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Examining the Roles of PsToc75 POTRA Domains in Chloroplast Protein Import

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I am submitting herewith a thesis written by Richard Franklin Simmerman entitled "Examining the Roles of PsToc75 POTRA Domains in Chloroplast Protein Import." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Dr. Barry Bruce, Major Professor

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Examining the Roles of PsToc75 POTRA Domains in Chloroplast Protein Import

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Richard Franklin Simmerman
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Dedicated to my family

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Abstract

During chloroplast formation via endosymbiosis most of the plastid genome was transferred to the host nuclear genome. Genomic and proteomic analysis suggests that >95% of the original plastid proteome is now encoded in the nucleus, and these now cytosolically fabricated proteins require a post-translational transport pathway back into the organelle. This process is not well understood, yet it has been shown to involve translocons at the outer and inner envelope of the chloroplast membranes (TOC & TIC). These translocons interact with a cleavable N-terminal extension of between 20 and 100 residues on chloroplast-bound precursor proteins known as the transit-peptide. Precursor proteins pass through the outer membrane via the outer chloroplast membrane β-barrel, Toc75. In addition to containing a transmembrane beta-barrel, Toc75 also contains three polypeptide transport (POTRA) domain repeats at the N-terminus. Despite widespread occurrence the role of POTRAs is poorly understood. One possibility is that they function to promote either homo- or heterotypic protein:protein interactions.

To investigate these possibilities, we modeled the psToc75 POTRA domains and purified recombinant POTRA domains. POTRA1, POTRA3, and POTRA1-3 have been used to investigate interactions. Homotypic POTRA interactions have been supported by crosslinking experiments and analytical ultra centrifugation (AUC). Crosslinking data shows POTRA1 and POTRA3 undergo oligimerization. AUC suggests that POTRA1 may homodimerize. Heterotypic interactions have been studied via pull-down assays, crosslinking, and AUC and demonstrate that POTRA1 and POTRA3 interact with transit peptide. Soluble POTRA1-3 seems to stimulate precursor protein import into isolated chloroplasts in an import assay..

Table of Contents

Table of Figures

Appendix of Supplementary Figures

Chapter 1 Introduction and Literature Review

1.1 Origin and Evolution of the Chloroplast

It has been more than a century since Mereschkowsky first proposed the endosymbiotic theory (Mereschkowsky 1905), and it is now widely accepted that contemporary chloroplasts are descendants of an ancient autonomous cyanobacteria (Martin and Kowallik 1999). Stromatolites and fossils provide evidence that oxygenic photosynthesis started ~3.5 Billion years ago (Ga) (Byerly et al. 1986; Walter et al. 1980), and that eukaryotic algae-like organisms existed 1.2 Ga (Butterfield 2000). Both symbiont and host cell underwent an incredible process, resulting in the transformation of a once free living cyanobacteria into chloroplasts, "little workers, green slaves" dependent on the host cell for function and replication (Figure 1.1, Mereschkowsky 1905).

One such adaptation was an enormous transfer of genetic material from the symbiont to the eukaryotic host cell (Weeden 1981). Modern chloroplast have a genome (plastome) that codes for less than 100 proteins (Leister and Kleine 2008), while having a proteome estimated between 1,900 and 4,500 (Abdullah et al. 2000; Leister 2003). The vast majority of the plastid DNA was either lost or transferred to the nucleus (Gabaldon and Huynen 2003). After a proto-symbiont lysed its DNA would have been free in the cytosol (Leister 2003), and DNA uptake from the cytosol seems to be a feature typical of eukaryotic cells (Howe et al. 2008). It has been estimated that *Arabidopsis thaliana* requisitioned between 12 and 18% of its current ~25,000 genes from plastid precursors (Abdullah et al. 2000; Martin et al. 2002).

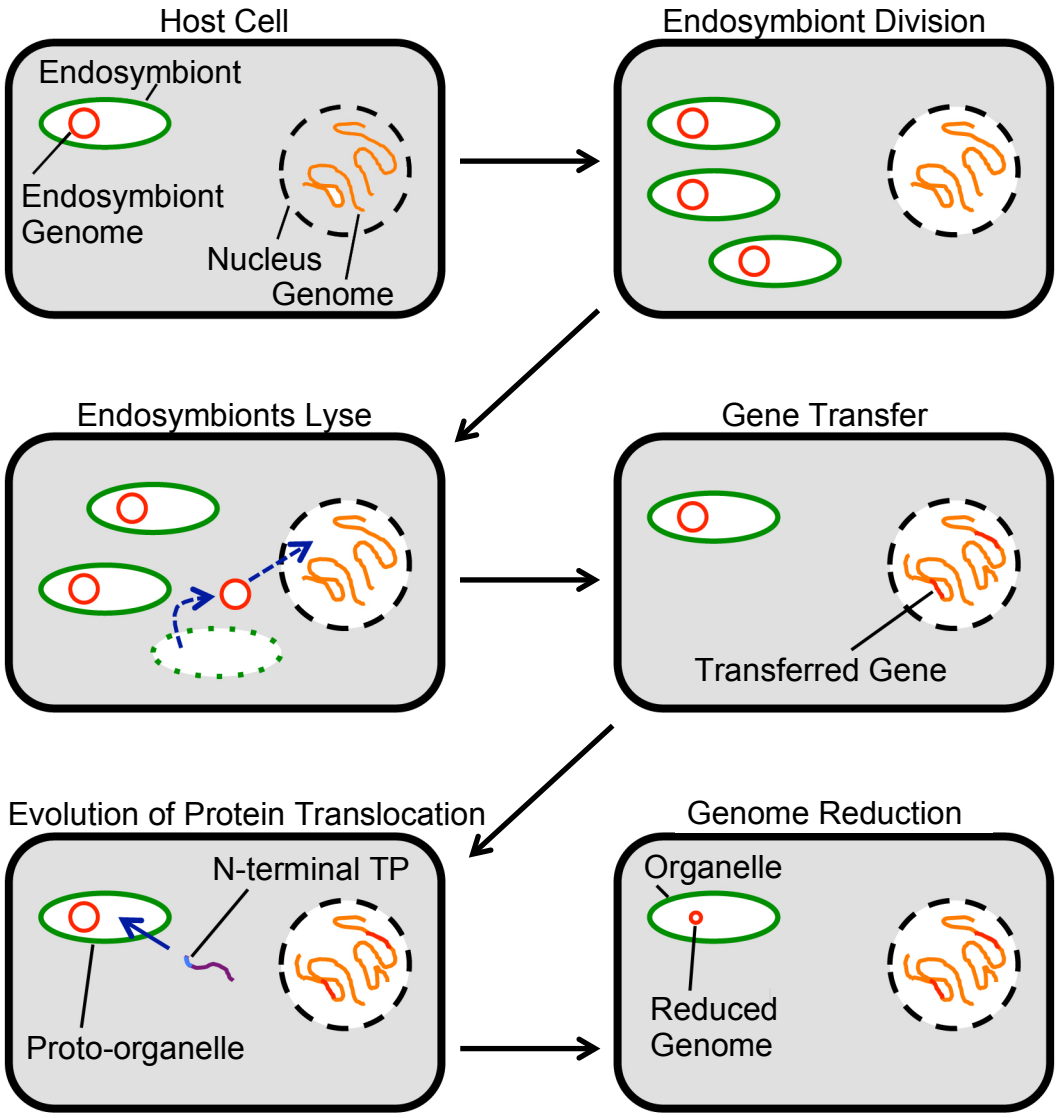


Figure 1.1 Endosymbiosis and Chloroplast Evolution

After endosymbiosis, chloroplast ancestors (green) could have replicated within the host (gray). Lysis of these endosymbionts would result in a transfer of DNA to the host cell nucleus. After a mechanism for protein translocation evolved the redundant DNA could be lost from the organelle with no negative effect on the organism. Adapted from Dyall et al. 2004.

This newly acquired DNA in the nucleus of the host cell could mutate and rearrange until it was functionally active with plastid promoters (Noutsos et al. 2005; Stegemann and Bock 2006). The proteins destined for the chloroplast would need to obtain chloroplast-targeting sequences and the chloroplast would have to develop machinery to translocate the cytosolically-translated chloroplast-bound proteins through the chloroplast envelope consisting of outer and inner membranes (Inoue 2007; Keegstra and Cline 1999; Keegstra et al. 1984; Ueda et al. 2006). All of these steps would have been prerequisites for the deletion of homologous genes by the pre-chloroplasts. Even without selection for DNA retention, DNA introduced into eukaryotes is hypothesized to be able to persist for up to a million years, giving the photosynthetic eukaryotes opportunity to evolve (Huang et al. 2005; Matsuo et al. 2005). Because not all of the still present chloroplast-derived DNA is targeted back to the chloroplast the endosymbiosis benefitted the host with new genetic material as well as an organelle that can create sugar from water, inorganic carbon, and sunlight (Martin et al. 2002). Not all chloroplast-derived DNA is targeted to the chloroplast, and not all chloroplast-targeted DNA is derived from one cyanobacterial ancestor. The sea slug *Elysia chlorotica* has acquired the psbO gene via horizontal gene transfer from algae that it eats, but is has not developed its own chloroplasts yet (Rumpho et al. 2008). There is evidence for eukaryote and even proteobacteria-derived proteins that are targeted to and used by the chloroplast (Martin and Schnarrenberger 1997).

1.2 General Import Pathway of Cytosolically Produced Chloroplast-Bound Proteins

The host cell incorporating and reshuffling genes from the chloroplast ancestor into locations suitable for expression did not solve the logistical problem of moving cytosolically produced proteins through two membranes and back into the proto-plastid. The majority of chloroplast-destined proteins are recognized by cleavable N-terminal targeting sequences termed the transit peptides (TPs) discovered over 30 years ago (Chua and Schmidt 1978; Highfield and Ellis 1978). There is very little homology in primary sequence or secondary structure in solution among the thousand of known and predicted TPs (Bruce 2000). Different precursor proteins are specifically targeted to six locations in the chloroplast: the outer membrane, intermembrane space, inner membrane, stroma, thylakoid membrane, or the lumen (Dyall et al. 2004). If the TP is the ticket into the chloroplast via the general import pathway (Figure 1.2) then the receptor protein complexes termed the Translocon at the Outer/Inner envelope of the Chloroplast (TOC/TIC) and soluble chaperones that ensure specificity and directionality of chloroplast protein import make up the turnstiles (Blobel 1980; Cline 2000). This network involved in protein trafficking across the membranes of the chloroplast includes proteins that originated from eukaryotes, eubacteria, and cyanobacteria (Dyall et al. 2004).

