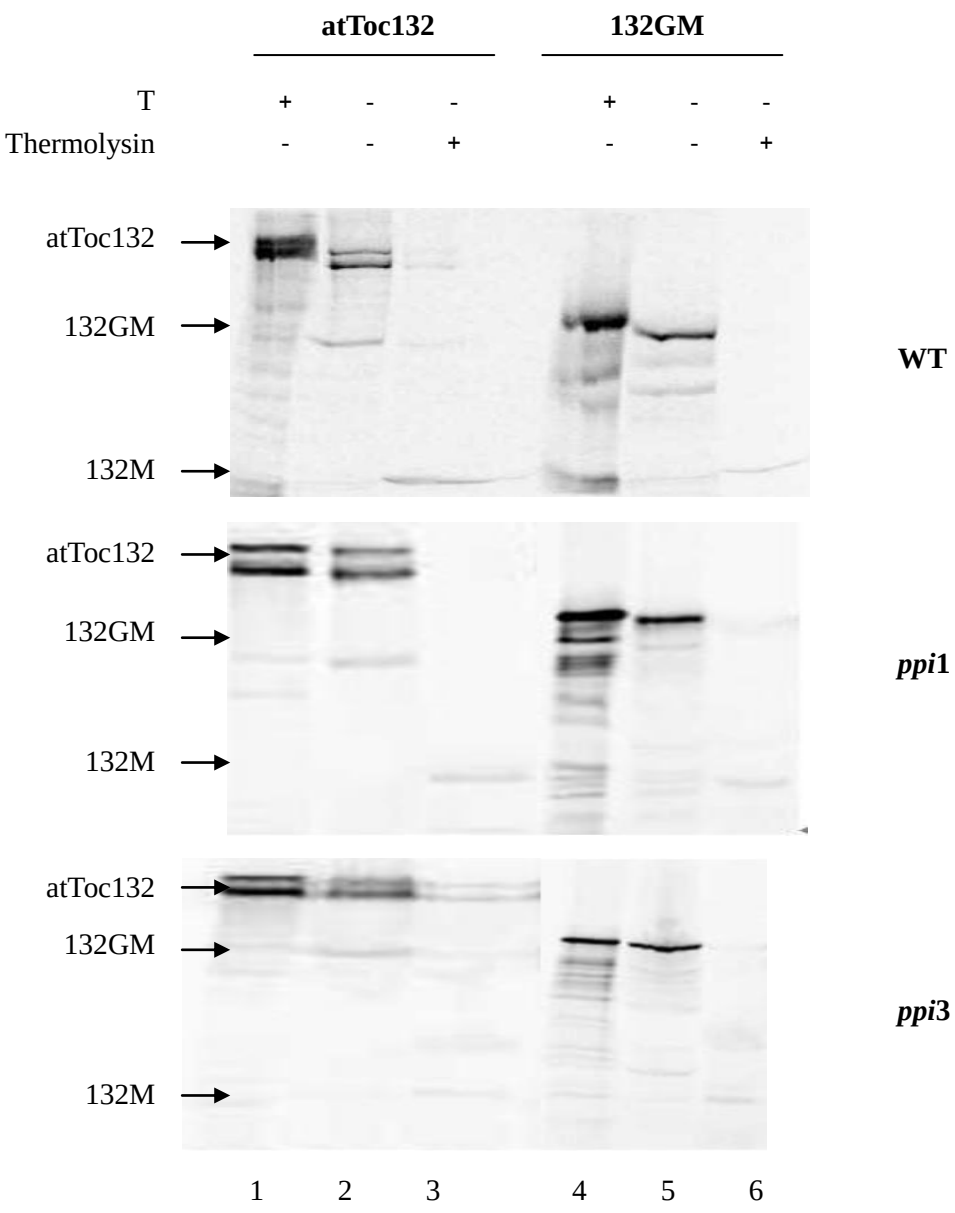
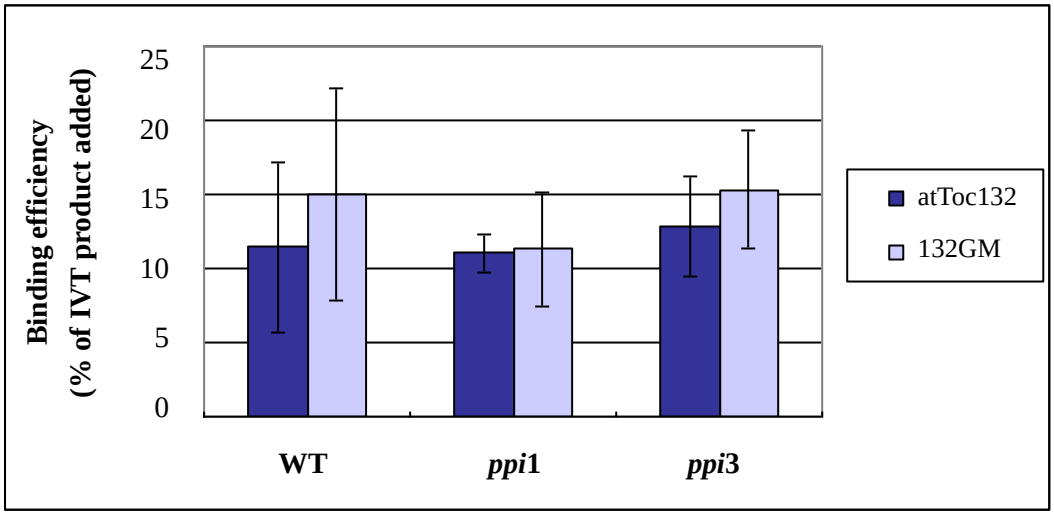


Figure 8. Targeting of atToc132 or 132GM to wild-type, *ppi*1 or *ppi*3 chloroplasts. (A) Schematic diagram illustrating the set-up of the chloroplast targeting assays; (B)Phosphorimager-visualized SDS-PAGE gels following *in vitro* chloroplast targeting assays comparing targeting efficiency of atToc132 and 132GM to isolated chloroplasts. Lanes 1 and 4 contain *in vitro* transcription/translation products corresponding to 10% of the amount used in each import reaction (T). Untreated chloroplasts (corresponding to bound proteins) are shown in lanes 2 and 5. Lanes 3 and 6 are loaded with intact chloroplasts treated with thermolysin following the targeting assay. 132M represents the M-domain of atToc132 which is protected from thermolysin due to insertion in the outer envelope membrane.

A.



B.

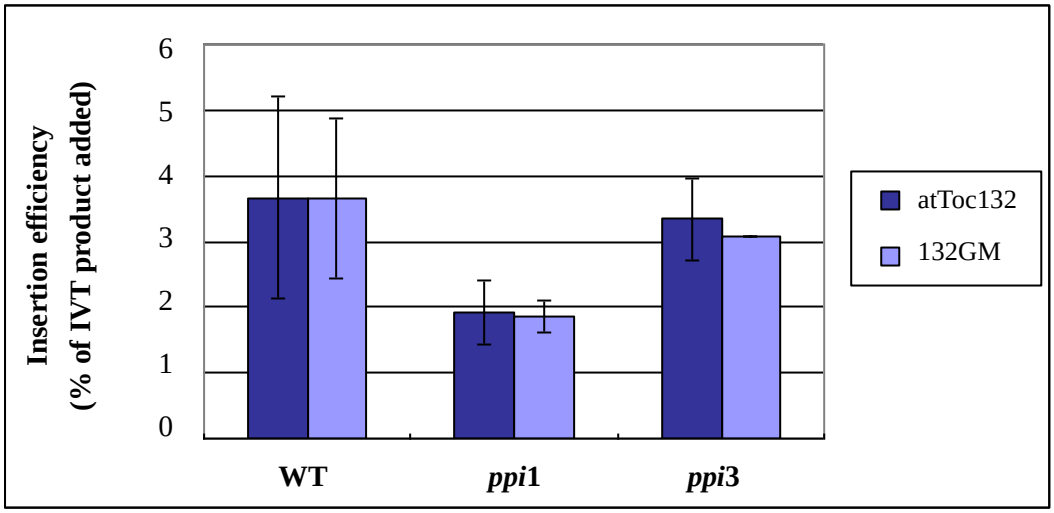


Figure 9. Quantitation of targeting efficiency of atToc132 receptor proteins to chloroplasts isolated from wild-type, *ppi*1 or *ppi*3 Arabidopsis. Binding (A) and insertion (B) efficiencies were quantitated and are presented as the percentage of *in vitro* transcription/translation (IVT) product added to the reaction. Error bars correspond to standard error of the mean for ≥2 repeats. The insertion efficiency of 132GM to ppi3 chloroplasts was measured only once in duplicate.

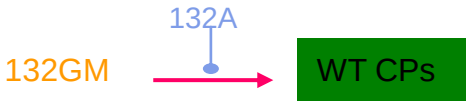
To clarify whether the A-domain affects the targeting of atToc132 to chloroplasts by inhibiting its binding to the membrane, a set of chloroplast targeting competition assays were designed. These experiments were performed by targeting 132GM to wild-type chloroplasts in the absence or presence of 0.5, 1.5 or 4 μM of purified recombinant $132A_{His}$ as a competitor (Figure 10). As shown in Figure 11 both binding and insertion efficiency of 132GM decline as an increasing concentration of $132A_{His}$ is added as competitor. The binding efficiency falls to 85.6% and 57.6% of control (binding efficiency of 132GM in the absence of $132A_{His}$), respectively, when 0.5 μM and 1.5 μM of $132A_{His}$ is applied (Figure 11). The binding efficiency further decreases to 22.3% in the presence of a high concentration (4 μM) of $132A_{His}$ (Figure 11). This result confirms that the A-domain inhibits the binding of atToc132 to the outer membrane of chloroplasts.

Interestingly, the insertion efficiency declines more sharply than the binding efficiency when 0.5 μM and 1.5 μM 132A is added (Figure 11). Insertion efficiency falls to 57.2% of control when 0.5 μM $132A_{His}$ is added, and to 25.4% and 12.8% at 1.5 μM and 4 μM of $132A_{His}$ competitor, respectively. These data suggest that the A-domain affects binding and insertion of atToc132, but might, in fact, have its strongest effect on insertion. The trend observed here is inconsistent with earlier observations by Richardson (2007), who found that there were no differences in the insertion efficiency between atToc132 and 132GM targeting to wild-type chloroplasts.

In summary, the deletion of the A-domain shows a stimulation function in binding of atToc132 to the outer membrane of chloroplasts (Figure 9), and the decreased binding

efficiency of 132GM in the presence of $132A_{His}$ added *in trans* (in a dose-dependent manner) demonstrates that the A-domain plays a role in regulating atToc132 targeting to chloroplasts. However, the exact mechanism by which the A-domain might have this effect, in particular how it might affect insertion, remains unclear.

A.



B.

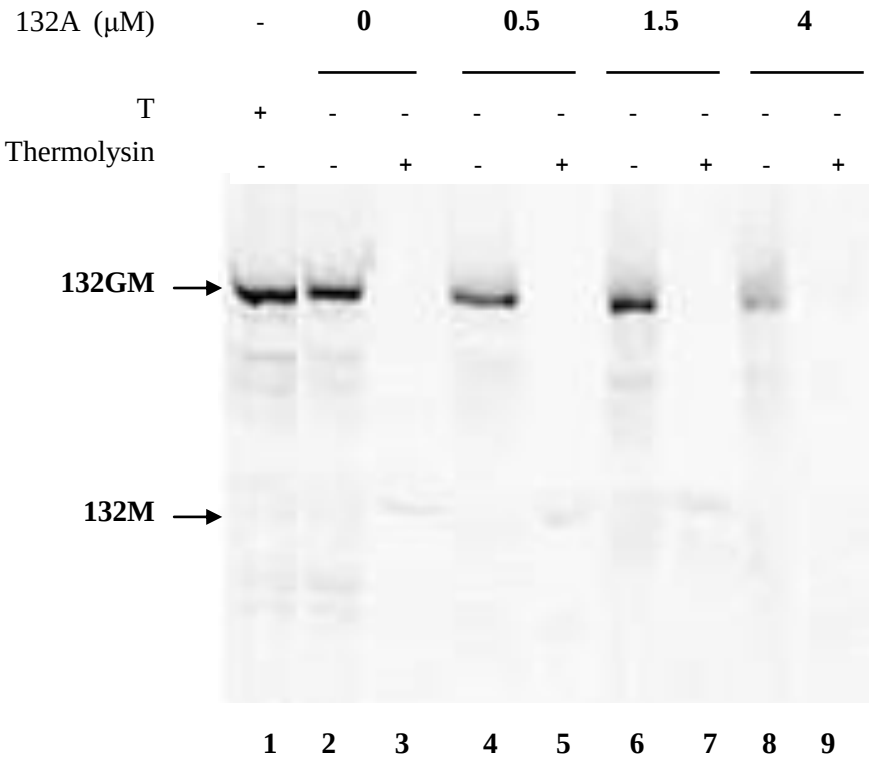
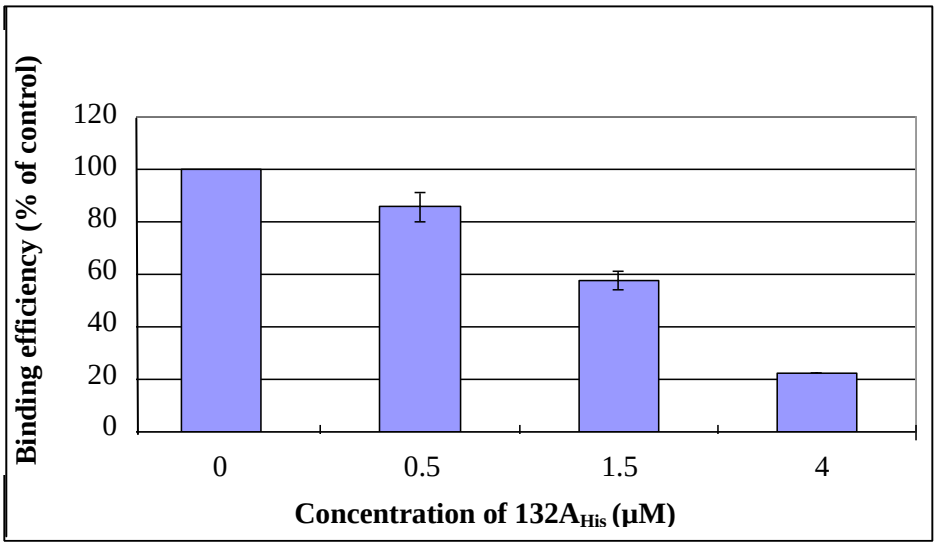


Figure 10. Targeting of 132GM to wild-type chloroplasts competed with $132A_{His}$. (A) Schematic diagram illustrating the set-up of the chloroplast targeting assays; (B) Phosphorimager-visualized SDS-PAGE gels following *in vitro* chloroplast targeting assays comparing the targeting efficiency of 132GM to wild-type chloroplasts in the presence of $132A_{His}$. Lane 1 is loaded with *in vitro* transcription/translation (IVT) products corresponding to 1/10 the amount used in each import reaction (T). Lanes 2, 4, 6 and 8 contain untreated chloroplasts corresponding to bound proteins. Lanes 3, 5, 7 and 9 contain chloroplasts following thermolysin treatment. 132M represents the M-domain of atToc132 which is protected from thermolysin due to insertion in the outer envelope membrane.