Nascent precursor proteins interact with cytosolic chaperones including Hsp70 and 14-3-3 that along with other proteins make up a guidance complex (May and Soll 2000; Waegemann et al. 1990). This guidance complex transports the unfolded precursor protein to the outer membrane of the chloroplast (Hinnah et al. 1997) and facilitates precursor docking with either lipids in the chloroplast outer membrane (Bruce 1998) or the TOC complex (Inoue 2007). The core TOC complex consists of two GTP-binding

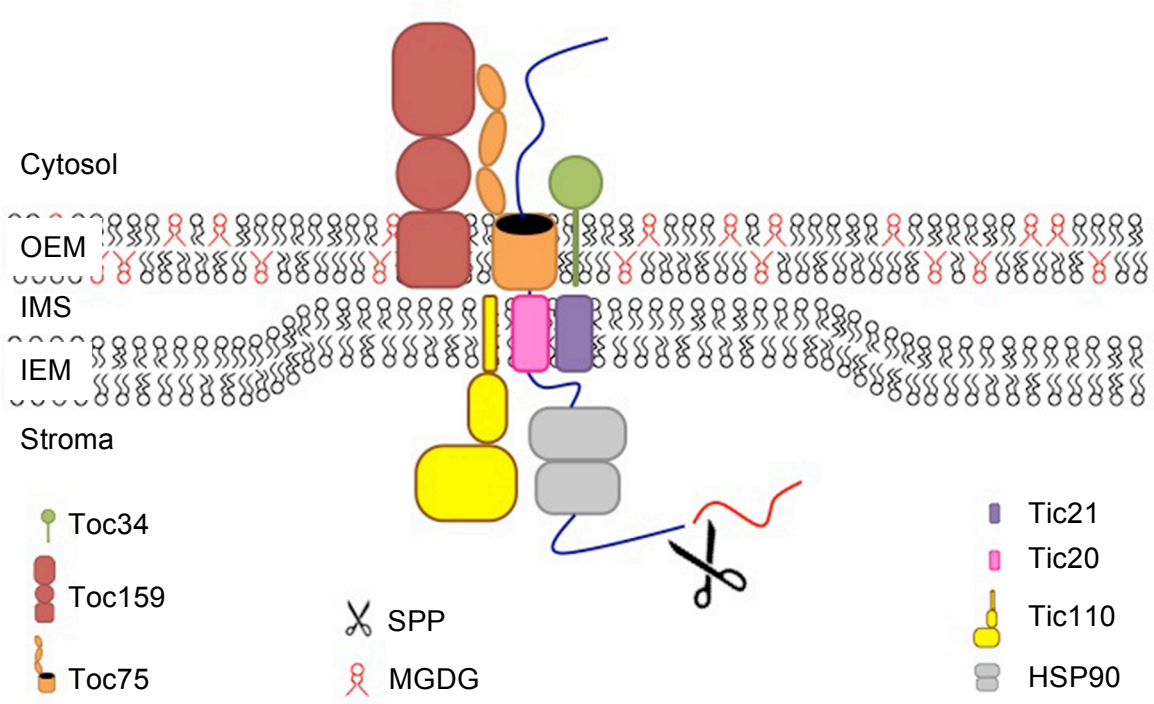


Figure 1.2 General Import Scheme

Schematic representation of precursor protein being translocated through the outer and inner membranes of the chloroplast via Toc75 (confirmed) and Tic20 (proposed). Transit peptide is cleaved during or directly after transport via the Stromal Processing Peptidase, leaving the mature protein. Area of the different subunits of import mechanism represents the number of residues that each subunit contains.

protein receptors the translocon at the outer envelope of the chloroplast of 159 kDa (Toc159) (Waegemann 1991) and translocon at the outer envelope of the chloroplast of 34 kDa (Toc34) (Kessler et al. 1994) along with a β-barrel pore translocon at the outerenvelope of the chloroplast of 75 kDa (Toc75) all first discovered in pea plants (Bauer et al. 2002; Waegemann and Soll 1991). Translocation across the outer membrane of the chloroplast requires low concentrations of GTP in the cytosol and ATP in the intermembrane space, and is mechanistically separable from inner envelope translocation *in vitro* (Olsen et al. 1989; Scott and Theg 1996; Theg et al. 1989).

In vivo studies have shown that although there can be a forced separation of the TOC and TIC import, in the cell these complexes work in a coordinated fashion (Alefsen et al. 1994; Kouranov et al. 1998; Schnell and Blobel 1993). The TIC complex is not as well understood as the TOC complex, but several members have been discovered. Tic110, the most abundant protein of the TIC complex (Block et al. 1983; Lubeck et al. 1996; Schnell et al. 1994), forms a cation-selective channel in proteoliposomes and isolated inner membranes (Balsera et al. 2009; Heins et al. 2002). Tic20 is another putative pore protein that uses four predicted α-helical transmembrane segments to span the inner membrane (Kouranov et al. 1998). A motor complex is formed by the stromal ATPase Hsp93 (Akita et al. 1997; Chou et al. 2006) and Tic40 (Chou et al. 2003; Nielsen et al. 1997; Stahl et al. 1999) that is thought to assist in translocation of the precursor protein. Once the precursor protein makes it way into the stroma the N-terminal transit peptide is cleaved by the Stromal Processing Peptidase (SPP) and further degraded inside the stroma (Richter and Lamppa 1998), and the stromal chaperone Cpn60 is thought to play a

role in refolding the mature protein in an ATP-dependent pathway (Kessler and Blobel 1996).

1.3 Transit Peptide

The large majority of nuclear-encoded precursor proteins that are destined to be translocated into the chloroplast possess a cleavable N-terminal targeting sequence of between 20-150 amino acids termed the transit peptide (TP) (Bruce 1998). Transit peptide acquisition by plastid-destined proteins via exon shuffling, alternative splicing or gene duplication (Arimura et al. 1999; Gantt et al. 1991) was a critical step in plastid evolution (McFadden 1999), and has been linked with the origin of the import channel Toc75 (Reumann et al. 1999). Thousands of proteins are targeted to the chloroplast, and it is hypothesized that over a thousand different transit peptides target different precursor proteins into the chloroplast (Bruce 2000). At the primary sequence level transit peptides are highly divergent with respect to amino acid composition, organization, and length (Bruce 2001). TP is largely unstructured in an aqueous environment (Bruce 1998; Wienk et al. 1999), although in membrane mimicking solutions such as 2,2,2-trifluorethanol (TFE) or micelles TP has an increased alpha-helical content (Endo et al. 1992; von Heijne and Nishikawa 1991).

The TP possesses all of the information necessary for import into the chloroplast. Without the TP, chloroplast-bound proteins are unable to be imported (Bruce 2000; von Heijne et al. 1989). The TP is also sufficient for translocation and correct localization to the chloroplast stroma of precursor proteins. Any protein not natively destined for chloroplast will still be imported with the addition of an N-terminal TP (Schreier et al.

1985; Van den Broeck et al. 1985). The average TP is 58 amino acids in length (Bhushan et al. 2006) and is rich in hydroxylated, hydrophobic, and positively charged amino acids, while being deficient in acidic ones (Zhang and Glaser 2002). It has been postulated that there are three distinct regions in TPs: an uncharged N-terminal domain of between 10-15 residues that is essential for correct targeting (Emanuelsson et al. 1999; Lee et al. 2006; Lee et al. 2002; Rensink et al. 2000), a central domain with no acidic residues but increased serine and threonine residues, and a c-terminal domain with a cleavage site (Bruce 2000; Claros et al. 1997). The TP is cleaved from the precursor protein by the Stromal Processing Peptidase (SPP) during or directly after translocation leaving the mature protein intact (Schmidt et al. 1981).

The transit peptidome can be grouped into multiple subgroups with distinct sequence motifs (Lee et al. 2008). Some sequence motifs display a high degree of functional redundancy (Lee et al. 2006) and are proposed to interact with lipids and/or translocation machinery (Lee et al. 2008). All membranes that transport proteins have high levels of lipids that prefer to adopt a non-bilayer structure (Bruce 2000), and in the case of the chloroplast monogalactosydiacylglycerol (MGDG), a galactolipid found in the outer envelope of the chloroplast, is a wedge-like molecule that would form an inverted micelle if it was the only lipid present (Endo and Schatz 1988; Rietveld et al. 1995). In fact, it has been shown that precursor proteins interact better with artificial membranes if they contain MGDG (Pinnaduwage and Bruce 1996; van 't Hof et al. 1991) and is postulated that the ability of TPs to form alpha-helices may promote interaction or even insertion into the membrane lipids (Bruce 1998). Translocation machinery (import

receptors, heat shock proteins, and guidance proteins) could also form interactions with the multiple domains of transit peptides (Lee et al. 2008; Lee et al. 2006).

1.4 The Translocon at the Outer Envelope of the Chloroplast

The precursor protein can be guided to the TOC complex by cytosolic factors. Hsp70 and 14-3-3 form a guidance complex that acts in a pathway specific to precursor proteins containing phosphorylated transit peptides, while un-phosphorylated precursor proteins can bind to cytosolic Hsp90 and a co-receptor Toc64 (Inoue and Akita 2008; May and Soll 2000; Qbadou et al. 2007; Qbadou et al. 2006). The TOC complex was originally isolated from peas in the early 1990s, and the core components first named Outer Envelope Proteins (OEPs) (Hirsch et al. 1994; Schnell et al. 1994; Seedorf et al. 1995; Waegemann and Soll 1991). OEP34, OEP75, and OEP86 later renamed Toc34, Toc75, and Toc159 (a proteolytically sensitive protein yielding an 86 kDa unit) are the components that make up the core TOC complex (Bolter et al. 1998; Schnell et al. 1994; Seedorf et al. 1995). Toc34 and Toc159 are integral GTPases that share high homology in their GTP-binding domains (G-domains) and are thought to be the primary receptors for precursor proteins (Andres et al. 2010). These GTPases work with the beta-barrel containing Toc75, which functions as the pore of the complex, to import precursor proteins (Gentle et al. 2004). The core TOC complex is sufficient for *in vitro* translocation into lipid vesicles and is estimated between 500 kDa and 1 MDa (Chen and Li 2007; Kikuchi et al. 2006; Kikuchi et al. 2009; Schleiff et al. 2003b; Schleiff et al. 2003c).

It has been shown that TOC subunits interact with themselves and each other, for example Toc75 interacts with Toc34 and Toc159, and Toc34 homodimerizes and heterodimerizes with Toc159 (Nielsen et al. 1997; Reddick et al. 2007; Seedorf et al. 1995). Isolated core complexes have a stoichiometry of 1:4:4 of Toc159:Toc75:Toc34 and were estimated to have a diameter of 120-140 angstroms and a height of 100-120 angstroms (Schleiff et al. 2003c). This information came from imaging a purified core TOC assembly by TEM with random conical tilt reconstruction performed to determine a low-resolution 3D structure of the TOC core complex (Figure 1.3). In *Arabidopsis* the presence of 2 paralogs of Toc34; *At*Toc33 and *At*Toc34, along with the 4 paralogs of Toc159; *At*Toc159, *At*Toc132, *At*Toc120, and *At*Toc90 mean that distinct classes of TOC complexes exist as combinations of those different paralogs (2000; Ivanova et al. 2004; Jackson-Constan and Keegstra 2001).