A.



B.

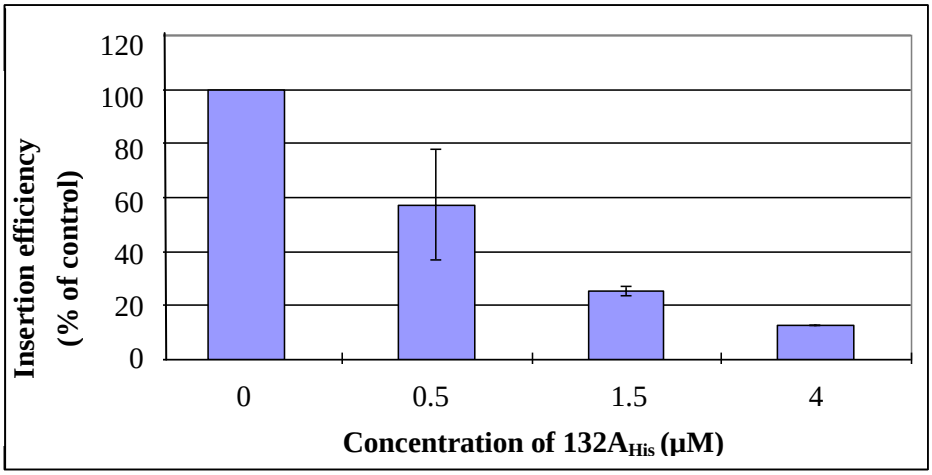


Figure 11. Quantitation of targeting efficiency of 132GM competed with increasing concentration of $132A_{His}$. Binding (A) and insertion (B) efficiency of 132GM in the presence of different amount of $132A_{His}$ are indicated as the percentage of *in vitro* transcription/translation (IVT) product added to the reaction. Error bars correspond to standard error of the mean for ≥3 repeats. The binding and insertion efficiency of 132GM in the presence of 4 µM $132A_{His}$ was measured only once in duplicate..

4. Specific interaction between members of the Toc159 and Toc34 families

4.1 Background

The members of the Arabidopsis Toc159 family, atToc159, atToc132 and atToc120, have been proposed to distribute into distinct Toc complexes based on immunoaffinity chromatography and solid-phase binding assays (Ivanova et al., 2004). In that paper, they illustrated 1) that atToc132 and atToc120 form different Toc complexes from those containing atToc159 using immunoprecipitation; 2) that atToc132/120 have a higher affinity than atToc159 for atToc34G; and 3) that atToc159 binds to atToc33G with a higher affinity than atToc132 or atToc120. Interestingly, the apparent affinity of atToc132 and atToc120 was approximately equal for atToc33 and atToc34 using *in vitro* binding assays; the apparent affinity of atToc159 for atToc33, however, was markedly higher than it was for atToc34 (Ivanova et al., 2004). These data raised the possibility that atToc159 may primarily and preferentially associate with atToc33 and atToc132/120 preferentially with atToc34 to form distinct Toc complexes. However, the mechanism by which this preferential association might occur remains untested.

In this chapter, three sets of *in vitro* chloroplast targeting competition assays will be described that were designed to investigate whether the members of the Toc159 family (atToc132 and atToc159) specifically interact with members of the Toc34 family. Briefly, radiolabeled full-length atToc132 or atToc159 were targeted to wild-type or *ppi*1 chloroplasts in the absence or presence of a G-domain fragment of one of these two homologues (132G and 159G) as competitor. It is reported that the G-domain of atToc159 binds to chloroplasts

with the same efficiency as full-length and 159GM-domain fragment of atToc159 (Smith et al., 2002b). Although a similar experiment has not been done for atToc132 or atToc120, it leads to the possibility that the presence of 132G or 159G may compete for the targeting of its own full-length polypeptide or may compete for the targeting of both homologues. If atToc132 and atToc159 preferentially associate with distinct members of the Toc34 family, 132G and 159G would be expected to specifically inhibit the targeting of atToc132 or atToc159, respectively, by capturing most of the G-domain interaction sites on Toc33/34 at the chloroplast surface.

4.2 Objective and Hypothesis

The objective of this study is to examine the specific interactions between members of Toc159 family, atToc132 and atToc159, and those of the Toc34 family, atToc33 and atToc34, using *in vitro* chloroplast targeting competition assays. It is hypothesized that atToc132 primarily associates with atToc34, while atToc159 mainly interacts with atToc33 at the chloroplast outer membrane.

4.3 Results

4.3.1 Expression and purification of atToc132G$_{His}$

atToc132G$_{His}$ (132G$_{His}$) was expressed and purified to be used in *in vitro* chloroplast targeting competition assays as a competitor. Ideally, the A-domain deletion mutant of atToc132 (132GM) would be used here as a competitor for determining the role of the A-domain. However, as mentioned in “Materials and Methods” section, attempts to express

recombinant 132GM$_{His}$ in *E. coli* failed. Since an interaction between the GTPase domain of members of the Toc159 and Toc34 families have been shown to be essential for Toc complex assembly (Bauer et al., 2002; Smith et al., 2002b), we surmised that the G-domain fragment of atToc132 would work as a competitor in these assays in a similar way as 132GM. For the same reason, 159G was also used instead of 159GM as a competitor in this set of experiments. 132G$_{His}$ was expressed in *E. coli* and purified using IMAC in the presence of 0.1 mM THP (IMAC-compatible reducing agent). The molecular weight of 132G is 37.5kDa (Figure 12).

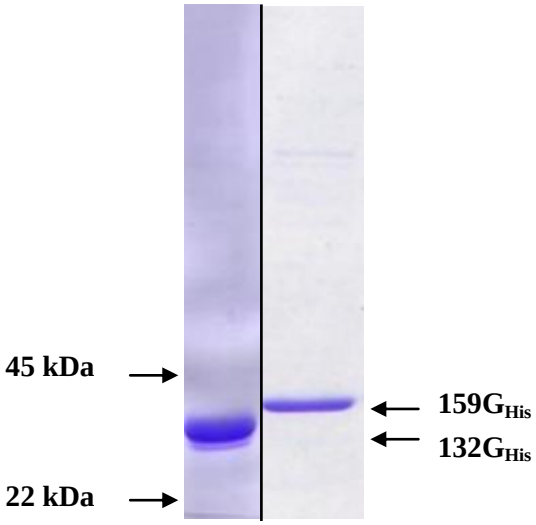


Figure 12. Expression and purification of $132G_{His}$. $132G_{His}$ was expressed in *E. coli* and purified using IMAC (pure $159G_{His}$ was obtained from Geetika Patel in our lab, and $132G_{His}$ was over-expressed ans purified according to the procedure in section 2.4.1). The protein was resolved using SDS-PAGE, and stained with Coomassie blue. Lane 1 and lane 3 are loaded with IMAC-purified $132G_{His}$ and $159G_{His}$, respectively. The black line separates lanes taken from different SDS-PAGE gels run under the same conditions.

4.3.2 Specific interaction between members of the Toc159 and Toc34 families

The first group of experiments in this section compares the targeting of full-length atToc132 or atToc159 to wild-type chloroplasts in the absence or presence of different amounts of 159G as the competitor (Figure 13A). We surmised that if 159G interacts specifically with atToc33, and if atToc132 interacts specifically with atToc34, 159G would compete for targeting of atToc159 to chloroplasts more efficiently than for targeting of atToc132. If, on the other hand, there is little specificity for interactions among the Toc GTPase domains, then 159G might be expected to compete for the targeting of atToc159 and atToc132 with approximately equal efficiency. 0, 0.5 μM, 2 μM and 4 μM of the 159G competitor (much higher concentrations than the radiolabeled atToc132 or atToc159 added to the reaction) was added to compete with the targeting of either atToc132 or atToc159 of the proteins to isolated chloroplasts. The results shown in Figure 14 indicate that 159G competes for the binding and insertion of both atToc132 and atToc159 in a dose-dependent manner.

The binding efficiency of atToc132 to wild-type chloroplasts declined to 77.3%, 64.3% and 32.2% of the efficiency in the absence of 159G when 0.5, 2 and 4 μM of the 159G competitor were added, respectively. Meanwhile, the binding efficiency of atToc159 was 71.4%, 40.5% and 22.0% (Figure 14A) in the presence of 0.5, 2 and 4 μM of the 159G, respectively. Therefore, 159G was slightly more effective at competing for the targeting of atToc159 than it was for atToc132; specifically, the decrease in binding of atToc159 to wild-type chloroplasts by 159G was approximately 6-10% more effective than for atToc132 binding efficiency. The lowest level of 159G competitor tested (0.5 μM) decreased insertion

of atToc132 by ~50%, and by ~60% for atToc159 (Figure 14B). When 2 μM 159G was used as the competitor, the insertion efficiency of atToc132 and atToc159 dropped to 37.0% and 27.4% respectively (Figure 14B). In summary, 159G competes for the targeting of both atToc132 and atToc159 to wild-type chloroplasts. The data suggest that atToc159, or at least 159G, does not exclusively associate with atToc33 or atToc34. However, whether looking at the efficiency of either binding or insertion, 159G is a slightly more effective competitor of atToc159 than of atToc132, which indicates that atToc159 or 159G may have a slightly higher affinity for one or the other member of the Toc34 family. Moreover, the fact that insertion is more dramatically affected by the competitor than is binding suggests that the G-domain of atToc159 may play a more important role in the insertion of the receptor into the membrane than is does in binding it to the outer membrane surface, which is consistent with findings from an earlier study on the targeting of atToc159 to chloroplasts (Richardson, 2007).

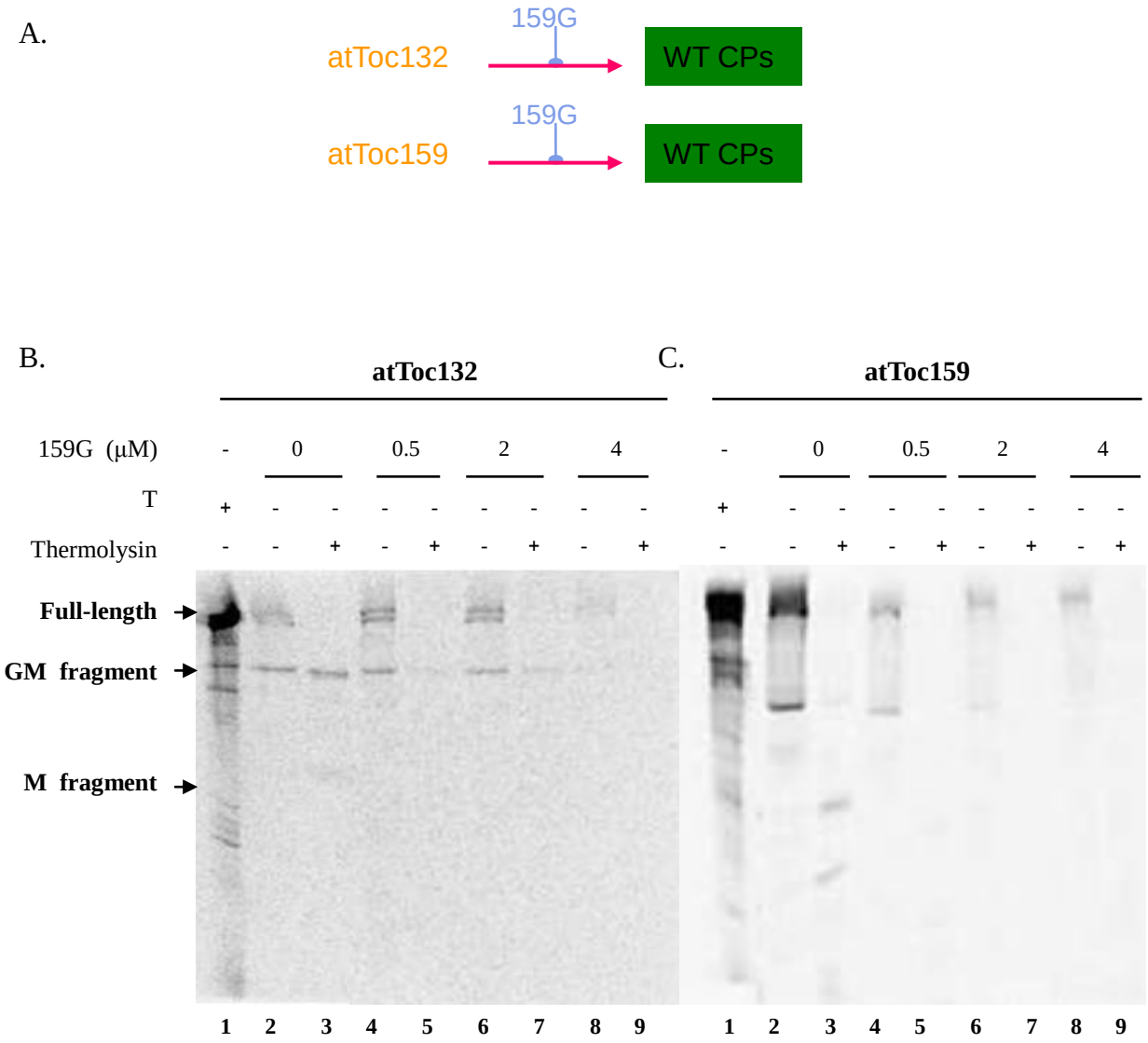
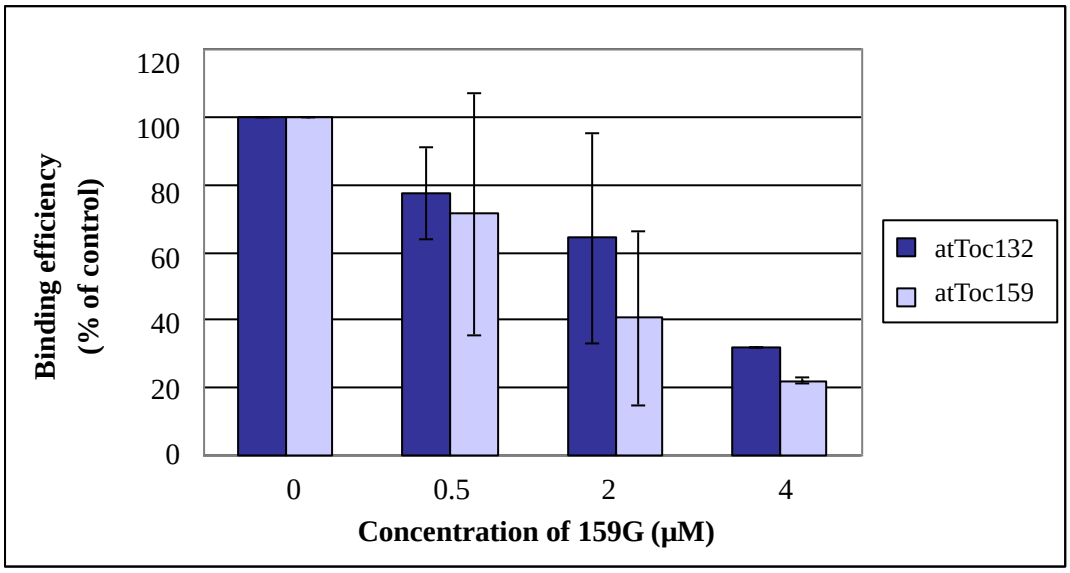


Figure 13. Targeting of atToc132 or atToc159 to wild-type chloroplasts competed with 159G. (A) schematic diagram illustrates how the chloroplast targeting assays were set up; (B,C) Phosphorimager-visualized SDS-PAGE of the chloroplast targeting assays representing targeting efficiency of atToc132 (B) and atToc159 (C) to chloroplasts isolated from wild-type plants competed with increasing concentrations of 159G. Lanes 1 contain *in vitro* transcription/translation products corresponding to 1/10 amount used in each import reaction (T). Lanes 2, 4, 6 and 8 contain untreated chloroplasts corresponding to bound proteins. Lanes 3, 5, 7 and 9 are loaded with intact chloroplasts following thermolysin treatment. The GM fragment is the product of endogenous proteolysis. The M fragment represents the thermolysin protected portion of atToc132 or atToc159 (132M or 159M)

A.



B.

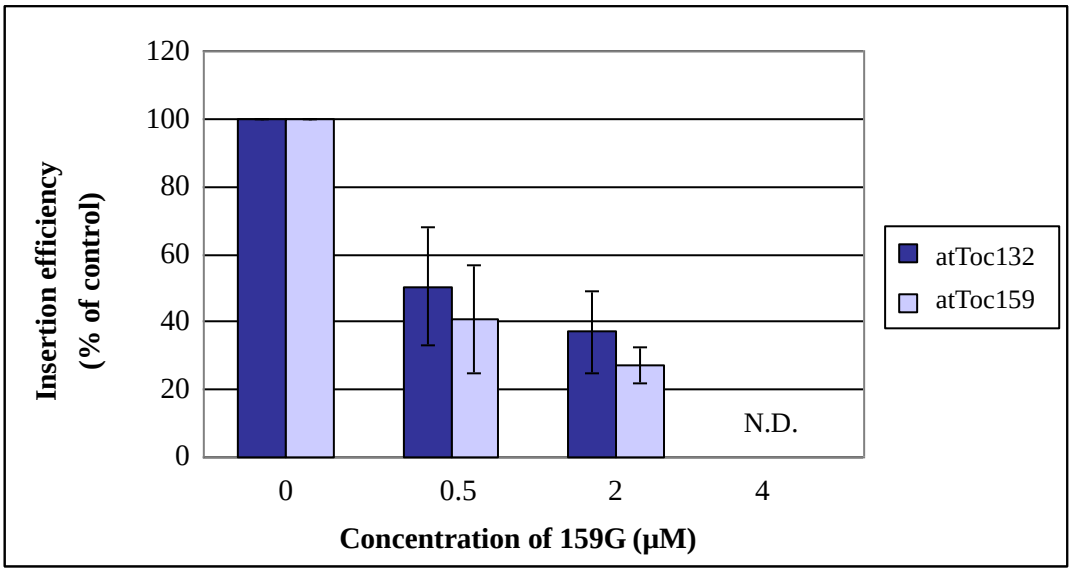


Figure 14. Quantitation of the targeting efficiency of atToc132 and atToc159 to wild-type chloroplasts competed with 159G. Binding (A) and insertion (B) efficiencies were quantitated and calculated as the percentage of *in vitro* transcription/translation (IVT) product added to the reaction. Error bars correspond to standard error of the mean for ≥2 repeats. The binding efficiency of atToc159 in the presence of 4μM 159G was measured only once in duplicate, and the insertion efficiency in the presence of 4μM 159G was not detected (N.D.).

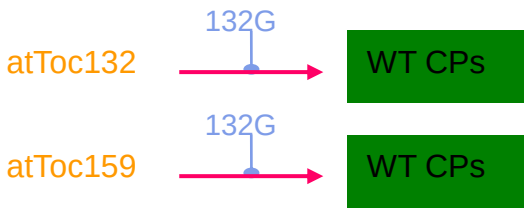
The second group of chloroplast targeting assays involved targeting atToc132 or atToc159 to wild-type chloroplasts competed with 132G (Figure 15A). As with the import assays competed with 159G (Figure 14), the effect of increasing concentrations of 132G as a competitor on targeting of the two preprotein receptors will shed light on whether atToc132 and atToc159 preferentially associate with a specific member of the Toc34 family. Specifically, if 132G competes more effectively with atToc132 than with atToc159, it will suggest that the two receptors have distinct binding sites at the chloroplast (presumably one of atToc33 and atToc34). If, on the other hand, 132G competes with equal effectiveness for the targeting of atToc132 and atToc159, it will indicate that the two receptors share binding sites at the chloroplast, and therefore likely do not preferentially interact with either atToc33 or atToc34.

The data shown in Figure 16 indicate that the presence of 132G competes for the binding and insertion of both atToc132 and atToc159. However, the trend here is distinct from that seen when using 159G as the competitor (Figure 14).

The binding efficiency of atToc132 declines to 80.5%, 71.7% and 53.9% of the efficiency in the absence of competitor in the presence of 0.5, 2 and 4 μM competitor, respectively. However, competition with 132G does not show a dose-dependent response for the binding of atToc159 to wild-type chloroplasts. Rather, the binding efficiencies drop off more dramatically to 23.4%, 40.8% and 23.0% of that without a competitor, with 0.5 μM, 2 μM and 4 μM 132G, respectively (Figure 16A). The competition of 132G with both atToc132 and atToc159 confirm that neither of these two receptors exclusively interacts with only one member of the Toc34 family. Assuming that atToc33/34 is the primary binding site for

atToc132 and atToc159, the data suggest that these receptors, or at least their GTPase domains, can interact with either member of the Toc34 family. The addition of 0.5 μM, 2 μM and 4 μM 132G reduces the insertion efficiency of atToc132 to 66.1%, 53.2% and 26.5% respectively, and reduced the insertion efficiency of atToc159 to 50%, 32.3% and 12.2% (Figure 16B). Interestingly, the effect of 132G on both the binding and insertion of atToc159 is more dramatic than that of atToc132. This might be due to the different amounts of atToc33 and atToc34 present in wild-type chloroplasts; specifically, atToc33 is much more abundant than atToc34 (Gutensohn et al., 2000). Another possibility is that the A-domain functions in inhibiting binding and insertion of atToc132 to atToc33, so that the lack of A-domain allows 132G to associate with atToc33 simply due to its higher abundance than atToc34. To clarify whether the larger amount of atToc33 causes these intriguing results, another chloroplast targeting assay was designed using *ppi*1 chloroplasts (atToc33 knockout mutant) competed by 132G.

A.



B.

C.

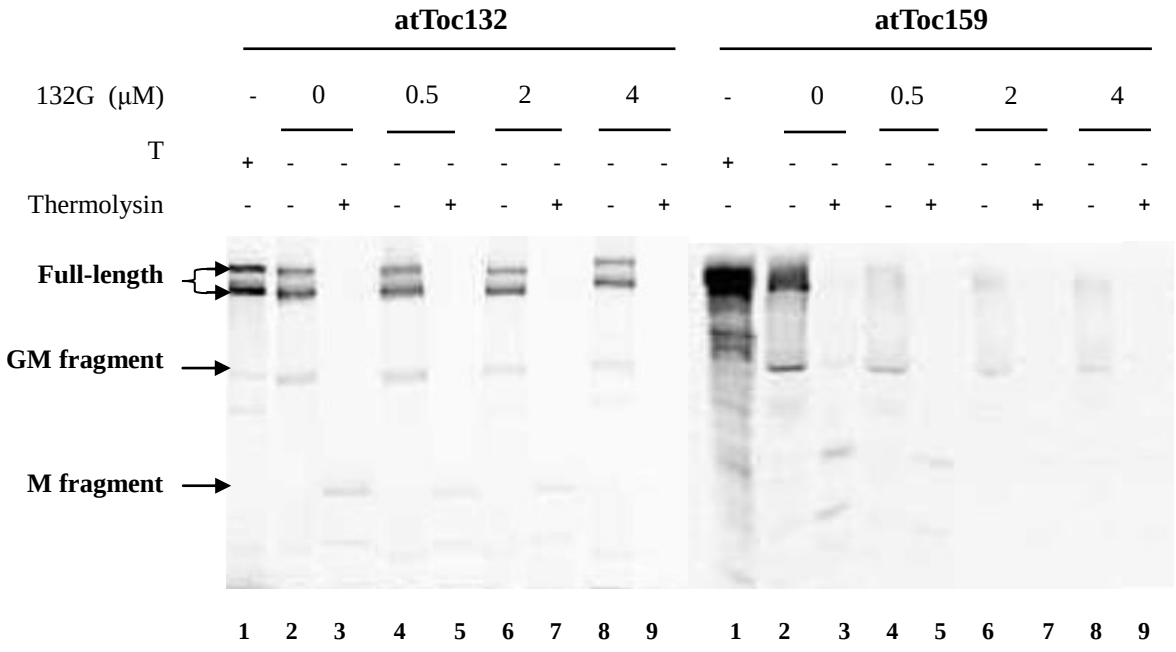
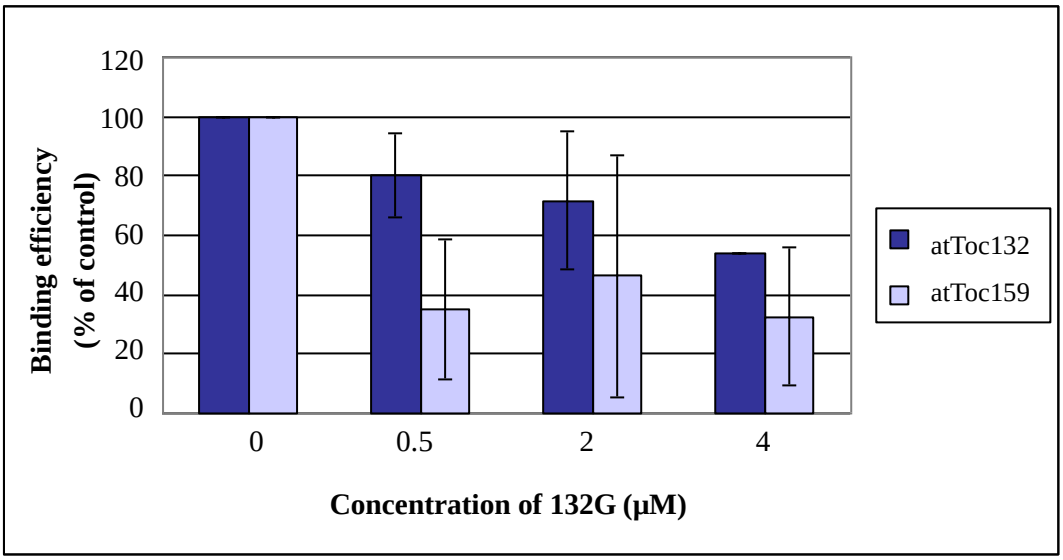


Figure 15. Targeting of atToc132 or atToc159 to wild-type chloroplasts competed by 132G. (A) schematic diagram illustrates how the chloroplast targeting assays set up; (B,C) Phosphorimager-visualized SDS-PAGE of the chloroplast targeting assays representing targeting efficiency of atToc132 (B) and atToc159 (C) to chloroplasts isolated from wild-type plants competed by increasing concentration of 132G. Lanes 1 contains *in vitro* transcription/translation products corresponding to 1/10 amount used in each import reaction. Lanes 2, 4, 6 and 8 contain untreated chloroplasts corresponding to bound proteins. Lanes 3, 5, 7 and 9 are loaded with intact chloroplasts following thermolysin treatment. The GM fragment is the product of endogenous proteolysis. The M fragment represents the thermolysin protected portion of atToc132 or atToc159 (132M or 159M).

A.



B.

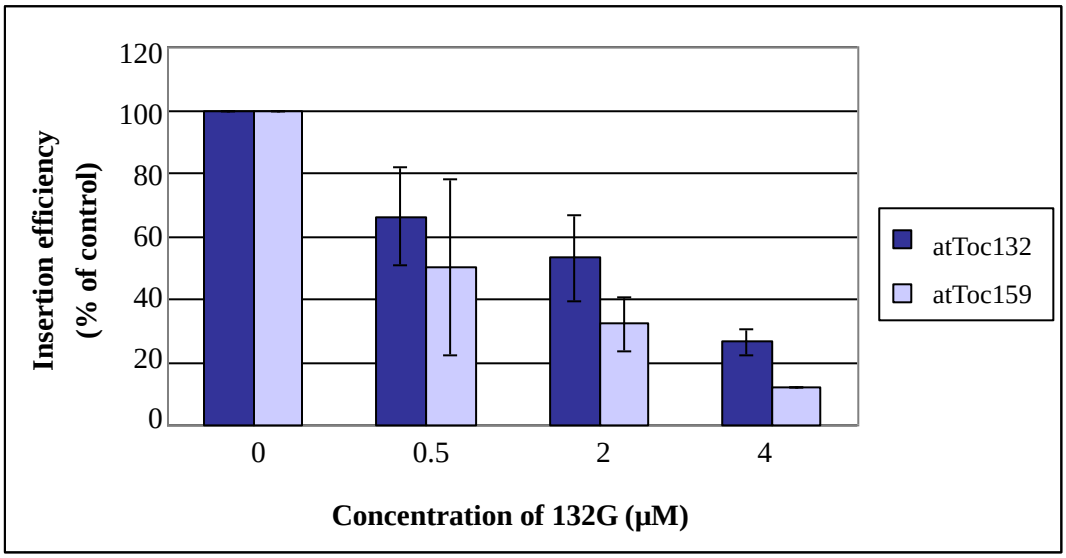


Figure 16. Quantitation of the targeting efficiency of atToc132 and atToc159 to wild-type chloroplasts competed with 132G. Binding (A) and insertion (B) efficiencies were quantitated and calculated as the percentage of *in vitro* transcription/translation (IVT) product added to the reaction. Error bars correspond to standard error of the mean for ≥3 repeats. The binding efficiency of atToc132 and insertion efficiency of atToc159 in the presence of 4µM 132G were measured only once in duplicat.

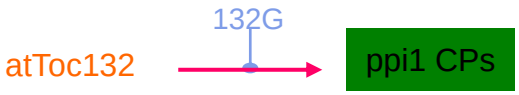
Chloroplasts isolated from *ppi*1 Arabidopsis, which lack atToc33 (Jarvis et al., 1998), were used instead of wild-type in an effort to avoid the complication of having different amounts of two members of the Toc34 family at the chloroplast surface as potential docking sites for atToc132 and atToc159 (Figure 17). Binding efficiency of atToc132 to *ppi*1 chloroplasts falls to 49.9%, 48.4% and 47.6% of that in the absence of a competitor in the presence of 0.5 μM, 2 μM and 4 μM 132G, respectively (Figure 18). The competition seen here is more dramatic for wild-type chloroplasts, which supports the idea that the difference observed between the effectiveness of 132G as a competitor for the targeting of atToc132 and atToc159 in the earlier experiment (Figure 16) may be due to the difference in the amount of atToc33 and atToc34 in wild-type chloroplasts. A less effective competition with 132G for atToc132 to wild-type chloroplasts, where much more atToc33 is located, also indicates that the A, M-domain deletion (132G) fragment of atToc132 has a higher affinity for atToc33 than full-length atToc132. This result supports the idea that the A-domain plays a role in restricting the targeting of atToc132 to atToc34 docking sites on the chloroplast surface by inhibiting its binding to atToc33.