1.5 The Small GTPase Toc34 Family

Toc34 has 2 domains: an N-terminal GTP-binding domain (G-domain) that is located in the cytosol and the C-terminal membrane anchor domain (M-domain) composed of a short hydrophobic stretch of amino acids (Kessler et al. 1994). GTP-binding proteins act as "molecular switches" by alternating between active GTP-bound and inactive GDP-bound states (Vernoud et al. 2003). The process regulated by TOC GTPases is the import of proteins into the chloroplast. *Ps*Toc34 binds to phosphorylated precursor proteins with higher affinity, and Toc34 can be phosphorylated to regulate its activity (Fulgosi and Soll 2002; Sveshnikova et al. 2000). Neither the G-activating protein (GAP), which causes hydrolysis of the GTP to GDP and inactivating Toc34, nor

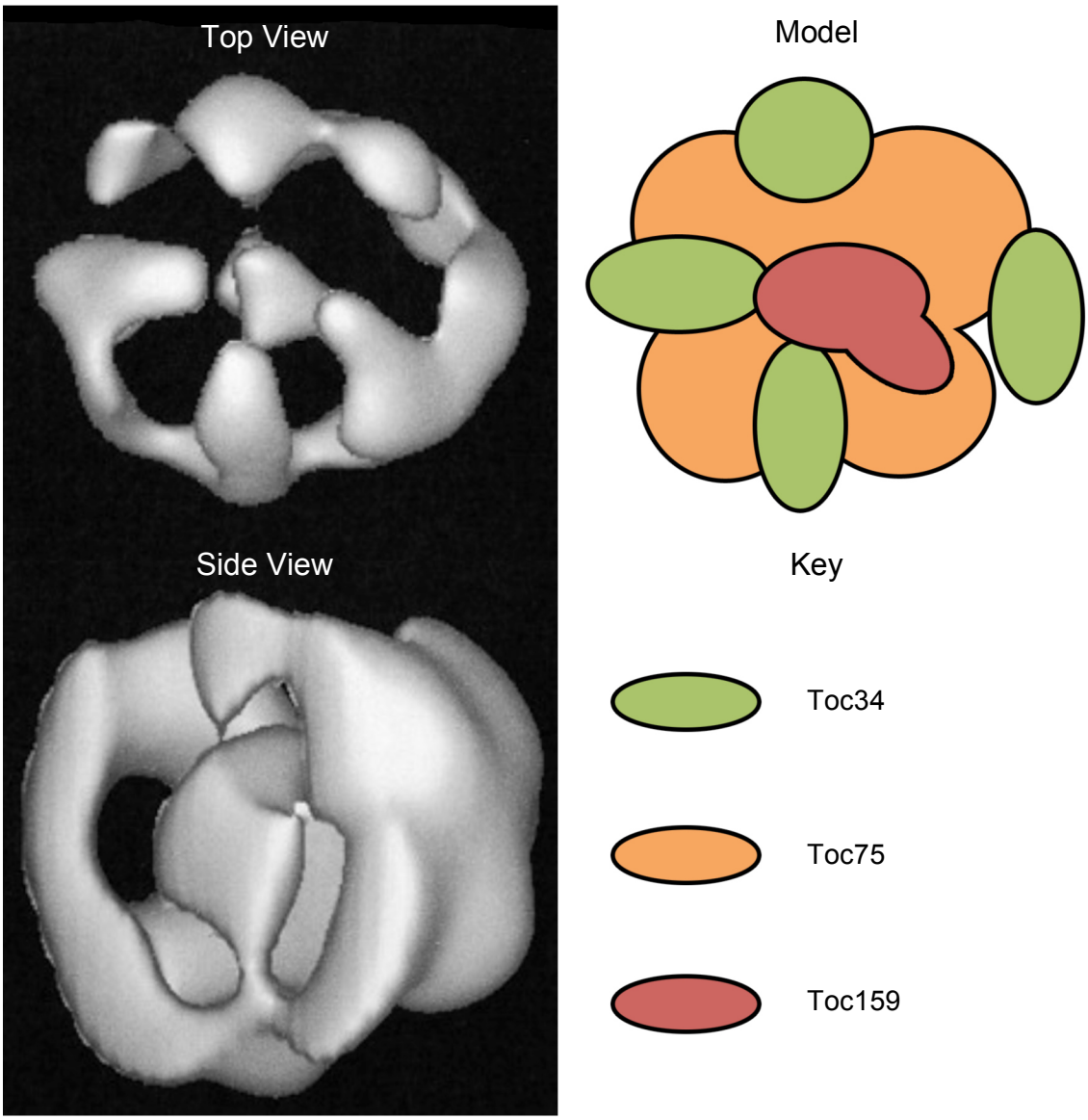


Figure 1.3 Reconstructed TEM of TOC core complex

Purified TOC complex was imaged with transmission EM and three dimensionally reconstructed (Schleiff et al. 2003c). The TOC complex has 4 independent pores, consistent with the proposed 4:4:1 stoichiometry of Toc75:Toc34:Toc159 in the complex.

the guanine exchange factor (GEF), which leads to exchange of GDP for GTP activating Toc34 have been identified with certainty. Although some work has shown that transit peptide can act as the GAP for Toc34 (Reddick et al. 2008). Toc34 was specifically co-isolated with precursor proteins at early and late import intermediate stages (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994; Seedorf et al. 1995). Crystalization of the G-domains of Toc34 shows this domain is responsible for dimerization and that each monomer has a nucleotide binding site (Koenig et al. 2008; Sun et al. 2002).

Arabidopsis has 2 Toc34 paralogs, *At*Toc33 and *At*Toc34 (Constan et al. 2004; Jarvis et al. 1998). *At*Toc33 shares more identity and is thought to share the same role as *Ps*Toc34 (Constan et al. 2004). A knockout mutation of *At*Toc33, plastid protein import #1 (*ppi1*), gives a plant with a pale green phenotype and abnormal chloroplasts with smaller and less developed thylakoids (Gutensohn et al. 2004; Jarvis et al. 1998). *Ppi1* even results in a decrease of expression of nuclear encoded photosynthetic proteins, suggesting that there is regulation of the protein expression by the TOC (Kubis et al. 2003). An *At*Toc34 mutant with an insert into an exon, *ppi3*, yields plants with short root length (Constan et al. 2004). While neither mutation is lethal to the plant, a combination of *ppi1* and *ppi3* will not allow the plants to develop past the embryo stage (Constan et al. 2004). These homologs have partially overlapping, but non-redundant functions with respect to substrate import specificity.

1.6 The Large GTPase Toc159 Family

Toc159 has a highly acidic N-terminal domain (A-domain) located in the cytosol, a central GTP-binding domain (G-domain) that shares very high identity with the G-domain of Toc34, and a C-terminal domain (M-domain) that anchors the protein to the outer membrane (Richardson et al. 2009). The GTP dependence of the TOC as evidenced by inhibition of import with non-hydrolyzable GTP analogs (Schnell et al. 1994) is due to the Toc34 and Toc159 (Leipe et al. 2002; Weirich et al. 2008). Toc159 was originally isolated from pea as an 86-kDa species and dubbed OEP86 (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994; Seedorf et al. 1995). The 86-kDa protein was a fragment consisting of the G and M domains (Bolter et al. 1998; Chen and Li 2007). The null mutant of Toc159 (*ppi2*) has shown that the A-domain is dispensable because the mutant is rescued by the A-domain deleted Toc159-GM (Agne et al. 2009; Lee et al. 2003). Toc159 has been cross-linked to the precursor protein at the binding phase and early import phase (Ma et al. 1996; Perry and Keegstra 1994). Toc159 preferentially binds to only non-phosphorylated precursor proteins (Becker et al. 2004).

It has been demonstrated that Toc159 has specificity for photosynthetic precursor proteins, which helps explain the pale phenotype and decreased transcription of photosynthesis-regulated genes in the mutant *ppi2* (Bauer et al. 2000; Jarvis 2008). Toc132 and Toc120 seem to have at least partially overlapping functions as double mutant knock-outs of both genes is embryo lethal, and overexpression of either Toc120 or Toc132 complement the double mutant, but not *ppi2* (Ivanova et al. 2004; Kubis et al. 2004). Toc132 and Toc120 are suggested to import housekeeping genes and to be more

involved in non-photosynthetic tissues, it is these members of the Toc159 family that are thought to play a role in gravitropism while possibly interacting with actin (Friedman et al. 2003; Jouhet and Gray 2009; Kubis et al. 2004; Stanga et al. 2009). It is interesting to note that Toc132 and Toc120 are not found in the same complex with Toc159, and that Toc132 and Toc120 locate with Toc34 while Toc159 locates with Toc33 (Ivanova et al. 2004; Smith et al. 2004).

1.7 The Translocation Channel Toc75

Toc75 is the only TOC component of cyanobacterial origin, Toc34 and Toc159 were added later on, but still early in evolution because they are common to all higher plants, algae, and mosses (Kalanon and McFadden 2008; Reumann et al. 2005; Reumann and Keegstra 1999). Toc75 is the most abundant protein in the outer membrane of the chloroplast and interacts with Toc159 and Toc34 (Nielsen et al. 1997; Seedorf et al. 1995). Toc75 is the only known chloroplast outer membrane protein with a cleavable bipartite N-terminal targeting sequence, where the most N-terminal part is a classical transit peptide, cleaved by the SPP, and the C-terminal portion is cleaved in the inner membrane space by an envelope-bound type I signal peptidase (Inoue and Keegstra 2003). The mature portion of Toc75 has two unique domains typical of the outer membrane protein of 85 kDa (Omp85)/ two partner secretion (TPS) superfamily; an N-terminal portion composed of three polypeptide-transport associated (POTRA) domains, and a C-terminal pore made up of twelve to eighteen β-strands, TPS protein FhaC has been crystallized and is shown in Figure 1.4 (Ertel et al. 2005; Sanchez-Pulido et al. 2003).

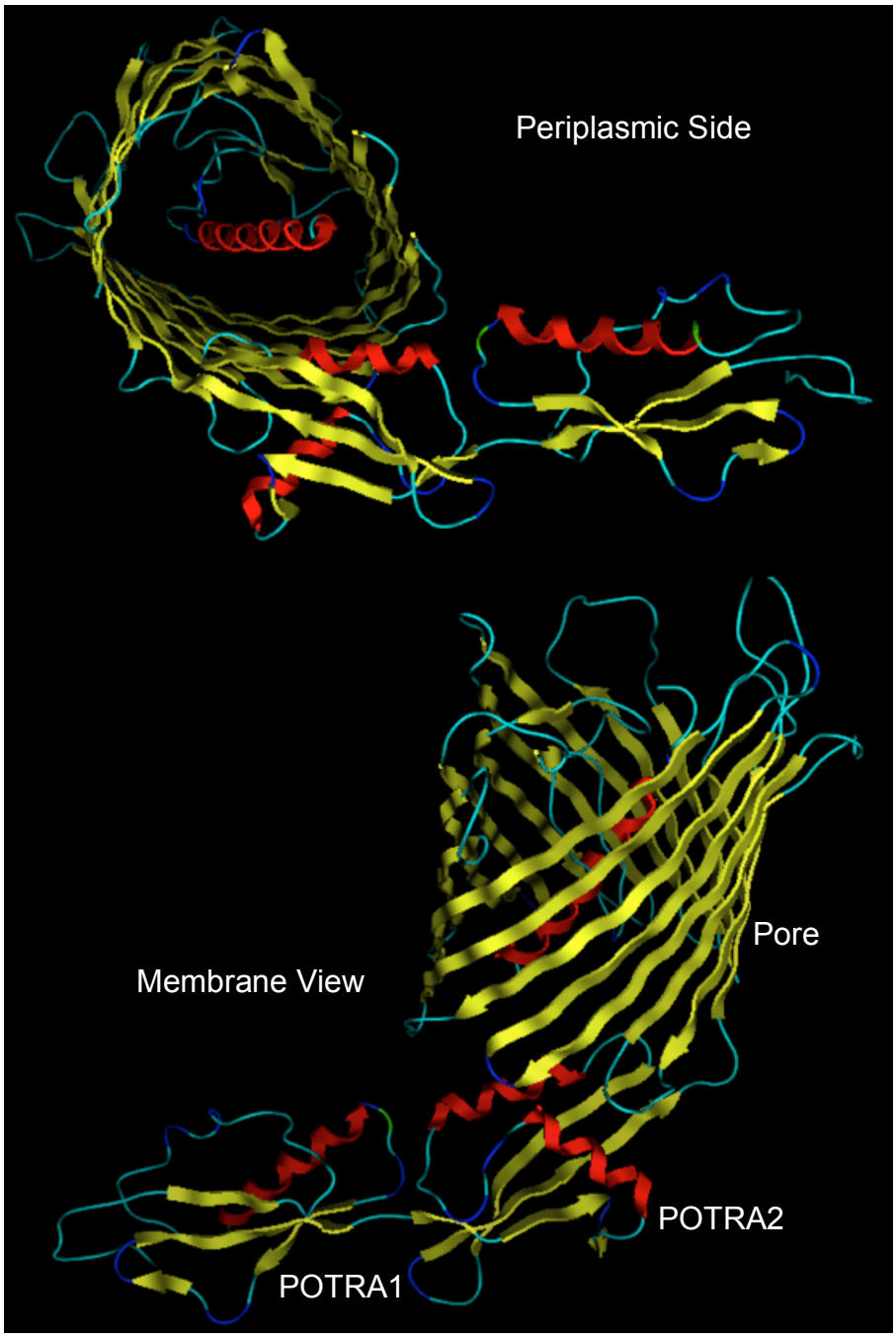


Figure 1.4 Crystal Structure of FhaC

FhaC, the TpsB exporter of filamentous hemagluttinin A (FHA), crystalized by Clantin et al. 2007 demonstrates the C-terminal pore and N-terminal POTRA domains that interact with FHA (Delattre et al. 2011).