The insertion efficiencies of atToc132 at 0.5 μM, 2 μM and 4 μM of the 132G competitor were 61.9%, 60.7% and 37.1% (Figure 18B), which is similar to the trend observed when competing for targeting to wild-type chloroplasts (Figure 16B). An interesting point is that the data on 132G's effect on targeting of atToc132 to *ppi*1 chloroplasts (Figure 18B) is similar to its effect on targeting of atToc159 to wild-type chloroplasts (Figure 16B). The sharp decrease and stable level of binding in the presence of different concentrations of

competitor may illustrate that 132G associates with both members of the Toc34 family. Since the G-domain of atToc132 may have a similar affinity for atToc33 and atToc34 (Ivanova et al., 2004), if our hypothesis that there are distinct Toc complexes at the outer membrane of chloroplasts is correct, the A-domain will play a role in regulating the atToc132-containing Toc complexes by restricting the interaction between atToc132 and a specific member of the Toc34 family.

In conclusion, experiments described in this chapter indicate that atToc132 and atToc159 do not exclusively associate with specific members of the Toc34 family. However, they may have slightly different affinities for atToc33 or atToc34 in order to form structurally distinct Toc complexes. In addition, the last set of chloroplast targeting assays raises the possibility that the A-domain might affect the targeting of atToc132 to chloroplasts by inhibiting its association with a specific member of the Toc34 family, atToc33.

A.



B.

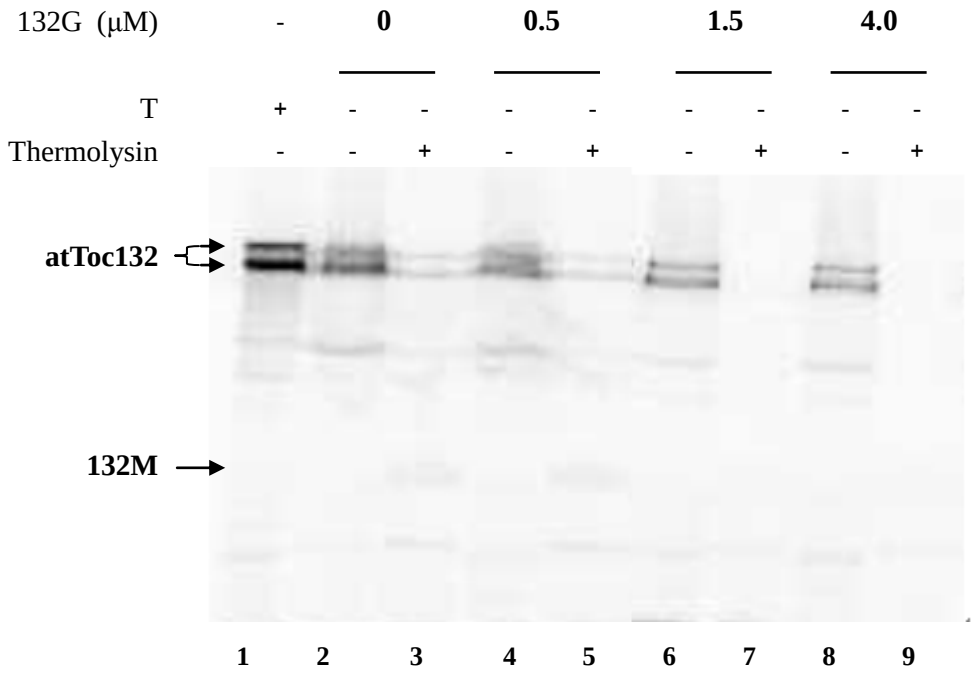
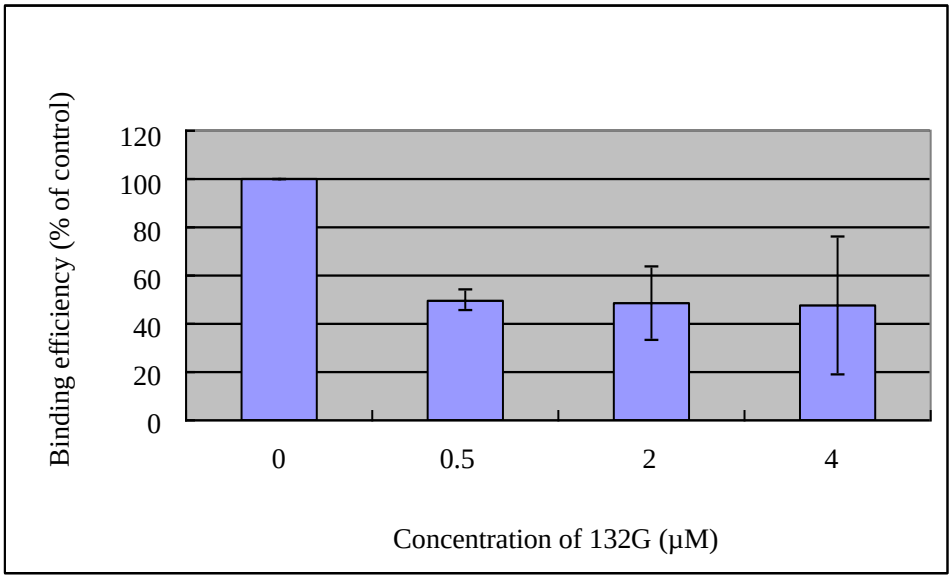


Figure 17. Targeting of atToc132 to *ppi*1 chloroplasts competed with 132G. (A) schematic diagram illustrates how the chloroplast targeting assays set up; (B) Phosphorimager-visualized SDS-PAGE of the chloroplast targeting assays representing targeting efficiency of atToc132 to chloroplasts isolated from *ppi*1 plants in the presence of increasing concentrations of 132G. Lane 1 contain *in vitro* transcription/translation products corresponding to 1/10 amount used in each import reaction (T). Lanes 2, 4, 6 and 8 contain untreated chloroplasts corresponding to bound proteins. Lanes 3, 5, 7 and 9 are loaded with intact chloroplasts following thermolysin treatment. 132M represents the M-domain fragment of atToc132.

A.



B.

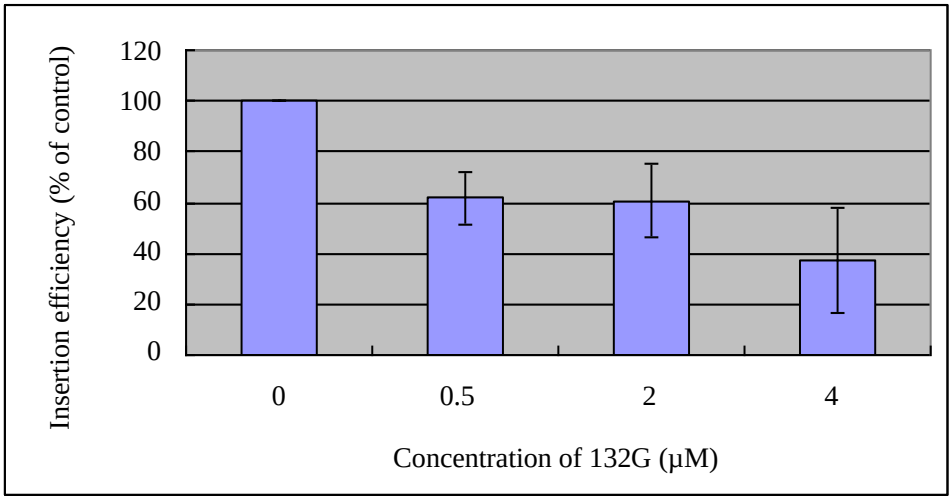


Figure 18. Quantitation of the targeting efficiency of atToc132 to *ppi*1 chloroplasts competed with 132G. Binding (A) and insertion (B) efficiencies were quatitated and calculated as the percentage of *in vitro* transcription/translation (T) product added. Error bars correspond to standard error of the mean of ≥3 repeats.

5. The role of the A-domain in the assembly of distinct Toc complexes

5.1 Background

Although distinct Toc complexes, atToc132-atToc34 or atToc159-atToc33, have been shown to play roles in recognizing non-photosynthetic and photosynthetic preproteins, respectively (Ivanova et al., 2004), data presented in Chapter 4 suggest that atToc132 and atToc159 do not exclusively interact with specific members of the Toc34 family. The data do, however, indicate that there might still be a difference in the affinity of atToc132 and atToc159 for atToc33 and atToc34. In addition to the conclusion of Chapter 3 that the A-domain inhibits the binding of atToc132 to wild-type chloroplasts, there is another prediction that can be made based on experiments done in Chapter 4, which is that the A-domain may affect the targeting of atToc132 to chloroplasts by restricting its binding to atToc33.

To clarify how the A-domain affects the interaction of atToc132 with the members of the Toc34 family, two more sets of experiments were designed: 1) comparison of the binding efficiency of full-length atToc132 and 132GM to 33G or 34G (G-domain fragments of atToc33 or atToc34) using *in vitro* solid-phase binding assays; and 2) *in vitro* chloroplast targeting competition assays in which 132GM was targeted to *ppi*1 or *ppi*3 (a atToc34 deletion mutant) chloroplasts competed by different concentrations of 132A. Basically, both assays presented in this chapter are designed to eliminate the drawbacks of using wild-type chloroplasts in which two members of the Toc34 family are present at the same time, but in very different amounts (i.e. atToc33 is much more abundant than atToc34) (Gutensohn et al.,

2000). More specifically, the use of solid-phase binding assays avoids the effect of all other proteins in chloroplasts isolated from plants. In solid-phase binding assays, the binding efficiency of radiolabeled proteins to immobilized "bait" proteins can easily be detected and quantitated. The radiolabeled proteins used as prey were *in vitro* transcription/translation products of full-length atToc132 and 132GM. $33G_{His}$ and $34G_{His}$, which represent recombinant versions of the GTPase-domains of atToc33 and atToc34, respectively, with C-terminal His-tag fusion, were the bait in these assays.

5.2 Objective and hypothesis

Experiments described in this section were designed to clarify the role of the A-domain in binding atToc132 to distinct Toc complexes. It is hypothesized that the A-domain restricts the binding of atToc132 to members of the Toc34 family by inhibiting its association with atToc33, so that the atToc132-atToc34-atToc75 Toc complex is formed preferentially to function in recognizing non-photosynthetic "housekeeping" preproteins.

5.3 Results

5.3.1 Expression and purification of atToc34G$_{His}$

Recombinant atToc34G with a C-terminal His-tag ($34G_{His}$) was expressed and purified to be immobilized on His-Bind Ni-charged resin as a bait for *in vitro* solid-phase binding assays. Both members of the Toc34 family in *Arabidopsis thaliana* (atToc33 and atToc34) include a short α-helical C-terminal membrane anchor domain and a GTPase domain which

plays an essential role in the core Toc complex (Gutensohn et al., 2000). It is reported that the interactions between GTPase domains of atToc159 and atToc33 mediate the targeting of Toc159 to chloroplasts as well as its interaction into the Toc complex (Smith et al., 2002b; Wallas et al., 2003).

In order to avoid degradation of $34G_{His}$, all steps of the IMAC purification process took place in a cold room (4℃), and THP (an IMAC-compatible reducing agent) was added into each buffer to a final concentration of 0.1 mM to prevent oxidation. IMAC purification yielded good concentrations of adequately pure recombinant protein (Figure 19).

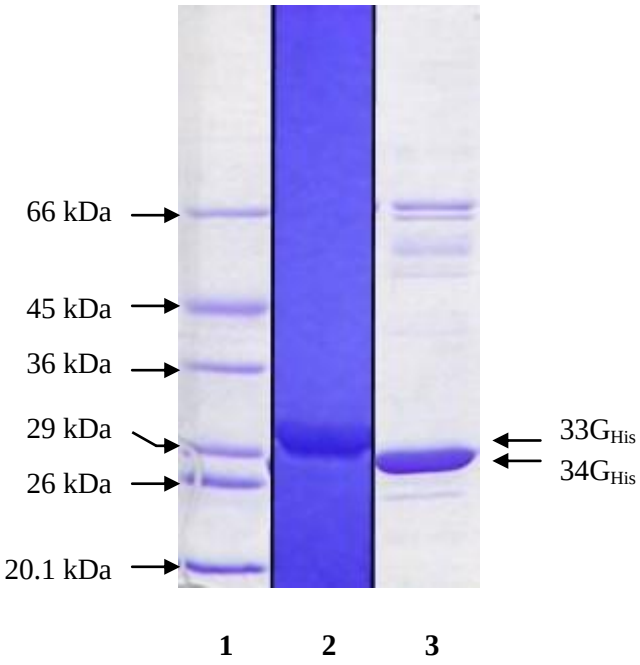


Figure 19. Expression and purification of 33G$_{His}$ and 34G$_{His}$. Recombinant 33G$_{His}$ and 34G$_{His}$ were expressed in *E. coli* and purified using IMAC (33G$_{His}$ was abteined from Kyle Weston in our lab; expression and purification of 34G$_{His}$ were done as section 2.4.3). Samples were separated using SDS-PAGE gel and stained with Coomassie blue. Lane 2 is loaded with IMAC-purified 33G$_{His}$. Lane 3 contains IMAC-purified 34G$_{His}$. The black line separates lanes taken from different SDS-PAGE gels run under the same conditions.

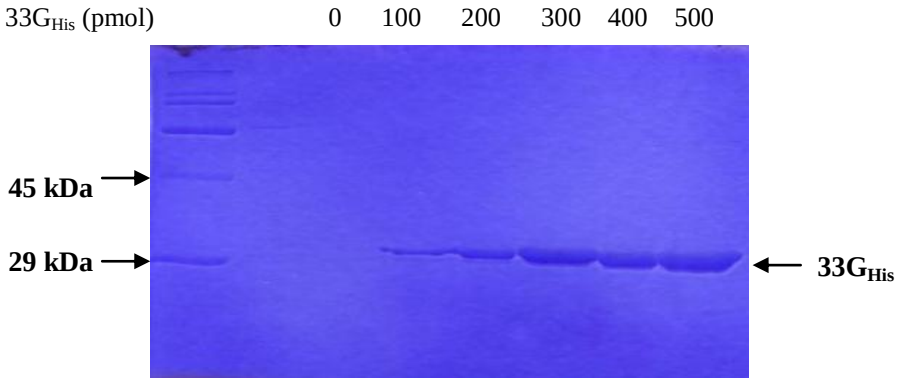
5.3.2 Effect of the A-domain on binding of atToc132 to specific members of the Toc34 family

Solid-phase binding assays were used to compare the binding of radiolabeled full-length atToc132 and 132GM to increasing amounts of immobilized $33G_{His}$ or $34G_{His}$ (Figure 20A & Figure 21A). 100 , 200 , 300, 400 and 500 pmol of $33G_{His}$ or $34G_{His}$ were immobilized on the His-Bind Ni-charged resin and incubated with the same amount of radiolabeled atToc132 (Figure 20B & Figure 21B) or 132GM (Figure 20C & Figure 21C). The binding efficiency was calculated as the percent of *in vitro* transcription/translation radiolabeled proteins added to the original reaction. If the interaction between the "bait" and the "prey" is genuine, it is expected that the amount of prey "pulled down" by the bait will increase as the amount of bait increases. If, on the other hand, the bait-prey interaction is non-specific in nature, then the amount of prey that is pulled down should not change with the amount of bait, as the bait is always in excess in this experiment.