The Omp85 family has proteins that function to insert other β-barrel proteins into the outer membranes of gram-negative bacteria, mitochondria, and chloroplasts (Gentle et al. 2005; Patel et al. 2008). TPS systems consist of two proteins, secreted "TpsA" proteins and their specific "TpsB" transporters (Jacob-Dubuisson et al. 2009; Kajava and Steven 2006).

The pea has two Toc75 paralogs; Toc75 (the translocation pore) and Toc75-V (a direct ortholog of cyanobacterial ancestor (Eckart et al. 2002). *Arabidopsis* has three that are represented by expressed sequence tags; atToc75-I (shares 60% identity with atToc75-III), atToc75-III (the main pore with 74% identity to psToc75), and atToc75-IV (half the size with 407 amino acids, that align with the C-terminus of the other Toc75s, versus the 819 present in atToc75-III) (Jackson-Constan and Keegstra 2001). AtToc75-I is a pseudo-gene with a transposon inserted inside the sequence, meaning there is no real expression in the cell (Baldwin et al. 2005). AtToc75-IV does not have a cleavable N-terminal transit peptide, and most likely lacks POTRA domains, as it was not sensitive to protease treatment (Baldwin et al. 2005). The atToc75-III knock-out mutant is embryo lethal (Baldwin et al. 2005). The OEP80 (originally named atToc75-V) is responsible for insertion of outer membrane proteins into the chloroplast envelope is a member of the Omp85 family and is thought to have diverged from Toc75 before the endosymbiotic event (Inoue and Potter 2004).

The evidence for Toc75 acting as the pore for protein translocation includes; cross-linking and patch-clamp experiments showing Toc75 interacts with precursor proteins during import, crosslinking data showing an association with envelope-bound

import intermediates, and arrest of import into purified chloroplasts by antibodies against Toc75 (Ma et al. 1996; Perry and Keegstra 1994; Schnell et al. 1994; Tranel et al. 1995). Toc75 forms a cationic-selective voltage-gated ion channel that interacts specifically with precursor proteins, with a pore of 14 angstroms at its narrowest point (Hinnah et al. 1997; Hinnah et al. 2002).

1.8 Polypeptide-Transport Associated (POTRA) Domains

The polypeptide-transport associated (POTRA) domain is found in the N-terminus in members of the outer membrane protein of 85 kDa (Omp85) and two partner secretion (TPS) superfamily and is involved in the assembly of proteins into or translocation across the outer membrane of mitochondria, chloroplasts, and gram negative bacteria (Delattre et al. 2011; Gentle et al. 2004; Gentle et al. 2005; Knowles et al. 2009; Moslavac et al. 2005; Paschen et al. 2003; Reumann et al. 1999; Sanchez-Pulido et al. 2003; Voulhoux et al. 2003). POTRA domains are counted increasingly from the N-terminus (Sanchez-Pulido et al. 2003). POTRA domains are often recognized by antibodies raised against the entire protein (e.g.: D15) and have been suggested for possible use in vaccines against: cholera (Ruffolo and Adler 1996), meningitis (Manning et al. 1998; Yang et al. 1998), gonorrhea (Manning et al. 1998), and syphilis (Cameron et al. 2000; Poolman et al. 2000; Robb et al. 2001). Antibodies against POTRA domains could be developed in people without risk of catching the disease the bacteria give, and could provide protection against future infections. All members of the Omp85/TPS

superfamily have between one and seven POTRA domain repeats (Figure 1.5) (Arnold et al. 2010; Sanchez-Pulido et al. 2003).

TpsA proteins all harbor a conserved, 250-residue long N-terminal "TPS" domain essential for secretion (Clantin et al. 2004; Hodak et al. 2006) and are involved in adhesion (Borlee et al. 2010), cytolysis, contact-dependent growth inhibition (Aoki et al. 2005), biofilm formation, proteolysis, and host-cell invasion (Jacob-Dubuisson et al. 2004). The POTRA domains present in the N-terminus of the TpsB proteins interact with the secreted TpsA proteins (Clantin et al. 2007; Hodak et al. 2006).

Beta-barrel proteins in the outer membrane involved in protein transport termed polypeptide-transporting β-barrel proteins (PTBs) can be divided in two classes via their functions (Inoue and Potter 2004; Jacob-Dubuisson et al. 2009; Moslavac et al. 2005). Class I PTBs are involved in secretion through the outer bacterial membrane, while Class II proteins function to insert β-barrel proteins into outer membranes (Koenig et al. 2010). Class I PTBs have two POTRA domains, while class II proteins have between one and seven (Arnold et al. 2010; Kim et al. 2007; Koenig et al. 2010; Sanchez-Pulido et al. 2003).

POTRA domains show very little primary sequence homology, in-fact, only glycine and hydrophobic residues forming the core of the domain are conserved between POTRAs (Sanchez-Pulido et al. 2003). However, the secondary structure was both predicted (Sanchez-Pulido et al. 2003) to be conserved and shown by crystallization and/or NMR of POTRA domains of BamA (Kim et al. 2007), FhaC (Clantin et al. 2007), FtsQ (van den Ent et al. 2008), *Thermosynechococcus elongatus* Omp85 (*Te*Omp85)

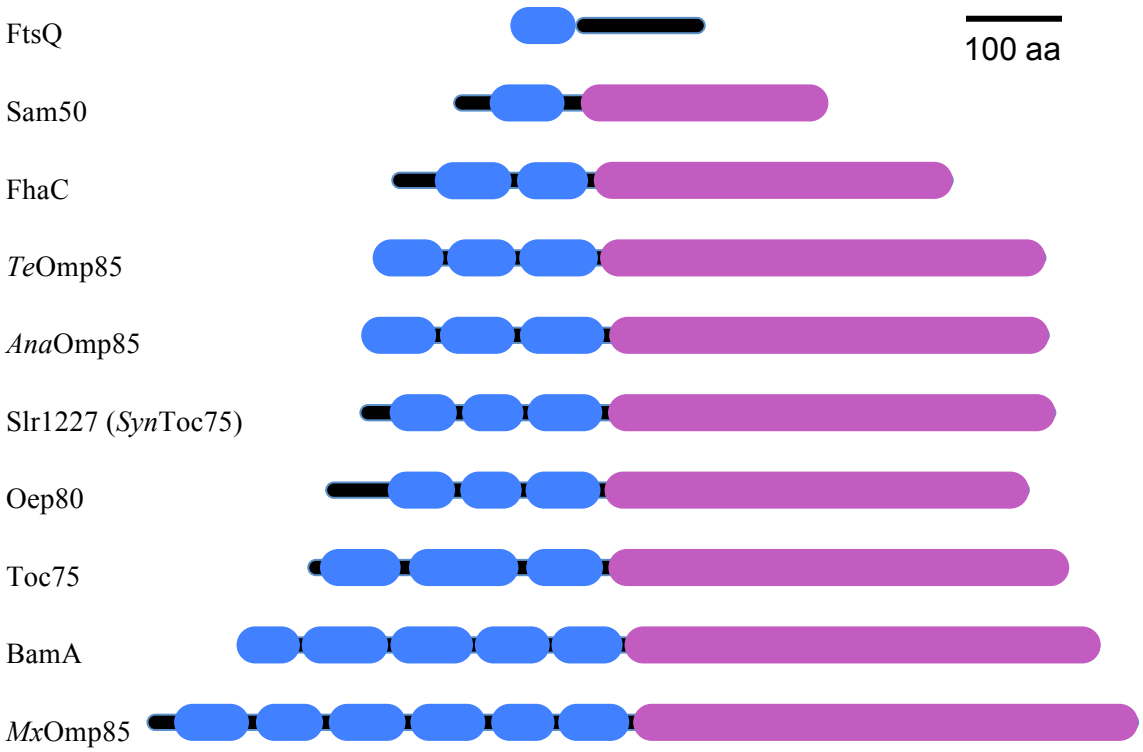


Figure 1.5 POTRA Domains of Omp85/TspB Superfamily

Conserved POTRA domains are found in *E. coli*; BamA - involved outer membrane biogenesis and FtsQ - the only POTRA containing protein that does not have a β-barrel involved in cell division. POTRA domains are also present in cyanobacteria; *T. elongatus*, *Anabaena*, and *Synechocystis* - these proteins are proposed to function in membrane biogenesis. Mitochondrial Sam50 has one copy of the POTRA domain, while the Omp85 of *Myxococcus xanthus* has six POTRA copies, both proteins function in membrane biogenesis. The Oep80 protein functions in chloroplast membrane biogenesis as well. FhaC is a protein exporter in N. meningitides with two POTRA copies. Toc75 is the protein importer in the outer chloroplast membrane and possesses three POTRA repeats. Figure adapted from Sanchez-Pulido. (2003) and Hsu and Inoue (2009)

(Arnold et al. 2010), and *Anabaena* Omp85 (*Ana*Omp85) (Koenig et al. 2010) to have a conserved structure, which was experimentally determined to have the consensus β-α-α-β-β (Figure 1.6). The orientation of tandem POTRA domains differ among crystal structures (Ward et al. 2009), and in-solution structures of POTRA domains reveal a dynamic structure (Knowles et al. 2008). Generally the POTRA domain closest to the pore tends to have a fixed arrangement with respect to the pore (Koenig et al. 2010).

In Omp85 the POTRA domains are thought to be involved in protein-protein interactions during substrate recognition or in formation of the heteroligomer transporter complexes (Ertel et al. 2005; Kalanon and McFadden 2008; Kim et al. 2007). A suggested form of interaction between the POTRA domains with each other or other proteins is β-augmentation (Harrison 1996), which has been seen in crystal structures (Gatzeva-Topalova et al. 2008; Kim et al. 2007; Koenig et al. 2010). β-sheet augmentation occurs when a free peptide or part of a protein possesses clusters at the edge of a β-sheet, and the free peptide adopts a β-strand conformation stabilizing the interaction between peptide and β-sheet by the addition of another β-strand (Harrison 1996).

The Omp85 family is essential for the assembly of outer membrane β-barrel proteins in gram negative outer membranes (Genevrois et al. 2003; Voulhoux et al. 2003; Wu et al. 2005) and has been found in the genome of all sequenced bacteria (Gentle et al. 2004; Voulhoux and Tommassen 2004). Omp85 homologs in *Arabidopsis thaliana* include: sorting assembly machinery of 50 kDa (Sam50) in the mitochondria outer membrane (Gentle et al. 2004; Kozjak et al. 2003; Paschen et al. 2003), outer envelope

protein of 80 kDa (OEP80), which mediates assembly of chloroplastic outer membrane proteins (Schleiff et al. 2003a) and is closely related to bacterial and cyanobacterial Omp85s (Eckart et al. 2002; Inoue and Potter 2004), and Toc75-III (Hinnah et al. 1997; Schleiff et al. 2003c; Schnell et al. 1994).

While certain POTRA domains are essential for cell viability, e.g. POTRA5 of Omp85 in *Neisseria meningitidis* (Bos et al. 2007), deletion of any of them show a decrease in function of the protein machinery they are part of or a decrease in cell viability (Bos et al. 2007; Bredemeier et al. 2007; Clantin et al. 2007; Ertel et al. 2005; Habib et al. 2007; Kim et al. 2007). The single POTRA domain of Sam50 is not necessary for targeting or correct insertion of Sam50 into the mitochondrial outer membrane, but it is required for optimal function of Sam50 and normal growth of yeast cells (Habib et al. 2007). Less β-barrel is imported at lower import efficiencies into the outer mitochondrial membrane with less POTRA domains, and POTRA domains has been shown to specifically bind β-barrel proteins destined for the mitochondrial outer membrane (Habib et al. 2007). The N-terminal portion (containing the POTRA domains) of Omp85 of *Nostoc* sp. *PCC7120* (*n*Omp85) is responsible for complex formation in native membranes, its deletion yields only one stabilized assembly of monomeric Omp85 while the wild-type protein is part of a homo-trimeric complex (Bredemeier et al. 2007). The POTRA domains of *n*Omp85 have an affinity for the C-terminal pore of the protein, and act as a docking site for incoming precursor proteins (Bredemeier et al. 2007). POTRAs of *n*Omp85 have a structural and functional influence and can even regulate the flux through the pore of the protein (Bredemeier et al. 2007).

POTRA domains one through four of BamA crystallized as a dimer with individual domains interacting via β-augmentation, even though they act as a monomer in solution (Kim et al. 2007). POTRA5 is necessary for interactions with YfiO, NlpB, and SmpA (all proteins involved in the outer membrane β-barrel protein biogenesis), while POTRA1 is required for an interaction with YfgL (Kim et al. 2007). POTRA1 also interacts with SurA (Sklar et al. 2007; Vuong et al. 2008), a periplasmic chaperone, with an arginine residue of the POTRA domain crucial for the interaction (Bennion et al. 2010). Cells harboring deletions of POTRA1 or POTRA2 of BamA lead to poor cell growth, while cells with deletions of POTRA3 or POTRA4 do not survive wild-type BamA depletion, and cells without POTRA5 do not survive even when they possess copies of wild type BamA (Kim et al. 2007).

POTRA domains are very important for the function of FhaC the *Neisseria meningitidis* exporter of filamentous hemagglutinin (FHA) as deletion of either POTRA domain will abolish secretion of FHA, and insertion of residues into the POTRA domains strongly affects secretion (Clantin et al. 2007). The POTRA domains (specifically helix 2 of POTRA1) are involved in FHA recognition (Clantin et al. 2007). The isoelectric points of POTRA1 and FHA are 4.56 and 9.99 suggesting that their 3.9 ± 0.5 μM interaction may be electrostatic (Delattre et al. 2011). Functionally essential POTRA2 is presumed to use β-augmentation during the transport of FHA, and substitions in this domain have the most severe affect on secretion (Delattre et al. 2011).

POTRA3 of BamA shows a β-bulge of two residues as well as an additional ten amino acids in between the α-1 and α-2 helices (Gatzeva-Topalova et al. 2008), and

POTRA2 of *Anabaena* has equivalent features with the ten residues forming a 3-10 helix (Koenig et al. 2010). Phylogenetic analysis shows that POTRAs cluster by their positional number, which supports the hypothesis that their order remains important (Bos et al. 2007). In-solution structures of POTRA domains reveal dynamic domain orientations (Knowles et al. 2008) that are supported by the substantial deviation between different crystal structures of tandem POTRA domains (Ward et al. 2009). Although spectroscopic studies suggest that there is more restriction among domain-domain orientations between POTRA1 and POTRA2 than NMR data does (Ward et al. 2009). *Ana*Omp85 shows a stable interaction for the POTRA3-POTRA2 region, and more flexibility at the POTRA2-POTRA1 interface (Koenig et al. 2010).

Toc75 POTRAs were not shown to have affinity for the C-terminal pore by blue-native PAGE, cross-linking, or liposome titration (Bredemeier et al. 2007). Toc75 has a precursor protein-binding site (Hinnah et al. 1997). The N-terminus of *Ps*Toc75 was shown to interact with precursor to the small subunit of RuBisCO (pSSU) but not the mature form of the protein (mSSU) (Ertel et al. 2005). An interaction of the N-terminus of *Ps*Toc75 with Toc34ΔTM has been noted (Ertel et al. 2005). The most C-terminal POTRA has been demonstrated to be responsible for gating of the translocons (Ertel et al. 2005; Kim et al. 2007).

POTRA domains seem to be important for homooligomerization (Bredemeier et al. 2007; Ertel et al. 2005; Moslavac et al. 2005). Similarities between proteobacterial and cyanobacterial POTRAs include interfaces on POTRAs that are proposed to be involved in protein-protein interactions along with a flexible “hinge” region between a pair of

domains (Koenig et al. 2010). The L1-loop is situated between β-1 and α-1 in the most C-terminal POTRA domain, and has been identified to be important in gating in *Ana*Omp85 (Koenig et al. 2010) and has been predicted to be in Toc75-III (Baldwin et al. 2005), OEP80 (Eckart et al. 2002), and in Sam50 (Gentle et al. 2004; Kozjak et al. 2003; Paschen et al. 2003). There is a three amino acid insertion found in cyanobacterial and Toc75 (L1) that is not present in mitochondria or proteobacterial Omp85s (Koenig et al. 2010). It seems that each different POTRA domain of a protein have unique roles in the insertion or translocation of proteins into or through outer membranes.

The goal of this work is to produce individual and combinations of recombinant POTRA domains from *Ps*Toc75, and test their interactions with themselves, other recombinant POTRA domains, and precursor proteins/transit peptides. The possible interactions will be tested with glutaraldehyde and BMH cross-linking, analytical ultracentrifugation, pull-down assays, and an *in vitro* chloroplast protein import assay. This will allow an *in vitro* dissection of each POTRA domain's contribution to the TOC complex and chloroplast protein import, and possible conserved roles for specific POTRA domains.

Chapter 2 Materials and Methods

2.1 Generation of POTRA1-3-His

Primers introducing an NdeI (CAT ATG) restriction site 5' and an XhoI (CTC GAG) restriction site 3' from the predicted POTRA1-3(small) codon-optimized DNA of *ps*Toc75 were designed.

P1-FKKY-FORW 5' GGT GGT CAT ATG TTT AAG AAA TAC AAA ATC TCA G 3'

POTRA6-Xho1 Reverse 5' GGT GGT GGT CTC GAG TTT CTG CTC CA 3'

All primers were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) received as lyophilized powder, and resuspended to 50 μM in deionized, autoclaved H_2O (ddH_2O). These 5x stocks were aliquoted and kept frozen at -20ºC. Working stocks of primers were made by diluting a small aliquot to 10 μM to prevent degradation and contamination of primer stocks. PCR using the high fidelity DNA polymerase ExTaq (Takara Bio Inc., Shiga, Japan) was set up according to appendix 1. PCR was performed in a Mastercycler Gradient Cycler, (Eppendorf, Hamburg, Germany) using the cycling conditions in appendix 2.

The PCR product was run on a 0.8% low melting agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified PCR product was then ligated into the pGEM-T Easy (Promega, Madison, WI) vector using the conditions in appendix 5 for 2 hours at 25ºC. The ligated product was transformed into *E. coli* GC5 competent cells (Genesee Scientific, San Diego, CA) and spread on 1.5% Luria Broth (LB) agar plates containing 150 μg/ml ampicillin, 100 μl of

20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galctopyranoside (X-gal), and 100 µl of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

This method allows for a blue-white colony screen in which blue colonies have a functional β-Galactosidase gene allowing hydrolysis of X-gal to 5-bromo-4-chloro-indoxyl which is spontaneously oxidized to the bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo causing these negative colonies to appear blue. White colonies have PCR product inserted into the β-Galactosidase gene causing the positive colonies to appear white. Seven white colonies and one blue colony were screened by colony PCR and re-streaked on 1.5% LB agar plates with 150 µg/ml ampicillin. The colony PCR set up is listed in appendix 4 and was run under the following cycling conditions listed in appendix 5

The colony PCR products were run on a 0.8% agarose gel and were visually screened to confirm a positive product around 1kb (300 bp for vector and 700 bp for insert), the negative product was ~ 300 basepairs. Positive colonies were sequence confirmed with NdeI and XhoI. A positive colony was picked, grown overnight in LB with 150 µg/ml ampicillin and a midi-prep (Wizard Plus, Promega, Madison, WI) was performed to isolate the pGEM-T Easy-POTRA1-3 plasmid.

The pGEM-T Easy-POTRA1-3 vector along with pET-30a were digested with XhoI and NdeI for 90 minutes at 37ºC, and enzymes were heat-killed at 65ºC for 20 minutes. Digested pET-30a (Promega, Madison, WI) was incubated with 1 µl of calf-intestine alkaline phosphatase (CIAP) (Promega, Madison, WI) for 1 hour at 37ºC to remove phosphate overhangs to inhibit self-ligation. Digested POTRA1-3 and CIAP

treated pET-30a were run on a 0.8% low melting agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). POTRA1-3 was then ligated into the pET-30(a) vector using the conditions in appendix 6 for two hours at 25ºC.

The ligation product was transformed into GC5 competent cells and plated on a 1.5% LB agar plate with 30 µg/ml kanamycin. The colony PCR set up is listed in appendix 7 with the only difference that the primers are now T7 Promoter F and T7 Terminator R instead of M 13 F and M 13 R and was run under cycling conditions listed in appendix 8.

The colony PCR products were run on a 0.8% agarose gel and were visually screened to confirm a positive product around 1kb (300 bp for vector and 700 bp for insert), the negative product was ~300 bp. Positive colonies were sequence confirmed with NdeI and XhoI. A positive colony was picked, grown overnight in LB with 30 µg/ml kanamycin and a midi-prep was performed to isolate the pET30a-POTRA1-3 plasmid. This results in a POTRA1-3 protein produced with a 6xHis tag at the C-terminus.

2.2 Expression and Purification of Proteins Using the Impact System

Competent *E. coli* ER2566 cells were transformed with pTYB2-SStp*Ps*, pTYB2-POTRA1, or pTYB2-POTRA3 constructs and grown at 37ºC with shaking at 225 rpm in three 1L LB flasks with 150 µg/ml ampicillin to an OD_{600} of 0.2, the cells were then moved to 25ºC and grown until OD_{600} of 0.4. IPTG was added to a final concentration of 1 mM for SStp*Ps*, 0.5 mM for POTRAs to induce fusion protein production, and cells

were grown for 14-16 hours. Cells were harvested by centrifugation, and resuspended in 30 ml of Tris buffer (10 mM Tris-HCl pH 8.0, 0.3% v/v Triton X-100) per liter of cell culture and lysed with three passes through a French Press (Thermo Electron Corporation, Waltham, MA) at an internal pressure of 25,000 psi. The lysate was cleared by centrifugation at 36,000 g for 15 minutes in a Sorvall SS-34 rotor. The supernatant was purified by anion exchange chromotography as described in FPLC Purification section. The flow through was then loaded onto a chitin matrix column (5 ml matrix/liter of cells, New England Biolabs, Beverly, MA) at 1 ml/min. The column was washed with twenty column volumes of ice-cold column buffer (20 mM Na-phosphate pH 9.0, 0.5 M NaCl, 1 mM EDTA) to remove unbound proteins. The column was then washed with three column volumes of ice-cold phosphate buffer (1 mM Na_2HPO_4, 0.17 mM KH_2PO_4, pH 7.5, for SStp*Ps*) or ice-cold column buffer (for POTRAs) supplemented with 50 mM β-mercaptoethanol (β-ME) to induce cleavage of the self-splicing intein tag. The column was then incubated at 4ºC for 24 hours. The proteins were eluted with the buffer that they were cleaved in without any additional β-ME in three 6 ml elutions. SStp*Ps* elutions were frozen in liquid nitrogen and lyophilized as a β-ME removal and concentration step. Transit peptide is stored as a lyophilized powder at -80ºC. POTRA elutions were dialyzed to remove β-ME, and stored at 4ºC. All proteins were analyzed on 19.2% Tris-Tricine gels stained with Coomassie Brilliant Blue as described in the gel electrophoresis section to assess purity.