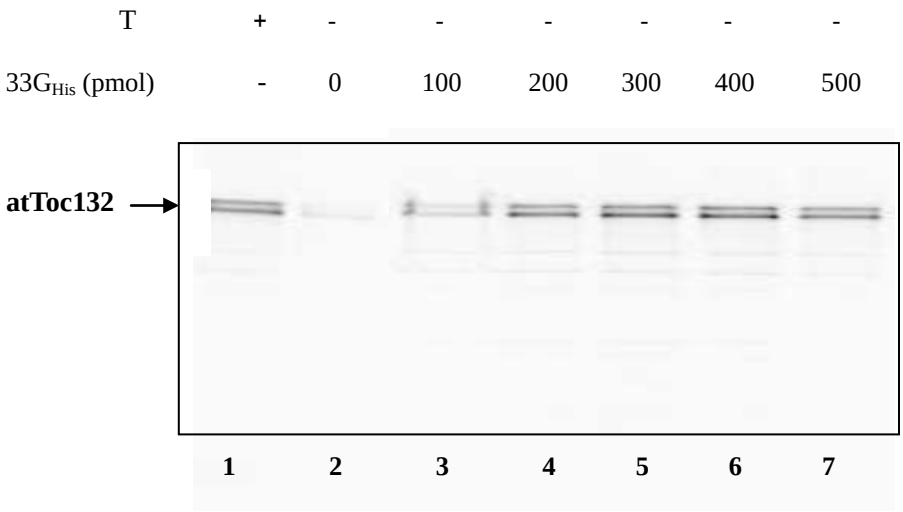
When full-length atToc132 binding to 33G was tested, binding efficiency increased from 1.2% to 11% as the amount of bait (33G) increased from 100 to 500 pmol (Figure 22A). In contrast, when 300 , 400 and 500 pmol of 33G was used as bait, the binding efficiency of 132GM were 21.0%, 18.8% and 18.3%, respectively (Figure 22A), which are dramatically higher than the binding efficiency of full-length atToc132. These data are consistent with the earlier observation that the deletion of the A-domain of atToc132 stimulates its binding to 33G. In other words, the presence of the 132A-domain prevents atToc132 from interacting with atToc33. On the other hand, the same general trends are observed for binding of atToc132 and

132GM to 34G. atToc132 binding efficiency increased from 4.9% to 23.5% as the 34G bait increases, whereas efficiency of 132GM binding to 34G increases from 16.2% to 29.2% for 132GM (Figure 22B). The overall binding efficiency of full-length atToc132 is higher for 34G than for 33G, which is consistent with atToc132 having a higher affinity for atToc34 than for atToc33. However, the increase in efficiency of binding to 34G upon removal of the A-domain is not as dramatic as observed for 33G. This result suggests that the A-domain primarily inhibits the association of atToc132 with atToc33.

A.



B.



C.

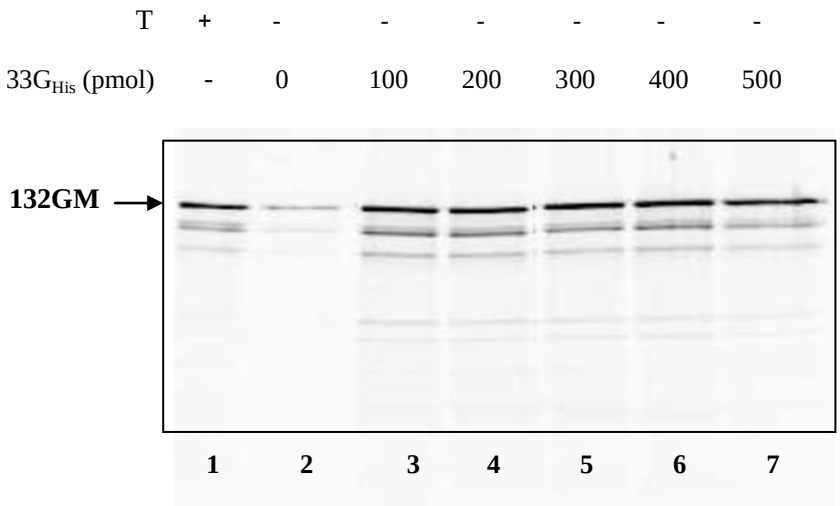
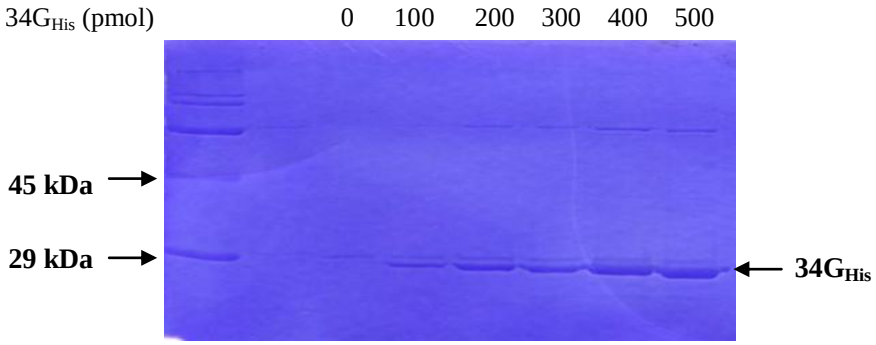


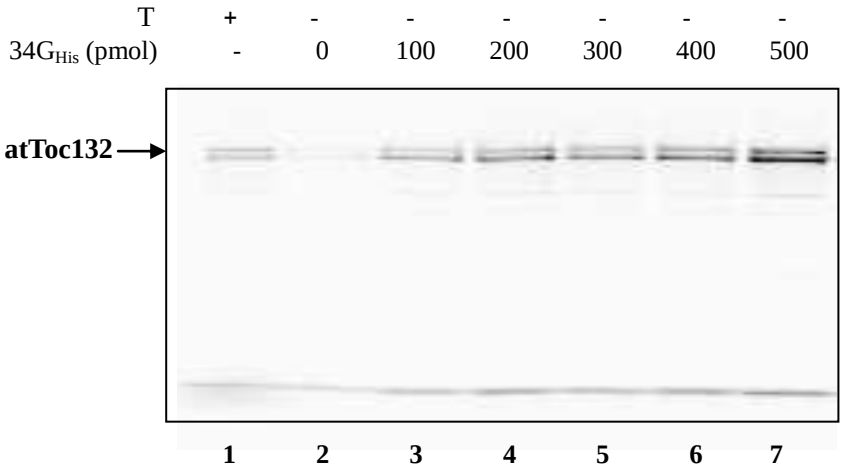
Figure 20.Solid-phase binding assay: binding of atToc132 or 132GM to immobilized $33G_{His}$. (A) SDS-PAGE showing increasing amount of bait, $33G_{His}$, immobilized on the resin. (B) Phosphorimager-visualized SDS-PAGE of binding full-length atToc132 to $33G_{His}$. (C) Phosphorimager-visualized SDS-PAGE of binding

132GM to $33G_{His}$. Lanes 1 in (B)(C) are loaded with 1/10 amount of radiolabeled protein added in each reaction (T). Lanes 2-6 represent binding of radiolabeled protein to 0-500 pmol $33G_{His}$.

A.



B.



C.

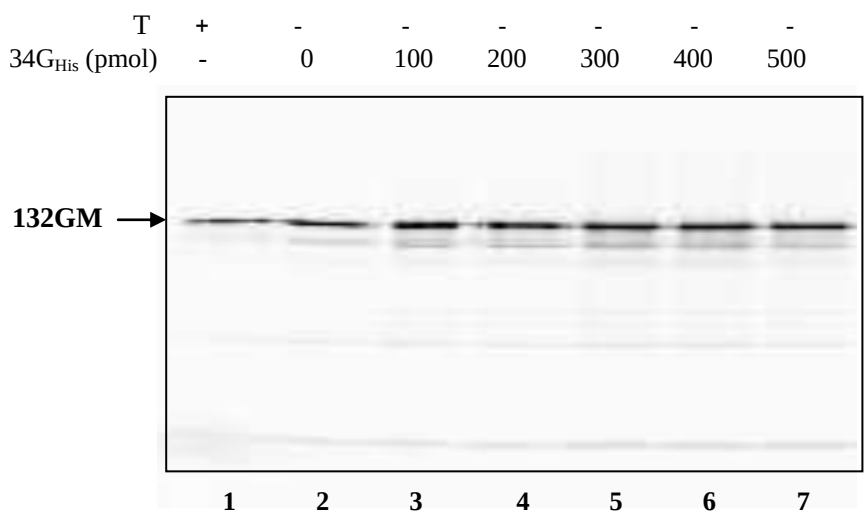
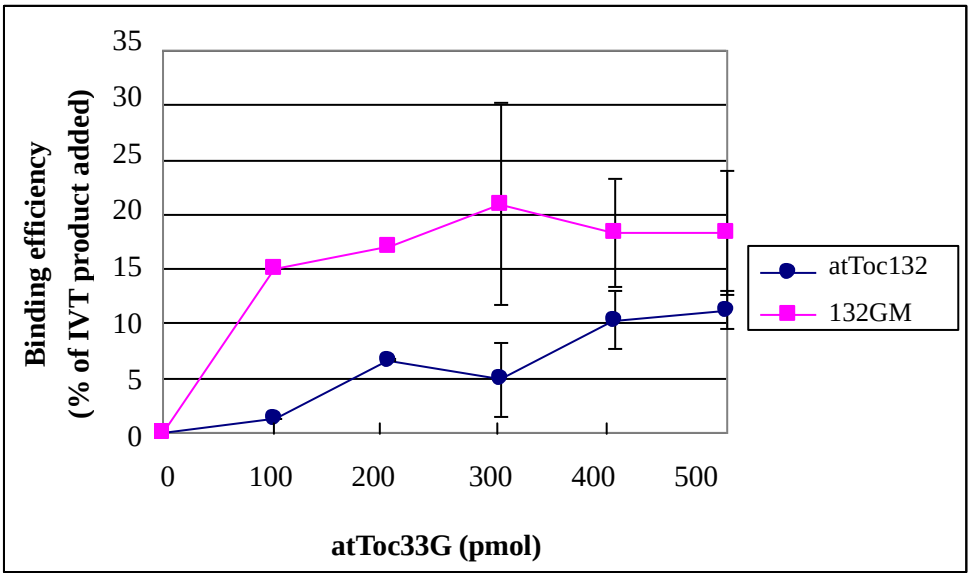


Figure 21. Solid-phase binding assay: binding of atToc132 or 132GM to immobilized $34G_{His}$. (A) SDS-PAGE showing increasing amount of bait, $34G_{His}$, immobilized on the resin. (B) phosphorimager-visualized SDS-PAGE of binding full-length atToc132 to $34G_{His}$. (C) phosphorimager-visualized SDS-PAGE of binding 132GM to $34G_{His}$. Lanes 1 in (B)(C) are loaded with 1/10 amount of radiolabeled protein added in each reaction

(T). Lanes 2-6 represent binding of radiolabeled protein to 0-500 pmol $34G_{His}$.

A.



B.

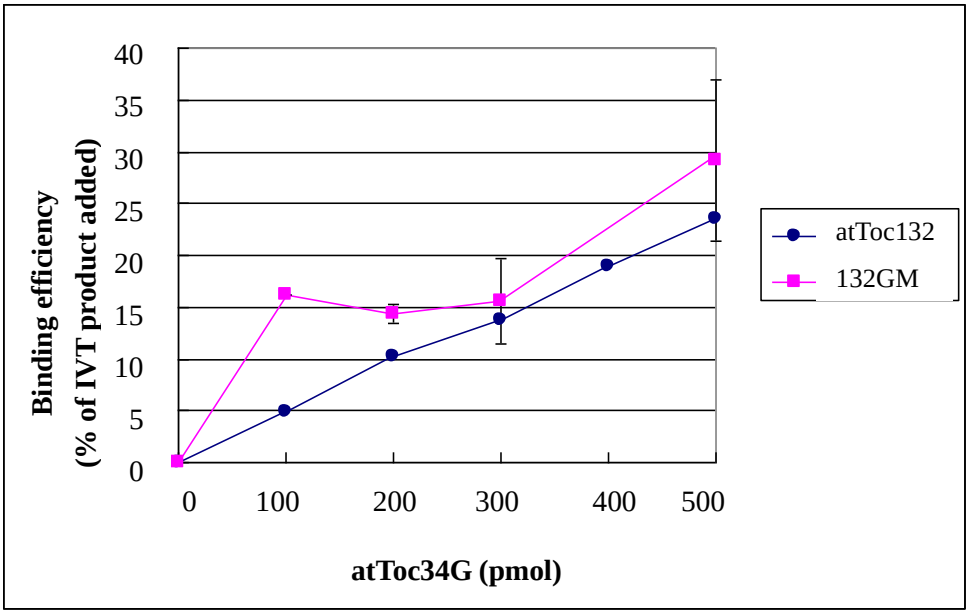


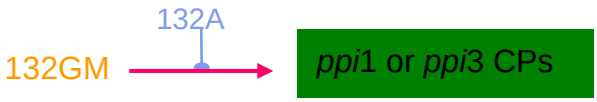
Figure 22 Binding efficiency of different Toc132 constructs to $33G_{His}$ (A) or $34G_{His}$ (B). The efficiency of binding of radiolabeled full-length atToc132 or 132GM was quantitated and is presented as the percentage of *in vitro* transcription/translation (T) product added. Error bars correspond to standard error of the mean of ≥2-time repeats. However, the lack of some error bars were due to that they were only detected once, and data of some binding reactions were not detected.

Chloroplast targeting competition assays were performed as described earlier. Targeting of 132GM to chloroplasts isolated from *ppi*1 or *ppi*3 mutant Arabidopsis (rather than wild-type as in the earlier assays) was competed by different concentrations of $132A_{His}$ (Figure 23). The deletion of atToc33 or atToc34 from chloroplasts was expected to give a clearer picture of how the A-domain affects the targeting of atToc132 to chloroplasts. Again, the binding and insertion efficiency of each reaction is presented as the percent of the efficiency of 132GM targeted in the absence of a competitor.

The binding efficiency of 132GM to *ppi*1 chloroplasts was reduced to 79.3%, 86.4% and 53.1% when 0.5, 1.5 and 4 μM of $132A_{His}$ was added as a competitor, and the insertion efficiency fell to 84.2%, 74.8% and 53.1%, respectively (Figure 24). Generally, the effect of $132A_{His}$ on targeting of 132GM to *ppi*1 chloroplasts was not as obvious as it was for wild-type chloroplasts, which supports the idea that the A-domain may not influence the association between atToc132 and atToc34. To further test this hypothesis, *ppi*3 chloroplasts were used as the target in similar competition assays. These data show that the binding efficiency of 132GM decreases to 73.6%, 61.5% and 38.1%, and that the insertion efficiency decreases to 54.9%, 59.3% and 29.8% when $132A_{His}$ is included at concentrations of 0.5, 1.5 and 4 μM, respectively (Figure 24). A relatively more dramatic declining trend is evident in targeting to *ppi*3 chloroplasts, which is consistent with the results of the solid-phase binding assays indicating that the A-domain inhibits the binding of atToc132 to atToc33. Interestingly, however, the decrease in 132GM targeting to *ppi*3 is still smaller than that to wild-type chloroplasts.