2.3 Expression, Purification, and Refolding of POTRA1-3

Competent *E. coli* BL21(DE3) cells were transformed with pET30a-POTRA1-3 and grown at 37ºC with shaking at 225 rpm in three 1 L flasks of LB with 30 µg/ml kanamycin to an OD_{600} of 0.4. IPTG was added to a final concentration of 1 mM to induce his-tagged protein production for 8 hours at 37ºC. Cells were harvested by centrifugation, and resuspended in 30 ml of lysis buffer (20 mM sodium phosphate pH 9.0, 0.5 M NaCl, 1 mM EDTA). The cells were lysed with three passes through a French Press at an internal pressure of 25,000 psi. The lysate was cleared by centrifugation at 36,000 g for 15 minutes in a Sorvall SS-34 rotor. The pellet was then resuspended via homogenizer with 5 ml of ice-cold denaturing lysis buffer (20 mM sodium phosphate pH 7.8, 0.5 M NaCl, 8 M urea), and centrifuged at 36,000 g for 15 minutes at 4ºC. The supernatant was dialyzed at 25ºC for 4 hours with 3 buffer changes of 2 L of column buffer (20 mM sodium phosphate pH 8.0, 0.5 M NaCl, 1 mM EDTA).

2.4 FPLC Purification of Fusion Proteins

Supernatant cleared by centrifugation at 36,000 g for 15 minutes in a Sorval SS-34 containing fusion proteins pTYB2-SStp*Ps*, pTYB2-POTRA1, or pTYB2-POTRA3 in ~30 ml of Tris buffer (10 mM Tris-HCl pH 8.0, 0.3% v/v Triton X-100) was diluted to a total of 300 ml with 10 mM Tris pH 8.0. The diluted supernatant was loaded onto a column containing a matrix of POROS HQ-20 (Invitrogen, Carlsbad, CA) using a BioCad perfusion chromatography workstation (S/N 500, SpectraLab Scientific, Ontario,

CA). The flow through containing the fusion protein was collected and analyzed by 15% tris-glycine gel. The column was washed and regenerated.

2.5 Circular Dichroism of POTRA Domains

Circular dichroism spectroscopy was performed on an Aviv Series 202 circular dichroism spectrophotometer (Aviv Instruments, Lakewood, NJ). POTRA1, POTRA3, or refolded POTRA1-3 was extensively dialyzed into CD buffer (10 mM potassium phosphate pH 7.0, 50 mM Na_2SO_4). Final concentration of the proteins was 10 μM for POTRA1 and POTRA3, and 3 μM for POTRA1-3. Circular dichroism spectra were collected at 25ºC as 5-second averages at 1 nm intervals from 185 to 285 nm. Three spectra were averaged, corrected for buffer contributions, smoothed, and converted to molar ellipticity using the Aviv software, version 2.71. Deconvolution was performed using the CDPro software with IBase3, a reference set of 37 soluble proteins (Sreerama, N. and R.W. Woody, 2000). From these data the secondary structural features of the POTRA domains were solved by CDPro. CDPro uses three algorithms to deconvolute measured secondary structure: Self-Consistent method for CD analysis, version 3 (Selcon3 - Sreerama N, Venyaminov, et. al. 1999); Contin/LL (Provencher, S. W., and J. Glo¨ckner, 1981); CDSSTR (Johnson. W.C., Jr. 1999).

2.6 MALDI-TOF of POTRAs

POTRA3 and POTRA1-3 were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to confirm purity, size, and

correct cleavage. MALDI-TOF MS was performed with a Bruker Daltonics Microflex™ mass spectrometer, with β-chain insulin, ubiquitin I, cytochrome C, and β-chain myoglobin used as an internal mass standard for calibration with POTRA3; trypsinogen, protein A, and bovine serum albumin used as external mass standards for calibration with POTRA1-3. Lyophilized standard protein was resuspended to a final concentration of 50 μM in 10 μl of 50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid. POTRA3 and standards were mixed with 10 μl of 60% (v/v) CHCA (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, St. Louis, MO) with 1% (v/v) nitrocellulose, POTRA1-3 and standards were mixed with 10 μl of 60% (v/v) sinapinic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid, Sigma-Aldrich, St. Louis, MO). Plates were spotted with 1 μl of the mixtures and dried thoroughly in a vacuum dessicator overnight at room temperature. Mass spectra were acquired in positive ion mode using 300 nitrogen laser pulses/spectrum. The resulting mass/charge data was analyzed using the FindPept program, which is freely accessible on the web at http://ca.expasy.org/tools/findpept.html. Users are able to enter predicted amino acid sequence and possible modifications along with the mass/charge values measured by MS to identify the peptide (intact or fragmented) that correspond to the experimentally obtained mass/charge values.

2.7 Plant Growth and Chloroplast Isolation

Dry dwarf pea (*Pisum sativum* progress number 9) seeds (approximately 200 ml/flat) were imbibed overnight with aeration and planted in autoclaved horticultural vermiculite in 12 x 24 x 5 inch flats. Plants were grown in environmental growth

chambers (EGC, Chagrin Falls, OH) with 160 μE/m2/sec cool white fluorescent light at 28ºC during day and 25ºC during night. Plants were grown using a 8 hr light/16 hr dark cycle to help reduce starch accumulation for 14 days. Leaves were harvested at the end of a dark cycle and chopped in a Cuisinart food processor with 2 second bursts until they were reduced to pieces no larger than 5-6 mm. The leaf tissue was then suspended with (3 x volume per weight of leaves harvested) ice-cold grinding buffer (GB: 330 mM Sorbitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM EDTA, 0.2% w/v BSA, 50 mM HEPES-KOH, pH 7.3) and homogenized with 4 cycles of 3-second bursts with a Polytron. The lysed tissue was then filtered through 2 layers of cheesecloth and 2 layers of MiraCloth. The filtrate was centrifuged at 900 g for 5 minutes in a Sorvall GS-3 swinging bucket rotor using a Sorvall RC26 Plus centrifuge at 4ºC to pellet the chloroplasts. The chloroplasts were gently resuspended in 5 ml of ice-cold import buffer (IB: 330 mM Sorbitol, 50 mM HEPES-KOH, pH 8.0) and gently layered on top an 80%:40% percoll (in IB) step gradient (2 ml of 80% percoll at the bottom of a 15 ml falcon tube carefully overlayed with 4 ml of 40% percoll). Chloroplast loaded gradients were centrifuged at 3,400 g for 15 minutes at 4ºC with 60% maximum acceleration and deceleration. Intact chloroplasts were collected from the interface of the step gradient with a large-bore syringe needle, and to remove percoll were diluted to a final volume of 14 ml with IB. The chloroplast-containing solution was centrifuged at 900 g for 5 minutes at 4ºC to pellet the chloroplasts. The chloroplasts were gently resuspended in 5 ml of ice-cold IB and the gradient centrifugation step was repeated. After chloroplasts were centrifuged at 900 g for 5 minutes at 4ºC, resuspended in a total volume of 1.5 ml of IB with 2% BSA and the

chloroplast suspension was diluted to a final total chlorophyll concentration of 1.0 mg/ml with IB with 2% BSA.

2.8 Chlorophyll Measurement

Chlorophyll concentration was measured by chlorophyll extraction with 990 µl of 80% acetone and 10 µl of chloroplast suspension. Acetone suspension was vortexed and centrifuged at 20,000 g for 1 minute in a microcentrifuge to remove debris. Absorbance measurements were taken at 663 nm for chlorophyll a and 645 nm for chlorophyll b and used to calculate total chlorophyll content using the following equation (Arnon, D. 1949).

$$\text{Chlorophyll (mg/ml)} = \frac{((8.02 \times A_{663}) + (20.2 \times A_{645}))}{0.01 \text{ ml} \times 1000 \text{ µg/mg}} \times 1 \text{ ml}$$

Chlorophyll measurement was averaged for two samples of chloroplast suspension in acetone.

2.9 Protein Measurement

Protein concentrations of all POTRA domains, B2Ims, and SStp*Ps* were determined using the BCA (bicinchoninic acid) reagents (Pierce, Rockford, IL). Bovine serum albumin (BSA, Pierce, Rockford, IL) was used as the protein standard for all protein concentration assays. Protein solutions that had concentrations of DTT or β-ME too high for the BCA assay to correctly measure were precipitated by the Compat-Able kit (Pierce, Rockford, IL) and resuspended in a buffer compatible with the standard BCA assay.

2.10 Gel Electrophoresis and Staining

All proteins were added to 4 x sample solubilizing buffer (4 x SSB, 400 mM DTT, 40 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS) vortexed and boiled for 3 minutes. POTRA1, POTRA3, SStp*Ps*, and B2Ims are less than 10,000 daltons, and a 19.2% Tris-Tricine gel (Schagger, H. and G. von Jagow, 1987) that gives much better resolution of low molecular weight species was used to visualize these smaller samples. A gel casting apparatus used was 0.75 mM thick spacer plates and short plates from the Protean-3 system (Bio-Rad Hercules, CA). Recipes for the resolving, separating, and stacking gel can be found in appendix 9. All three solutions are prepared and the 10% w/v ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) are added to the resolving solution, the resolving solution is then mixed and 2.5 ml is poured. TEMED and APS are added to the separating solution, mixed and 850 µl of separating gel is poured. Isopropanol is layered on top of the separating solution and the gels are allowed to polymerize (this usually takes ~ 5 minutes). After polymerization the isopropanol is removed and the stacking solution is mixed with APS and TEMED. The mixture is poured until the solution is at the top of the short plate. A comb is then inserted into the stacking solution and the gel is allowed to polymerize (this usually takes ~ 2 minutes.) The outer chamber is filled with anode buffer (0.2 M Tris-HCl pH 8.9) and the inner chamber is filled with cathode buffer (0.1 M Tris-HCl pH 8.25, 0.1 M Tricine, 0.1% SDS). The gels are run at 100 V until the bromophenol blue reaches the bottom (usually ~ 3 hours) before they are removed and either stained or used for Western blotting.

Fusion proteins and POTRA1-3 are all 30,000 daltons or larger and were run using a 15% acrylamide resolving and 5% acrylamide stacking gel using a Tris-glycine system. The resolving solution is mixed with APS and TEMED and 3.2 ml is poured, overlaid with isopropanol and allowed to polymerize (this takes ~ 5 minutes). After polymerization the isopropanol is removed and the stacking solution is mixed with TEMED and APS and poured until the solution is at the top of the short plate. A comb is then inserted into the stacking solution and the gel is allowed to polymerize (this takes ~ 2 minutes). The inner and outer chambers are filled with SDS Running buffer (25 mM Tris, 275 mM glycine, (added until pH is 8.3) 0.1% SDS). Gels are run at 20 mA/gel until the bromophenol blue reaches the bottom (usually ~ 1.5 hours) before they are removed for staining or Western blotting.