In summary, results of the assays presented in this section are consistent with each other, and show that the A-domain influences the formation of distinct Toc complexes by inhibiting binding of atToc132 to atToc33, thereby increasing the likelihood of binding to atToc34.

A.



B.

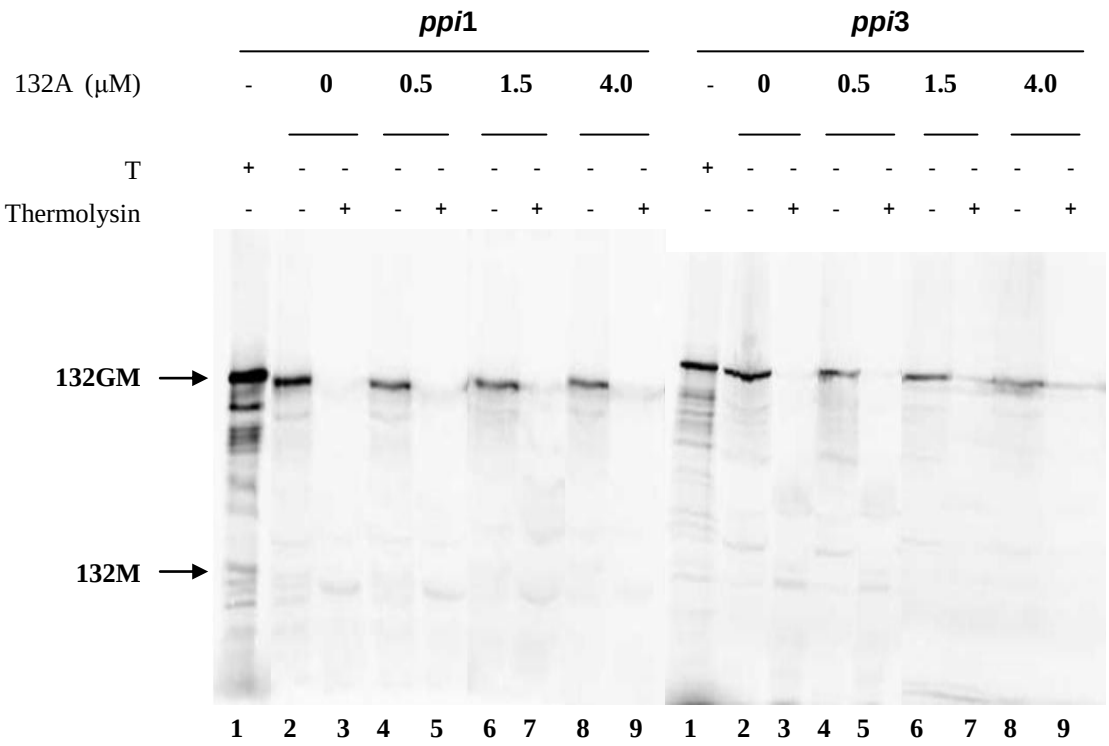
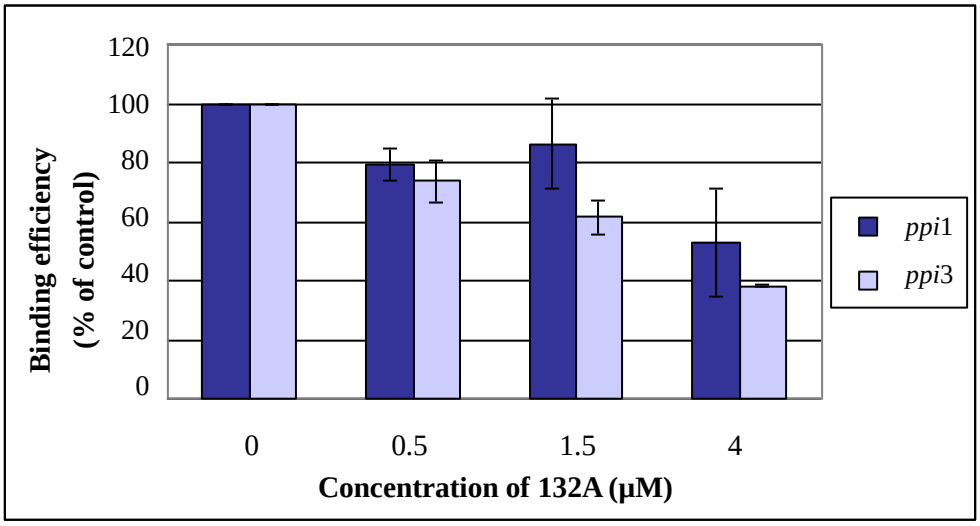


Figure 23. Targeting of 132GM to *ppi*1 or *ppi*3 chloroplasts competed with $132A_{His}$. (A) schematic diagram illustrates how the chlorolplast targeting assays were set up; (B,C) Phosphorimager-visualized SDS-PAGE of the chloroplast targeting assays representing targeting efficiency of 132GM to *ppi*1 and *ppi*3 in the presence of increasing concentration of $132A_{His}$. Lanes 1 contain *in vitro* transcription/translation products corresponding to 1/10 amount used in each import reaction (T). Lanes 2, 4, 6 and 8 contain untreated chloroplasts corresponding to bound proteins. Lanes 3, 5, 7 and 9 are loaded with intact chloroplasts following thermolysin treatment. 132M represents the M-domain fragment of atToc132.

A.



B.

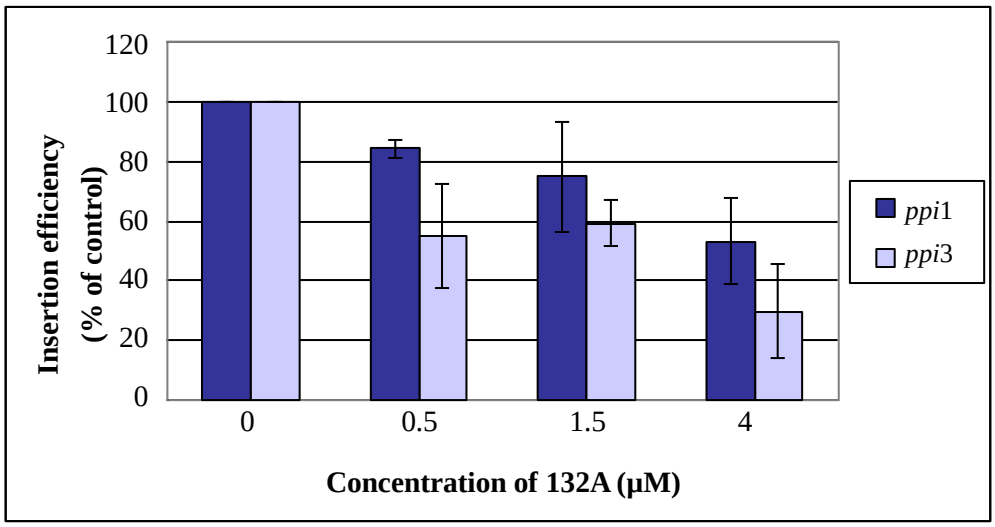


Figure 24. Quantitation of the targeting efficiency of 132GM to *ppi*1 and *ppi*3 chloroplasts competed with $132A_{His}$. Binding (A) and insertion (B) efficiencies were quantitated and calculated as the percentage of *in vitro* transcription/translation (T) product added. Error bars correspond to standard error of the mean of ≥3 repeats.

6. Discussion

6.1 The role of the A-domain in targeting of atToc132 to chloroplasts and in Toc complex assembly

Targeting of the Toc159 homologue, atToc159, to chloroplasts can be divided into two distinct steps: binding of atToc159 to the outer membrane and insertion into the membrane (Smith et al., 2002b). The stages can be differentiated following an *in vitro* targeting assay using thermolysin treatment to detect the insertion of Toc159 or other members of the family by the production of a characteristic thermolysin-protected fragment corresponding to the M-domain. This treatment allows us to determine the efficiency of both steps. During targeting of Toc159 to chloroplasts as part of the Toc complex assembly, it was demonstrated by Hiltbrunner et al. (2001b) that atToc159 binds to the G-domain of atToc33 through a homotypic interaction between GTPase domains. Later studies confirmed that integration of atToc159 to chloroplasts is mediated by the interaction between the GTPase domains of atToc159 and atToc33 (Bauer et al., 2002; Smith et al., 2002b; Wallas et al., 2003). In addition to the observation that the first step, binding of atToc159 to the chloroplast outer membrane via an interaction with atToc33, does not consume either GTP or ATP, while the insertion step requires the binding of GTP (Smith et al., 2002b), insertion efficiency is more representative for the Toc complex assembly. Since the G-domain of atToc132 is homologous to the G-domain of atToc159, it has been assumed, but not confirmed, that it also plays a crucial role in binding atToc132 to the chloroplast outer membrane and to its assembly into functional Toc complexes.

A previous study illustrated that the deletion of the A-domain does not affect the insertion of atToc132 into wild-type chloroplasts, rather removal of the A-domain stimulates the binding (Richardson, 2007; Figure 6A & B, atToc132 and 132GM). Viewed another way, one can say that the presence of the A-domain inhibits the targeting of atToc132 to wild-type chloroplasts (Richardson, 2007; Figure 6B, atToc132 and 132GM). Since atToc33 is much more abundant in wild-type chloroplasts than atToc34, it seems that the A-domain restricts the binding of atToc132 to atToc33. The current study was designed based on the earlier observation by Richardson (2007) that the A-domain plays a role in targeting of atToc132 to chloroplast outer membranes and in the formation of distinct atToc132-containing Toc complexes.

In order to confirm the earlier findings on the effect of the A-domain on targeting atToc132 to chloroplasts, the targeting efficiency of full-length atToc132 and 132GM to wild-type was compared as was described previously (Richardson, 2007; chapter 3). As was observed by Richardson (2007), the binding efficiency of atToc132 increases when the A-domain is deleted (Figure 6A, atToc132 and 132GM; Figure 9A), suggesting that the A-domain may play a role in regulating the targeting of atToc132. It is important to note, however, that the difference observed in the current study (~4% on average with the error bar of ±6) was not as dramatic as that observed by Richardson (2007) (~5% in average with the error bar of ±1). The binding and insertion efficiency of atToc132 and 132GM in this study was overall more efficient than in the previous one. Specifically, in the earlier study (Richardson, 2007), binding efficiencies of ~2-8% were achieved (Figure 6), as compared to

efficiencies on the order of ~10-15% in this study (Figure 9). This phenomenon might be due to improvement in the technique used for the *in vitro* chloroplast targeting assays. Nevertheless, both studies show that the deletion of the A-domain stimulates the targeting of atToc132 to isolated wild-type chloroplasts.

Since the amount of atToc33 and/or atToc34 expressed in wild-type, *ppi*1 and *ppi*3 plants are different, and the concentration of chloroplasts used in each set of assays are not exactly the same, the targeting efficiency to chloroplasts isolated from these three plants are not directly comparable. The results in the current study show that the binding efficiency of atToc132 to chloroplasts isolated from *ppi*1 mutant plants does not change as compared to the A-domain deletion fragment (132GM), while the efficiency of targeting to *ppi*3 chloroplasts increases slightly when the A-domain is deleted (Figure 9A). However, as with targeting to wild-type chloroplasts, the increase in the binding efficiency is not dramatic (~3% with the error bar of ±3). This result indicates that the A-domain may specifically inhibit the binding of atToc132 to atToc33. More data to confirm this interpretation were obtained using solid-phase binding and chloroplast targeting competition assays (chapter 5).

Another group of chloroplast targeting competition assays, involving targeting 132GM to wild-type chloroplasts competed by increasing concentration of $132A_{His}$, were designed to test the effect of the A-domain on targeting atToc132 to chloroplasts. A clear decrease in both binding and insertion was observed as higher concentrations of $132A_{His}$ were added in the 132GM targeting assay (Figure 11), which confirms that the A-domain has an inhibitory effect on targeting of atToc132 to chloroplasts. An intriguing point here is that the inhibitory effect

is increased as more $132A_{His}$ was applied, leading to a much more dramatic effect compared with its effect on full-length atToc132 targeting (compare Figures 9 and 11). The data can be interpreted to mean that the A-domain restricts the targeting of atToc132 to chloroplast outer membranes by occupying specific docking sites at the chloroplast membrane thereby preventing atToc132 from gaining access to its binding site (presumed to be the G-domain of atToc33 and atToc34). If this is the case, the A-domain might regulate the targeting of atToc132 through steric hindrance.

In summary, a set of chloroplast targeting competition assays, in which the targeting of 132GM was tested in the presence of different concentrations of $132A_{His}$, together with import assays comparing the targeting efficiency of full-length atToc132 and 132GM, indicate that the A-domain plays a role in restricting the targeting of atToc132 to chloroplasts isolated from wild-type plants, and might in particular be inhibiting the interaction between atToc132 and atToc33. The experiments described in chapter 4 were designed to investigate whether members of the Toc159 family, atToc132 and atToc159, associates with specific members of the Toc34 family.

6.2 Specific interaction between members of the Toc159 and Toc34 families

Although the two homologues of Toc34 in Arabidopsis, atToc33 and atToc34, have been shown to be functionally redundant *in vivo* (Jarvis et al., 1998; Wang et al., 2008; Balsera et al., 2009), they are expressed differently in the plant. atToc33 mRNA is detected at a higher level in young tissues, while the expression of atToc34 is similar in young and old plants

(Jarvis et al., 1998; Gutensohn et al., 2000; Constan et al., 2004). These findings raise the possibility that the atToc33 and atToc34 proteins are more prominent in green tissues and non-green tissues (e.g. root), respectively (Gutensohn et al., 2000; Jarvis, 2008). Each member of the Toc159 GTPase family is proposed to function primarily in recognizing distinct preproteins: atToc159 preferentially recognizes photosynthetic proteins, while atToc132/120 recognizes non-photosynthetic proteins (Bauer et al., 2000; Ivanova et al., 2004; Smith et al., 2004; Lee et al., 2009). Connecting these two hypotheses/findings gives rise to the possibility that there are multiple protein import pathways at the chloroplast outer envelope membrane. The study by Ivanova et al. (2004) provided evidence for this using immunoprecipitation experiments and solid-phase binding assays. It was demonstrated that atToc159 has a higher affinity for atToc33 than for atToc34, which confirms that atToc159 and atToc33 might associate with each other to act as photosynthetic protein receptors (Jarvis et al., 1998; Kubis et al., 2003). Interestingly, however, atToc132 associates with atToc33 and atToc34 with approximately equal affinity in the solid-phase binding assays (Ivanova et al., 2004). Experiments in chapter 4 were designed to investigate whether atToc132 and atToc159 exclusively or primarily interact with distinct members of the Toc34 family.