After gels were run they were incubated in Coomassie stain (10% acetic acid, 50% methanol, 0.25% Coomassie Brilliant Blue R 250) for 30 minutes. They were incubated in destain (10% acetic acid, 50% methanol) for 30 minutes. If a more sensitive staining technique was needed then silver staining (Heukshoren, J., and R. Demick, 1985) was performed. The proteins are fixed to the gel for 1 hour (with 2 buffer changes) with destain on an orbital mixer, they are then washed in ddH_2O for 30 minutes (with 2 buffer changes). The gel is exposed to silver nitrate (6 mM $AgNO_3$) for 30 minutes and rinsed with ddH_2O for 15 seconds. The gel is developed in developing buffer (236 mM Na_2CO_3, 0.02% formaldehyde) for 15 minutes and washed in ddH_2O for 20 minutes (with 1 buffer change). The gel is reduced with Farmer's reducer (12 mM $Na_2S_2O_3$, 685 μM $K_3Fe(CN)_6$) for 45 minutes, and washed with ddH_2O for 15 minutes (with 2 buffer

changes). The gel is then stained with silver nitrate again for 30 minutes and rinsed with ddH_2O for 15 seconds. The gel was incubated with developer and stopped when the signal was optimal by changing the buffer to 5% acetic acid.

2.11 Antibodies and Western Blotting

Rabbit antibodies against POTRA1 and POTRA3 were generated by Agriseria Inc. (Sweden) from lyophilized protein purified in our lab. Immobilon PVDF membrane (Millipore, Billerica, MA) was marked with a number 2 pencil and then activated in 100% methanol for 2 minutes and then soaked in transfer buffer (48 mM Tris, 390 mM glycine, (20% methanol - only used for Tris-glycine gels)) for at least 5 minutes. Gels were run with pre-stained markers (EZ-Run Prestained, Fisher Scientific, Fair Lawn, NJ; or SeeBlue, Invitrogen, Carlsbad, CA). Proteins were transferred to the pre-soaked PVDF using a Genie transfer apparatus (Idea Scientific, Minneapolis, MN) at 24 V for 1.5 hours at 4ºC. The blots were removed from the apparatus and incubated with Tris Buffered Saline with Tween-20 (TBST, 25 mM Tris-HCl pH 8.0, 137 mM NaCl, 3 mM KCl, 0.1% v/v Tween-20) containing 3% w/v non-fat milk (NFM) with rocking for 1 hour at room temperature (2 buffer changes). Blots were then incubated for 1 hour with 1:25,000 primary antibody : TBST-NFM with rocking. Blots were then washed with TBST-NFM for 30 minutes (with 2 buffer changes), and then incubated with 1:50,000 secondary antibody : TBST-NFM for 1 hour. Secondary antibodies used were donkey-anti-rabbit horseradish peroxidase conjugated (DAR-HRP) (Thermo Scientific, Rockford, IL). The blots were then washed 2 times with TBST alone and once with TBS for 5 minutes each.

The blots were then incubated with 1 ml total volume of a 1:1 mix of HRP substrate and Luminol (Millipore) for 5 minutes. The blots were then captured using a Chemidoc XRS (Bio-Rad, Hercules, CA) for 1.5 hours taking images every 15 minutes. The blots were analyzed with Quantity One software version 4.4 (Bio-Rad, Hercules, CA), and bands were quantitated by photon counting.

2.12 Chemical Crosslinking

Using the homobifunctional crosslinker bismaleimidohexane (BMH, Pierce, Rockford IL) that reacts with thiol groups, POTRA1 and SStp*Ps* could be specifically and positionally chemically crosslinked through a cysteine residue each protein possesses. POTRA3 and B2Ims crosslinking has to be done with glutaraldehyde because neither possess a cysteine. 100 µl reactions BMH crosslinking were run for 1 hour at room temperature in PBS pH 7.2 (1.47 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl) with 5 mM EDTA and 1 mM tris(2-carboxyethyl)phosphine (TCEP) and quenched with 5 mM DTT was done with the following set up :

POTRA1 (µM)	10	0	10	10
SStp*Ps* (µM)	0	100	100	100
BMH	-	-	-	+

Glutaraldehyde (Pierce, Rockford, IL) crosslinking was performed with POTRA1, POTRA3, SStp*Ps*, and B2Ims. 100 µl Crosslinking reactions were performed reactions for 5 minutes at room temperature in PBS pH 7.2, and quenched with 10 mM final volume Tris-HCl pH 8.0 with the following set up:

POTRA (μM)	5	0	5	0	5	5	5	10	10	10	10
Peptide (μM)	0	5	0	5	5	25	50	0	5	10	25
GA (0.1%)	-	-	+	+	+	+	+	+	+	+	+

(peptide is either SStp*Ps* or B2Ims)

All reaction products were mixed with 4 x SSB and run on a Tris-Tricine gel system to visualize oligomers via silver staining or Western Blot.

2.13 Fusion Protein – Transit Peptide Pulldown Assay

Chitin matrix (New England Biolabs, Beverly, MA) was used to co-purify POTRA1-fusion or POTRA3-fusion with SStp*Ps* or B2Ims. An N-terminal POTRA domain expressed as a protein with a self-splicing intein domain connected to a C-terminal chitin binding domain (fusion protein) (0.5 mg of 80% pure) purified by FPLC was flowed through a chitin column by gravity flow (chitin columns with only the intein tag and chitin binding domain of the fusion protein were used as a negative control). The columns were then washed with 10 column volumes of column buffer (20 mM Na-phosphate pH 9.0, 0.5 M NaCl, 1 mM EDTA) to remove unbound proteins. Columns were then incubated with 0.5 mg of SStp*Ps* or B2Ims for a negative control for 1.5 hours at 25ºC. Columns were washed with 5 column volumes of column buffer with 50 μM β-ME and left with one-half column volume for cleavage overnight at 4ºC. The column was eluted the following day and the elutant was mixed with 4 x SSB and ran on Tris-Tricine gels for silver-staining and Western blotting.

2.14 Analytical Ultracentrifugation

Analytical ultracentrifugation (AUC) analysis was used to follow the formation of POTRA homodimers and POTRA : Transit Peptide heterodimers. A Beckman Optima XL-1 analytical ultracentrifuge with both absorbance and interference optical detection systems was used to analyze sedimentation velocities. All samples were extensively dialyzed into AUC buffer (20 mM sodium phosphate pH 8.0, 50 mM NaCl) using Slide-A-Lyzer minidialysis units (Pierce, Rockford, IL) with a molecular weight cut-off (MWCO) of 3,500 daltons. For all experiments, dialysate was used as the reference buffer and loaded into the reference sector of the centerpiece. Sample cells were loaded into the rotor, placed in the ultra centrifuge, and allowed to equilibrate under vacuum to 25ºC for 1 hour. Samples were centrifuged at 50,000 rpm and data was collected using interference optics and/or absorbace at 280 nm. Data was analyzed by SedFit (Schuck 2000), allowing baseline, fit RI noise, fit time independent noise to float. The confidence level used was 0.80, and the resolution was 200.

2.15 Purification of prSSU*Nt* & mSSU*Nt* from Inclusion Bodies

Competent *E. coli* (BL21-DE3) cells were transformed with pET11-prSSU or pET11-mSSU constructs (Klein and Salvucci 1992). A transformed colony was grown in 1 L of LB at 37ºC with shaking at 225 rpm until a cell density of OD_{600} was 0.6 was reached. The cells were induced with 1 mM IPTG and grown for 6 hours. Cells were pelleted by centrifugation and resuspended in ice-cold buffer A (50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$). All constructs produced in this system are found primarily in inclusion bodies, allowing for easy purification (Reddick et al. 2008). Cells were lysed with 3

passes through a French Press at an internal pressure of 25,000 psi followed by centrifugation at 36,000 g for 20 minutes in a Sorvall SS 34 rotor. Inclusion bodies were isolated to homogeneity by 3 rounds of washing in buffer A with 0.1% Triton X-100 followed by 3 washes in buffer A without detergent. Pellets were resuspended with a dounce homogenizer and centrifuged at 36,000 g for 10 minutes in a Sorvall SS 34 after each wash. The final purified inclusion body pellets were then solubilized in urea buffer (8 M urea, 50 mM DTT, 20 mM Tris-HCl pH 8.0). Remaining impurities were removed by centrifugation at 36,000 g for 30 minutes. The concentrations of purified proteins were determined by BCA Compat-Able kit as described in the protein measurement section below, and stored at -80ºC.

2.16 *In Vivo* Radiolabeling of WT prSSU*Nt*

Precursor of the small subunit of rubisco of *Nicotiana tabacum* (prSSU*Nt*) was radiolabeled with ^{35}S by performing cell growth in methionine and cysteine-deficient DMEM (Dulbecco's Modified EAGLES Medium, MP Biomedicals, Santa Ana, CA). Cells were grown in LB until a cell density of OD_{600} 0.5 was reached, they were then pelleted, washed, and resuspended in Met-Cys-deficient DMEM. Cells were allowed to deplete any remaining methionine and/or cysteine supply by growth in Met-Cys-deficient DMEM for 2 hours, and induced using 1 mM IPTG. 200 µCi/ 5 ml of culture of Tran^{35}S-Label (MP Biomedicals, Santa Ana, CA) was added to the cells 15 minutes after induction. The ^{35}S is added after 15 minutes after induction to ensure most of the labeled cysteine and methionine is incorporated into the prSSU*Nt* instead of the T7 RNA polymerase, which is also induced at high levels with the addition of IPTG. Induction

was continued for 3 hours with the Tran^{35}S-cysteine and methionine Label being the only source of methionine and cysteine, forcing incorporation of the labeled amino acids into proteins translated after induction. ^{35}S labeled prSSU*Nt* was purified from inclusion bodies in the same manner as previously described, with the following changes. Cells were lysed with a probe sonicator (Misonix Sonic 550 probe ultrasonicator, Paulton, UK) on ice for 2 minutes total sonication time using a cycle of 5 seconds on and 10 seconds off. The specific activity was calculated and corrected for the purity of the protein as determined by Strom PhosphoImager (GE Healthcare, UK) of a 20-10% gradient Tris-glycine gel.

2.17 *In Vitro* Chloroplast Import Competition Assay

Chloroplasts are diluted to 2 mg/ml directly before the *In vitro* chloroplast import competition assay. Competition of import of ^{35}S-prSSU*Nt* was performed in 300 µl reaction with freshly isolated intact chloroplasts at 25 µg/ml chlorophyll final concentration and 100 nM final concentration of labeled ^{35}S-prSSU*Nt*. The concentration of the unlabeled competitors were varied at 0, 200, 500, 1,000, 2,000, 3,000, and 5,000 nM for 1 experiment and 0, 100, 200, 500, 700, 1,000, 1,500, and 2,000 nM for another 2 experiments. The reaction was supplemented with 2 mM of Mg-ATP from a freshly prepared 100 mM stock, and performed in the presence of 300 mM urea with 10 mM DTT. BSA was present at 0.5% in a final concentration, and the reactions were performed in IB. The assay was started by adding chloroplasts to the assembled import mixture and was incubated at room temperature for 15 minutes with occasional mixing to keep the chloroplasts suspended. The reaction was stopped with the addition of 700 µL of

ice-cold IB, and the tubes were placed on ice. Intact chloroplasts were re-isolated by layering the reactions on top of 700 µl of 40% percoll and centrifugation at 3,500 g for 5 minutes. The supernatant is removed and the chloroplasts are gently resuspended with 1 ml of ice-cold IB, 50 µl is saved for quantitation by BCA and the rest is centrifuged at 12,000 g for 1 minute. The pellet is resuspended in 100 µl of 2 x SSB and boiled for 3 minutes. Samples were brought to equal protein concentration by adding SSB; the volume added was determined by the BCA results. Samples were run at 6 mA per gel on 10-20% gradient Tris-glycine gels until the chlorophyll band is roughly 3 cm from the bottom of the gel (usually 12-15 hours). Gels were dried on a gel-dryer and placed on storage Phosphor screens (GE Healthcare, UK) for around 30 hours. Visualization was accomplished by scanning the screens with the Storm PhosphorImager (GE Healthcare, UK) and quantification of band intensity was performed using ImageQuant analysis software (GE Healthcare, UK).