Targeting of full-length atToc132 or atToc159 to chloroplasts isolated from wild-type plants was measured in the presence of increasing amounts of competitor, 132G or 159G. The results show that both 132G and 159G compete for the targeting of atToc132 and atToc159 to isolated chloroplasts (Figures 14 & 16). This suggests that the two receptors do not exclusively associate with specific homologues of Toc34. Interestingly, 132G and 159G are

more effective competitors of the targeting of atToc159 than they are for atToc132. There are two possible explanations for this observation: 1) atToc33 is more abundant in wild-type chloroplasts than atToc34, and 2) the deletion of the A-domain (i.e. use of G-domains alone as competitor) leads to an alteration in atToc132's affinity for homologues of Toc34. Since atToc159 preferentially associates with atToc33, and atToc132 shows similar affinity for 33G and 34G in solid-phase binding assays (Ivanova et al., 2004), most of the competitor added to the competition assays might bind with atToc33, which is notably more abundant in wild-type chloroplasts, thus resulting a more effective competition on targeting of atToc159. The latter reason is based on my hypothesis that the A-domain regulates the targeting of atToc132 by inhibiting its binding to atToc33. It is possible that the deletion of the A-domain stimulates the binding of atToc132 to atToc33, so that the A,M-domain deletion version, 132G, interacts with atToc33 with a higher efficiency, while does not compete the association between full-length atToc132 and atToc34 in isolated wild-type chloroplasts.

In order to clarify the somewhat ambiguous results from the competition assays, another set of chloroplast targeting competition assays were performed, in which full-length atToc132 was targeted to chloroplasts isolated from *ppi*1 Arabidopsis. The use of these chloroplasts should eliminate the effect of atToc33. A similar competition was observed in these assays (Figure 18), which confirms that 132G does not compete targeting of atToc132 to wild-type chloroplasts as effectively as with its targeting to *ppi*1 might be due to the more abundant atToc33 and a change in the affinity of atToc132 when the A-domain is deleted as mentioned above.

Since the G-domains alone of atToc132 and atToc159 were used as competitors in the chloroplast targeting assays, another important consideration here is Toc-GTPase dimerization. Heterodimerization between the G-domains of atToc33 and atToc159 has been shown to be an important aspect of the targeting of atToc159 to the chloroplast outer membrane (Smith et al., 2002b; Wallas et al., 2003; Weibel at al., 2003; Ivanova et al., 2004; Lee et al., 2009). In addition, atToc33 has been shown to homodimerize at the chloroplast outer membrane, although the precise function of this dimer formation is not completely defined (Weibel et al., 2003; Koenig et al., 2008; Lee et al., 2009). The tendency of GTPase domains to form dimers means that the G-domain of atToc132 or atToc159 might dimerize in solution when used as competitors and might therefore be unable to compete for the targeting of full-length atToc132 or atToc159 in the *in vitro* assays. Simultaneously, the G-domain competitor and the G-domain of the radiolabeled protein being targeted to chloroplasts might interact with each other to form homodimers or heterodimers, which would also affect (i.e. presumably inhibit or compete) the targeting efficiency detected.

To further investigate the preference of interaction between members of the Toc159 and the Toc34 families, another set of chloroplast targeting competition assays could be used, in which the targeting efficiency of full-length atToc132 or atToc159 to wild-type chloroplasts could be tested in the presence of excess 33G or 34G. This would be a more direct way to illustrate the interaction preference between Toc159 and Toc34. While these assays have not been performed as part of the current study, they should ideally be completed before the work is ready for publication.

In summary, the decline in targeting efficiency of atToc132 and atToc159 to chloroplasts isolated from wild-type plants caused by increasing concentrations of either 132G or 159G indicates that these two chloroplast protein receptors do not exclusively associate with specific members of the Toc34 family. This result is consistent with solid-phase binding assays reported previously by Ivanova et al. (2004). Furthermore, although the G-domains of atToc132 and atToc159 do not compete for targeting of full-length atToc132 as effectively as they do for the targeting of atToc159, competition assays done with chloroplasts isolated from *ppi*1 plants shed more light on this observation. This assay shows that this phenomenon is likely explained by the larger amount of atToc33 than atToc34 in wild-type chloroplasts. The speculated stimulatory effect of the deletion of the A-domain of atToc132 on its binding with atToc33 provides a possible additional explanation. To clarify the effect of the A-domain on forming distinct atToc132-containing Toc complexes, two more sets of experiments were designed and are described in Chapter 5.

6.3 The role of the A-domain in the assembly of distinct Toc complexes

Although the ectopic expression of atToc34 is able to rescue the pale phenotype of *ppi*1 (atToc33 null) mutant, another previous study also indicates that these two homologues of Toc34 might possess a certain degree of specificity in transporting chloroplast proteins (Jarvis et al., 1998; Kubis et al., 2003; Constan et al., 2004). In addition, the solid-phase binding assays performed by Ivanova et al. (2004) showed that atToc159 might have a higher affinity for 33G than for 34G, while atToc132 or atToc120 bind to 33G and 34G with approximately

similar efficiency. This suggests that atToc159 preferentially interacts with atToc33 to form structurally distinct Toc complexes (i.e. distinct from those containing atToc132/120 and atToc34). Since there is also evidence showing that atToc159 (Smith et al., 2004) and atToc33 (Kubis et al., 2003) serve as receptors for photosynthetic preproteins, it implies that these structurally distinct complexes are also functionally distinct; the atToc159-atToc33 containing complexes would be involved in recognizing and transporting photosynthetic proteins. In contrast, atToc132 is proposed to play a role in recognizing non-photosynthetic proteins in complexes with atToc34. Although the mechanism by which these distinct Toc complexes form remains undefined, Inoue et al.'s study (2010) indicates that the A-domain determines the specifity of atToc159 towards photosynthetic preproteins and that of atToc120/132 towards non-photosynthetic "housekeeping" preproteins.

The GTPase domains of Toc34 and Toc159 are homologous with one another, and have been shown to play essential roles in Toc complex assembly. Their similarity makes it difficult to imagine how they might specifically interact with only certain other G-domains. The A-domains of the Toc159 members, however, have been shown to be intrinsically disordered, which is a characteristic of proteins that are able to specifically interact with multiple binding partners (Dyson & Wright, 2005; Richardson et al., 2009). It seems possible, therefore, that the A-domain might have a function in mediating the binding of Toc159 to a specific member of the Toc34 family. The results in Chapter 3 confirm the inhibitory effect of the A-domain on targeting of atToc132 to chloroplasts isolated from wild-type plants. Experiments repeated in chapter 5 were designed to investigate whether the A-domain restricts the binding of atToc132

to a specific member of the Toc34 family.

Solid-phase binding assays were used to compare the binding efficiency of full-length atToc132 and 132GM to immobilized 33G or 34G. The results indicate that full-length atToc132 has a higher binding efficiency for 34G than for 33G (Figure 22). The data are the average of results from experiments repeated at least twice. Although the binding efficiency to 33G is not exactly consistent with that presented by Ivanova et al. (2004), the data in the current study confirms the hypothesis that atToc132 primarily associates with atToc34. The binding of the A-domain deletion mutant of atToc132 (132GM) to 34G is similar to that of full-length atToc132 (Figure 22B). In contrast, the binding efficiency of 132GM increases by ~10% as compared to atToc132 (Figure 22A), which indicates that the presence of the A-domain inhibits the association between atToc132 and atToc33. The use of solid-phase binding assays provides a more chemically defined approach for investigating the association between members of the Toc34 and Toc159 families. However, there are also drawbacks of these assays, which are the absence of other proteins (e.g. Toc75, Hsp70) involved in chloroplasts and the lack of a bilayers system. For making up these disadvantages, a set of *in vitro* chloroplasts targeting competition assays were also designed.

In the chloroplast targeting competition assays, the targeting efficiency of 132GM to *ppi*1 and *ppi*3 was tested in the presence of different concentrations of 132A. These experiments showed that the targeting of 132GM to *ppi*3 (atToc34 knockout mutant) chloroplasts was competed more effectively than the targeting of 132GM to *ppi*1 (atToc33 knockout mutant) by the addition of 132A (Figure 24). This result lends support to the idea

that the A-domain restricts the targeting of atToc132 to atToc33 more dramatically than to atToc34, which is consistent with the hypothesis that atToc132 primarily associates with atToc34.

A recent study indicates that 132GM can partially complement the *ppi*2 (atToc159 knockout mutant) mutation in Arabidopsis (Inoue et al., 2010). This suggests that the A-domain of atToc132 might not only function in regulating the specific association between atToc132 and the Toc34 homologues. Since atToc132 has been shown to be unable to rescue the *ppi*2 phenotype (Inoue et al., 2010), another possibility is that the presence of the A-domain inhibits the interaction between atToc132 and atToc33, so that blocks the way by which atToc132-containing Toc complexes is able to tranlocate photosythetic preproteins.

In summary, the results from the two groups of experiments described in chapter 5 are generally consistent with each other, and indicate that the A-domain affects the targeting of atToc132 to Toc complexes by inhibiting its binding to atToc33 thereby promoting its binding to atToc34.

6.4 Future recommendation

It should be noted that there is an obvious limitation to the *in vitro* chloroplast targeting competition assays. In particular, a negative control competitor protein was not included in any of the assays. Although the competitors lead to distinct decreases in targeting each time the assay was run, I cannot exclude the possibility that the competition was due to a non-specific effect of including high concentrations of protein in the targeting assays. To

exclude this possibility, control assays need to be run in which another unrelated protein, not expected to compete for the targeting of atToc132 or atToc159 to chloroplasts, is used as a negative control competitor. To date, I was not able to express and purify a soluble protein which is unrelated to proteins of the Toc-Tic complexes. The control experiment should be done using a suitable protein before the work is published to confirm that the observation in targeting efficiency is not simply a non-specific effect of adding any recombinant protein to the targeting assays. It will be important to run such controls before the work is ready for publication.

There is a disadvantage in the chloroplast targeting assays that the use chloroplasts isolated from wild-type, *ppi*1 and *ppi*3 plants, in that each plant (and therefore the chloroplasts isolated from them) contains different levels of atToc33 and atToc34. Indeed, the amount of atToc33 or atToc34 expressed in *ppi*3 or *ppi*1 plants, respectively, may be very different than in wild-type (Gutensohn et al., 2000). Furthermore, there are other chloroplast proteins, which may affect the interaction between homologues of the Toc159 and Toc34 families. In addition, the targeting efficiency of radiolabeled atToc132 will almost certainly be influenced by the presence of endogenous Toc159 family proteins, such as atToc159, 159GM, atToc132 and 132GM. This limitation can be overcome by using *in vitro* solid-phase binding assays (as described in the current study) or with *in vitro* proteoliposome-based targeting assays. In solid-phase binding assays, specific proteins produced recombinantly with a carboxy- or amino-terminal His tag are immobilized on Ni-charged resin, and used to monitor interactions with other “prey” proteins, that can be radiolabeled for ease of detection. In

proteoliposome-based targeting assays, specific Toc components are reconstituted into the liposome bilayers to mimic Toc complexes (Wallas et al., 2003). In this way, the Toc components (i.e. Toc33/34 and Toc75) and their amounts can be controlled more precisely than is possible with isolated chloroplasts. These two approaches make it possible to set up a protein binding system with precise amounts of specific "bait" proteins. In this study, the solid-phase binding assays were used in Chapter 5 to compare the binding efficiency of full-length atToc132 and 132GM to $33G_{His}$ or $34G_{His}$. The proteoliposome system has not been used as part of this study, this would, however, be a possible approach to pursue in the future as a way to corroborate the findings presented here.

7. Conclusions

Chapter 3 in this study illustrates that the A-domain inhibits the targeting of atToc132 to chloroplasts isolated from wild-type Arabidopsis. Since atToc33 is expressed at a higher level than atToc34 in wild-type Arabidopsis leaves (Jarvis et al., 1998), it is proposed that the inhibitory effect of the A-domain might be on the interaction between atToc132 and atToc33. However, no significant difference in the targeting efficiency of full-length atToc132 and 132GM was observed when chloroplasts isolated from *ppi*1 and *ppi*3 Arabidopsis were used.

To clarify the results of the targeting assays, interaction between members of the Toc159 and Toc34 families were tested using *in vitro* solid-phase binding assays. As shown in chapter 4 atToc132 and atToc159 do not exclusively associate with specific members of the Toc34 family (Figure 14 & 16). Of note is that competition with 132G is not as effective as competition with 159G on the targeting of full-length atToc132 and atToc159 to chloroplasts isolated from wild-type chloroplasts. A possible explanation for this observation is that the deletion of the A-domain stimulates the association of atToc132 with atToc33, which would be noticeable because of the abundance of atToc33 as compared to atToc34 in wild-type chloroplasts. The targeting of full-length atToc132 to chloroplasts isolated from *ppi*1 Arabidopsis competed with 132G supports this explanation (Figure 18).

To further clarify the hypothesis, *in vitro* solid-phase binding assays were used in addition to the chloroplast targeting competition assays reported in Chapter 5. The results shown in Figures 22 and 24 suggest that although there is still a slight effect on the interaction between atToc132 and atToc34, the A-domain influences the binding of atToc132 to atToc33

more so than to atToc34.

In conclusion, this study illustrates the inhibitory effect of the A-domain of atToc132, a member of the Toc159 chloroplast protein import receptor family in *Arabidopsis thaliana*, in its targeting to the chloroplast outer membrane and in distinct Toc complexes assembly. More specifically, 1) results in chapter 3 confirm the inhibitory effect of the A-domain on the targeting of atToc132; 2) experiments describted in chapter 4 ilusstrate that atToc132 and atToc159 do not exclusive associate with specific member of the Toc34 famlily; and 3) chapter 5 states that the A-domain restricts the targeting of atToc132 by inhibiting its binding to atToc33. To conclude, the A-domain regulates the specificity of atToc132 towards the members of the Toc34 family by inhibiting its interaction with atToc33 at the outer envelope membrane.

This study, focusing on investigating protein import into chloroplasts, makes use of knowledge of plant biology, cellular biology, molecular biology, and biochemistry. As one of the most important groups of organisms on earth, plants, especially the photosynthesis taking place in them, play an essential role in keeping the balance of the atmosphere. In order to further understand this crucial reaction, molecular and cellular biological tools are used for investigate the function of components in the organelle in which it takes place, the chloroplast. In addition, biochemical methods are also used frequently.

Future research should be aimed at investigating other possible function(s) of the A-domain of Toc159 homologues and identifying putative functional partners of the A-domain (i.e. other components of the Toc complex, transit peptides). These may further

clarify the data in this study and will be important for contributing to our understanding of the protein import into chloroplasts.

8. References

Agne B, Kessler F. 2009. Protein transport in organelles: The Toc complex way of preprotein import. Febs Journal 276(5):1156-1165.

Agne B, Andres C, Montandon C, Christ B, Ertan A, Jung F, Infanger S, Kessler F. 2010. The acidic A-domain of Arabidopsis Toc159 occurs as a hyperphosphorylated protein. Plant Physiology. 153(3):1016-1030.

Andres C, Agne B, Kessler F. 2010. The TOC complex: Preprotein gateway to the chloroplast. Biochemica Et Biophydoca Acta-molecular Cell Research. 1803(6):715-723.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts- Polyphenoloxidase in beta-vulgaris. Plant Physiology 24(1):1-15.

Baldwin A, Wardle A, Patel R, Dudley P, Park SK, Twell D, Inoue K, Jarvis P. 2005. A molecular-genetic study of the Arabidopsis Toc75 gene family. Plant Physiology 138(2):715-733.

Balsera M, Soll J, Bolter B. 2009. Protein import machineries in endosymbiotic organelles. Cellular and Molecular Life Sciences 66(11-12):1903-1923.

Bauer J, Chen KH, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F. 2000. The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403(6766):203-207.

Bauer J, Hiltbrunner A, Kessler F. 2001. Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting. Cellular and Molecular Life Sciences 58(3):420-433.

Bauer J, Hiltbrunner A, Weibel P, Vidi PA, Alvarez-Huerta M, Smith MD, Schnell DJ, Kessler F. 2002. Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. Journal of Cell Biology 159(5):845-854.

Becker T, Hritz J, Vogel M, Caliebe A, Bukau B, Soll J, Schleiff E. 2004. Toc12, a novel subunit of the intermembrane space preprotein translocon of chloroplasts. Mol. Biol. Cell. 15:5130-5144.

Bolter B, May T, Soll J. 1998. A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. Febs Letters 441(1):59-62.

Brock I, Hazell L, Michl D, Nielsen VS, Moller BL, Herrmann RG, Klosgen RB, Robinson C. 1993. Plant Molecular Biology. 23(4):717-725.

Caliebe A, Grimm R, Kaiser G, Lubeck J, Soll J, Heins L. 1997. The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. Embo Journal 16(24):7342-7350.

Chen KH, Chen XJ, Schnell DJ. 2000. Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiology 122(3):813-822.

Chen XJ, Smith MD, Fitzpatrick L, Schnell DJ. 2002. In vivo analysis of the role of atTic20 in protein import into chloroplasts. Plant Cell 14(3):641-654.

Chou ML, Fitzpatrick LM, Tu SL, Budziszewski G, Potter-Lewis S, Akita M, Levin JZ, Keegstra K, Li HM. 2003. Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. Embo Journal 22(12):2970-2980.

Constan D, Patel R, Keegstra K, Jarvis P. 2004. An outer envelope membrane component of the plastid protein import apparatus plays an essential role in Arabidopsis. Plant Journal. 38(1):93-106.

Dalbey RE, von Heijne G. 2002. Protein targeting transport & translocation.

Dhanoa PK, Richardson LGL, Smith MD, Gidda SK, Henderson MPA, Andrews DW, Mullen RT. 2010. Distinct Pathways Mediate the Sorting of Tail-Anchored Proteins to the Plastid Outer Envelope. Plos One 5(4).

Dyson HJ, Wright PE. 2005. Intrinsically unstructured proteins and their functions. Nature Reviews Molecular Cell Biology 6(3):197-208.

Fink AL. 2005. Natively unfolded proteins. Current Opinion in Structural Biology 15(1):35-41.

Gray MW. 1992. The endosymbiont hypothesis revisited. International Review of Cytology-a Survey of Cell Biology 141:233-357.

Gutensohn M, Schulz B, Nicolay P, Flugge UI. 2000. Functional analysis of the two Arabidopsis homologues of Toc34, a component of the chloroplast protein import apparatus. Plant Journal 23(6):771-783.

Heins L, Mehrle A, Hemmler R, Wagner R, Kuchler M, Hormann F, Sveshnikov D, Soll J. 2002. The preprotein conducting channel at the inner envelope membrane of plastids. Embo Journal 21(11):2616-2625.

Hiltbrunner A, Bauer J, Alvarez-Huerta M, Kessler F. 2001a. Protein translocon at the Arabidopsis outer chloroplast membrane. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 79(5):629-635.

Hiltbrunner A, Bauer J, Vidi PA, Infanger S, Weibel P, Hohwy M, Kessler F. 2001b. Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. Journal of Cell Biology 154(2):309-316.

Hirsch S, Muckel E, Heemeyer F, Vonheijne G, Soll J. 1994. A receptor component of the chloroplast ptoein translocation machinery. Science 266(5193):1989-1992.

Hormann F, Kuchler M, Sveshnikov D, Oppermann U, Li Y, Soll J. 2004. Tic32, an essential component in chloroplast biogenesis. Journal of Biological Chemistry 279(33):34756-34762.

Inoue K, Baldwin AJ, Shipman RL, Matsui K, Theg SM, Ohme-Takagi M. 2005. Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. Journal of Cell Biology 171(3):425-430.

Inoue H, Rounds C, Schnell DJ. 2010. The molecular basis for distinct pathways for protein import into Arabidopsis chloroplasts. Plant Cell. 22(6):1946-1960.

Ivanova Y, Smith MD, Chen KH, Schnell DJ. 2004. Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. Molecular Biology of the Cell 15(7):3379-3392.

Jarvis P, Chen LJ, Li HM, Pete CA, Fankhauser C, Chory J. 1998. An Arabidopsis mutant defective in the plastid general protein import apparatus. Science. 282(5386):100-103.

Jarvis P. 2008. Targeting of nucleus-encoded proteins to chloroplasts in plants. New Phytologist 179(2):257-285.

Jelic M, Sveshnikova N, Motzkus M, Horth P, Soll J, Schleiff E. 2002. The chloroplast import receptor Toc34 functions as preprotein-regulated GTPase. Biological Chemistry 383(12):1875-1883.

Kessler F, Blobel G. 1996. Interaction of the protein import and folding machineries in the chloroplast. Proceedings of the National Academy of Sciences of the United States of America 93(15):7684-7689.

Kessler F, Blobel G, Patel HA, Schnell DJ. 1994. Identification of 2 GTP-binding proteins in the chloroplast protein import machinery. Science 266(5187):1035-1039.

Kessler F, Schnell D. 2009. Chloroplast biogenesis: diversity and regulation of the protein import apparatus. Current Opinion in Cell Biology 21(4):494-500.

Kikuchi S, Oishi M, Hirabayashi Y, Lee DW, Hwang I, Nakai M. 2009. A 1-megadalton translocation complex containing Tic20 and Tic21 mediates chloroplast protein import at the inner envelope membrane. Plant Cell. 21(6):1781-1797.

Koenig P, Oreb M, Rippe K, Muhle-Goll C, Sinning I, Schleiff E, Tews I. 2008. On the significance of Toc-GTPase homodimers. Journal of Biological Chemistry 283(34):23104-23112.

Kouranov A, Schnell DJ. 1997. Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. Journal of Cell Biology 139(7):1677-1685.

Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D, Jarvis P. 2003. The Arabidopsis ppi1 mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. Plant Cell. 15(8):1859-1871.

Kuchler M, Decker S, Hormann F, Soll J, Heins L. 2002. Protein import into chloroplasts involves redox-regulated proteins. Embo Journal 21(22):6136-6145.

Lee J, Wang F, Schnell DJ. 2009. Toc receptor dimerization participates in the initiation of membrane translocation during protein import into chloroplasts. The Journal of Biological Chemistry. 284(45):31130-31141.

Lemeille S, Rochaix JD. 2010. State transitions at the crossroad of thylakoid signalling pathways. Photosynthesis Research 106(1-2):33-46.

Lopez-Juez E, Pyke KA. 2005. Plastids unleashed: their development and their integration in plant development. International Journal of Developmental Biology 49(5-6):557-577.

Ma YK, Kouranov A, LaSala SE, Schnell DJ. 1996. Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. Journal of Cell Biology 134(2):315-327.

May T, Soll J. 2000. 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12(1):53-63.

Pain D, Blobel G. 1987. Protein import into chloroplasts requires a chloroplast ATPase. Proceedings of the National Academy of Sciences of the United States of America 84(10):3288-3292.

Perry SE, Keegstra K. 1994. Envelope membrane-proteins that interact with chloroplastic precursor proteins. Plant Cell 6(1):93-105.

Pyke KA. 1999. Plastid division and development. Plant Cell 11(4):549-556.

Receveur-Brechot V, Bourhis JM, Uversky VN, Canard B, Longhi S. 2006. Assessing protein disorder and induced folding. Proteins-Structure Function and Bioinformatics 62(1):24-45.

Reumann S, Keegstra K. 1999. The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. Trends in Plant Science 4(8):302-307.

Rial DV, Ottado J, Ceccarelli EA. 2003. Precursors with altered affinity for Hsp70 in their transit peptides are efficiently imported into chloroplasts. Journal of Biological Chemistry 278(47):46473-46481.

Richardson LGL. 2007. Structure-function analysis of the acidic domain of the Arabidopsis Toc159 receptors. A thesis for the degree of Master of Science.

Richardson LGL, Jelokhani-Niaraki M, Smith MD. 2009. The acidic domains of the Toc159 chloroplast preprotein receptor family are intrinsically disordered protein domains. Bmc Biochemistry 10.

Schatz G, Dobberstein B. 1996. Common principles of protein translocation across membranes. Science 271(5255):1519-1526.

Schleiff E, Becker T. 2011. Common ground for protein translocation: access control for mitochondria and chloroplasts. Nature Reviews Molecular Cell Biology 12(1):48-59.

Schleiff E, Soll J, Kuchler M, Kuhlbrandt W, Harrer R. 2003. Characterization of the translocon of the outer envelope of chloroplasts. Journal of Cell Biology 160(4):541-551.

Schnell DJ, Blobel G, Keegstra K, Kessler F, Ko K, Sill J. 1997. A consensus nomenclature for the protein-import components of the chloroplast envelope. Trends in cell biology. 7(8):303-304.

Schnell DJ, Hebert DN. 2003. Protein translocons: Multifunctional mediators of protein translocation across membranes. Cell 112(4):491-505.

Schnell DJ, Kessler F, Blobel G. 1994. Isolation of components of the chloroplast protein import machinery. Science 266(5187):1007-1012.

Schulz A, Knoetzel J, Scheller HV, Mant A. 2004. Uptake of a fluorescent dye as a swift and simple indicator of organelle intactmess: import-competent chloroplasts from soil-grown Arabidopsis. Journal of Histochemistry & Cytochemistry. 52(5):701-704.

Smith MD. 2006. Protein import into chloroplasts: an ever-evolving story. Canadian Journal of Botany-Revue Canadienne De Botanique 84(4):531-542.

Smith MD, Fiuzpatrick L, Keegstra K, Schnell DJ. 2002a. *In vitro* analysis of chloroplast protein import. In: Current Protocols in Cell Biology. Eds. Bonifacina JS, Dasso M, Harford JB, Lippincott-Schwartz J, Yanada KM. New York: John Wiley and Sons Inc. 11.16.1-11.16.21.

Smith MD, Hiltbrunner A, Kessler F, Schnell DJ. 2002b. The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP. Journal of Cell Biology 159(5):833-843.

Smith MD, Rounds CM, Wang F, Chen KH, Afitlhile M, Schnell DJ. 2004. AtToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. Journal of Cell Biology 165(3):323-334.

Smith MD, Schnell DJ. Chloroplast protein targeting: multiple pathways for a complex organelle. In: Protein movement across membranes. Ed. Eichler J. 2004. Georgetown, TX: Landes Biosciences. 1-18.

Sohrt K, Soll J. 2000. Toc64, a new component of the protein translocon of chloroplast. Journal of Cell Biology. 148:1213-1221.

Soll J, Schleiff E. 2004. Protein import into chloroplasts. Nature Reviews Molecular Cell Biology 5(3):198-208.

Su HN, Xie BB, Zhang XY, Zhou BC, Zhang YZ. 2010. The supramolecular architecture, function, and regulation of thylakoid membranes in red algae: an overview. Photosynthesis Research 106(1-2):73-87.

Sun CW, Chen LJ, Lin LC, Li HM. 2001. Leaf-specific upregulation of chloroplast translocon genes by a CCT motif-containing protein, CIA2. Plant Cell 13(9):2053-2061.

Sveshnikova N, Soll J, Schleiff E. 2000. Toc34 is a preprotein receptor regulated by GTP and phosphorylation. Proceedings of the National Academy of Sciences of the United States of America 97(9):4973-4978.

Teng YS, Su YS, Chen LJ, Chen LJ, Lee YJ, Hwang I, Li HM. 2006. Tic21 is an essential translocon component for protein translocation across the chloroplast inner envelope membrane. Plant Cell. 18(9):2247-2257.

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K. 1989. Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. Journal of Biological Chemistry 264(12):6730-6736.

Tompa P. 2003. Intrinsically unstructured proteins evolve by repeat expansion. Bioessays 25(9):847-855.

Torres JH, Maldonado MAA, Chomilier J. 2007. Tandem duplications of a degenerated GTP-binding domain at the origin of GTPase receptors Toc159 and thylakoidal SRP. Biochemical and Biophysical Research Communications 364(2):325-331.

Tranel PJ, Froehlich J, Goyal A, Keegstra K. 1995. A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. Embo Journal 14(11):2436-2446.

Tsai LY, Tu SL, Li HM. 1999. Insertion of atToc34 into the chloroplastic outer membrane is assisted by at least two proteinaceous components in the import system. Journal of Biological Chemistry 274(26):18735-18740.

von Heijne, G, Steppuhn J, Herrmann TG. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. 180:535-545.

Vothknecht UC, Soll J. Protein import into chloroplasts: who, when, and how? In: The structure and function of plastid. Eds. Wise RR, Kenneth Hoober J. 2007. 53-74.

Wallas TR, Smith MD, Sanchez-Nieto S, Schnell DJ. 2003. The roles of Toc34 and Toc75 in targeting the Toc159 preprotein receptor to chloroplasts. Journal of Biological Chemistry 278(45):44289-44297.

Wang F, Agne B, Kessler F, Schnell DJ. 2008. The role of GTP binding and hydrolysis at the atToc159 preprotein receptor during protein import into chloroplasts. Journal of Cell Biology 183(1):87-99.

Waters MT, Langdale JA. 2009. The making of a chloroplast. Embo Journal 28(19):2861-2873.

Weibel P, Hiltbrunner A, Brand L, Kessler F. 2003. Dimerization of Toc-GTPases at the chloroplast protein import machinery. Journal of Biological Chemistry 278(39):37321-37329.

Wise RR. The diversity of plastid form and function. In: The structure and function of plastid. Eds. Wise RR, Kenneth Hoober J. 2007. 3-26.

Young ME, Keegstra K, Froehlich JE. 1999. GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. Plant Physiology 121(1):237-243.

Zhang XP, Glaser E. 2002. Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. Trends in Plant Science 7(1):14-21.

Appendix 1: Primers used for PCR amplification and sequencing of atToc132 constructs

	Primer Set	Purpose of Primers	Sequence
132GM	1	Incorporate 5' NdeI and 3' XhoI restriction enzyme sites for cloning	S 5'GATACATATGGGTCGTGCTTCTCC 3' AS 5'CTGTCTCGAGTTGTCCATATTGCGTTTG 3'
132GM	2	Approximately 20 ucleotides start from 500, 1000, and 1500 of 132GM sequence for sequencing	5'GATACATATGGGTCGTGCTTCTCC 3' 5'AGTGTGGAAGCCACATTTGTT 3' 5' GCCAGATTTATCTCTACCTGCG 3'
132G	3	Incorporate 5' NdeI and 3' SacI restriction enzyme sites for cloning	S 5'GATACATATGGGTCGTGCTTCTCC 3' AS 5'GCGTCTGCAGAGCTCTTGCAACTC 3'

Note: Restriction enzyme sites are underlined. S = sense primer; AS = antisense primer.