Chapter 3 Results

3.1 Introduction

With the vast majority of chloroplast proteins coded for in the nucleus (Abdullah et al. 2000; Martin et al. 2002) and cytosolically translated, the chloroplast is faced with protein trafficking problem. This logistics problem is solved via N-terminal transit peptide sequences present on precursor proteins destined for the chloroplast (Chua and Schmidt 1978), and two sets of translocation machinery at the outer and inner envelopes of the chloroplast membrane (TOC and TIC respectively) (Blobel 1980; Cline 2000). The TOC complex is responsible for transport of precursor proteins through the outer membrane of the chloroplast (Hirsch et al. 1994; Schnell et al. 1994). The core TOC complex is comprised of Toc159, Toc34, and Toc75 (Bolter et al. 1998; Schnell et al. 1994; Seedorf et al. 1995). Toc159 and Toc34 are GTPases and the initial receptors for the precursor protein. The most prevalent protein in the outer envelope of the chloroplast is Toc75. Mature Toc75 has an C-terminal pore domain through which the precursor proteins are transported (Hinnah et al. 1997; Schnell et al. 1994) and an N-terminal domain consisting of three N-terminal POTRA domain repeats (Sanchez-Pulido et al. 2003), which are thought to help with either TOC core assembly or precursor protein import (Ertel et al. 2005).

POTRA domains are present in the Omp85/TPS superfamily, and have been shown to be involved in the assembly of β-barrel proteins in the outer membranes or the translocation of proteins through outer membranes of mitochondria, chloroplasts and gram-negative bacteria (Delattre et al. 2011; Gentle et al. 2004; Gentle et al. 2005;

Knowles et al. 2009; Moslavac et al. 2005; Paschen et al. 2003; Reumann et al. 1999; Sanchez-Pulido et al. 2003; Voulhoux et al. 2003). All members of the Omp85/TPS superfamily possess between one and seven POTRA domains (Arnold et al. 2010; Sanchez-Pulido et al. 2003). POTRA domains have been shown to specifically interact with parts of substrate proteins that interact with the Omp85/TPS that they belong to (Clantin et al. 2007; Hodak et al. 2006). The secondary sequence of POTRA domains was both predicted (Sanchez-Pulido et al. 2003) and shown by crystallization and/or NMR of POTRA domains of BamA (Kim et al. 2007), FhaC (Clantin et al. 2007), FtsQ (van den Ent et al. 2008), *Thermosynechococcus elongatus* Omp85 (*Te*Omp85) (Arnold et al. 2010), and *Anabaena* Omp85 (*Ana*Omp85) (Koenig et al. 2010) to have a conserved structure, which was experimentally determined to have the consensus β-α-α-β-β. While certain POTRA domains are essential for cell viability, e.g. POTRA5 of Omp85 in *Neisseria meningitidis* (Bos et al. 2007), deletion of any of them show a decrease in function of the protein machinery they are part of or a decrease in cell viability (Bos et al. 2007; Bredemeier et al. 2007; Clantin et al. 2007; Ertel et al. 2005; Habib et al. 2007; Kim et al. 2007).

3.2 Generation of POTRA2-His and POTRA1-3-His

The first step towards in vitro testing of homotypic and heterotypic interactions of POTRA domains is to produce POTRA domains recombinantly. Toc75, and more specifically POTRA2 and POTRA3 were modeled after POTRA1 and POTRA2 of the TpsB exporter of FhaC (Clantin et al. 2004), and POTRA1 was predicted by sequence alignment (Dave, A. 2010). POTRA1, POTRA2, POTRA3, and POTRA1-3 (POTRA1,

POTRA2, and POTRA3 all expressed as one peptide) had already been cloned and inserted into pTYB2 by Ashita Dave in our lab (Dave, A. 2010). The DNA sequence for POTRA2 incorporated many rare codons, and a synthetic gene was synthesized to allow codon-optimization for *E. coli* expression by Ashita Dave (Dave, A. 2010).

Our modeling suggest that Toc75 POTRA2 is much larger than any other POTRA domains (Figure 1.5). The initial POTRA domain predictions done in our lab (Dave, A. 2010) included a twenty-two-residue linker between POTRA1 and POTRA2. In analyzing all structurally solved POTRA domains, typically the linker between adjacent POTRA domains was between two and four amino acids long, and almost always included a proline or a glycine. This analysis of conserved linkers led to the amendment of our POTRA domain model and the majority of the initial, yet improperly assigned, linker residues were included in POTRA2. Figure 3.1 shows a schematic representation of the different POTRA domains. DNA coding for POTRA2 and POTRA1-3 was amplified via PCR, cut with NdeI and XhoI, and ligated into identically cut pET-30(a).

Colony PCR screens confirmed POTRA1-3 (Figure 3.2) and POTRA2 (not shown) were successfully inserted into pET-30(a). PsT75(P2)PET30 and PsT75(P1-3)PET30 were transformed into competent *E.coli* BL21(DE3) cells, with protein expression induced with IPTG. POTRA1-3-His is almost totally insoluble, but can been purified and refolded from inclusion bodies.

Production of POTRA2 and POTRA1-3 proved very difficult using the IMPACT system. POTRA2 could not be expressed at a detectable level (Figure 3.3). POTRA1-3 could be expressed at a very low level (Figure 3.4), but was insoluble in all conditions

A

B

LLGRLSGFKKYKISDILFFDRNKKSKVETQDSFLDMVSLKPGGVYTKAQLQ
KELESLATCGMFEKVDMEGKTNADGSLGLTISFAESMWERADRFRCINVGL
MGQSKPVEMDPDMSEKEKIEFFRRQEREYKRRISSARPCLLPTSVHEEIKD
MLAEQGRVSARLLQKIRDRVQSWYHEEGYACAQVVNFGNLNTREVVCEVVE
GDITKLSIQYLDKLGNVVEGNTEGPVVQRELPKQLLPGHTFNIEAGKQALR
NINSLALFSNIEVNPRPDEMNEGSIIVEIKLKELEQKSAEVSTEWSIVPGR
GGRPTLASLQPGGTITFEHRNLQGLNRSLTGSVTTSNFLNPQDDLAFKMEY
AHPYLDGVDNPRNRTLRVSCFNSRKLSPVFTGGPGVDEVPSIWVDRAGVKA
NITENFSRQSKFTYGLVMEEIITRDESNHICSNGQRVLPNGAISADGPPTT
LSGTGIDRMAFLQANITRDNTRFVNGTIVGSRNMFQVDQGLGVGSNFPFFN
RHQLTVTKFLQLMSVEEGAGKSPPPVLVLHGHYGGCVGDLPSYDAFTLGGP
YSVRGYNMGEIGAARNILELAAEIRIPIKGTHVYAFAEHGTDLGSSKDVKG
NPTVVYRRMGQGSSYGAGMKLGLVRAEYAVDHNSGTGAVFFRFGERF

Figure 3.1 POTRA Domains and Colony PCR of POTRA1-3

A) Mature *Ps*Toc75 schematic with POTRA and the β-barrel are represented and labeled with their size in residues. B) The mature sequence is listed below the cartoon POTRA1-2 is 204, POTRA2-3 is 201, and POTRA1-3 is 288 residues. B2IMS is part of the beta barrel and is underlined, and is 70 residues.

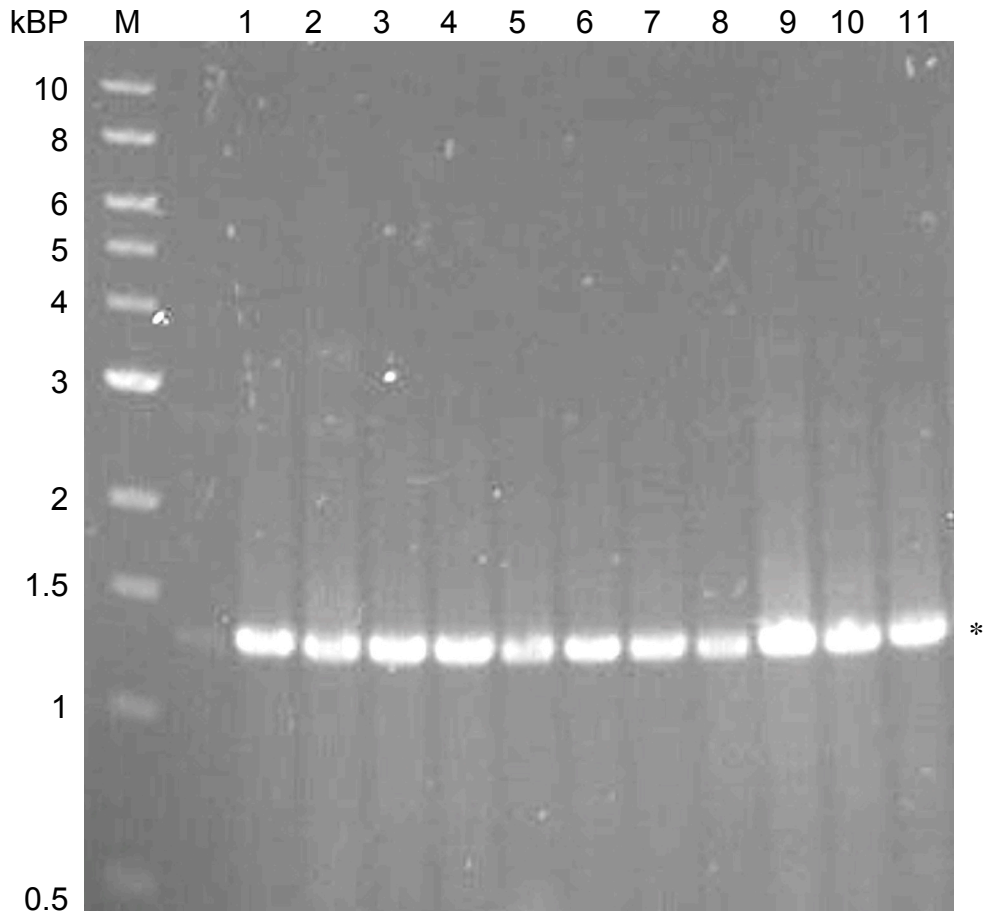


Figure 3.2 Colony PCR of POTRA1-3

Colony PCR confirmation of eleven positively inserted POTRA1-3 colonies into the pET-30(a) vector. Lane M is a 1kb marker, and lanes 1-11 are colonies screened for the insert (∗). The expected size of the insert is 1084 bp.

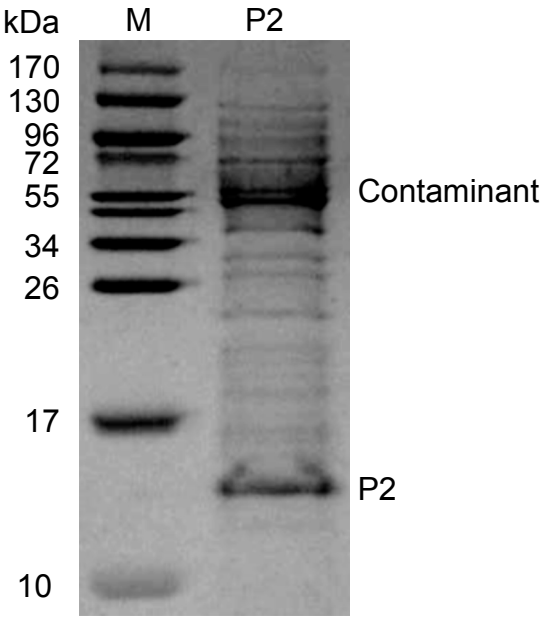


Figure 3.3 POTRA2-pTYB2 Production Problems

Lane M is EZ-run pre-stained standards. Lane P2 is lysed *E.coli* cells expressing POTRA2-pTYB2. POTRA2 is a minority species of the proteins produced by the cells.

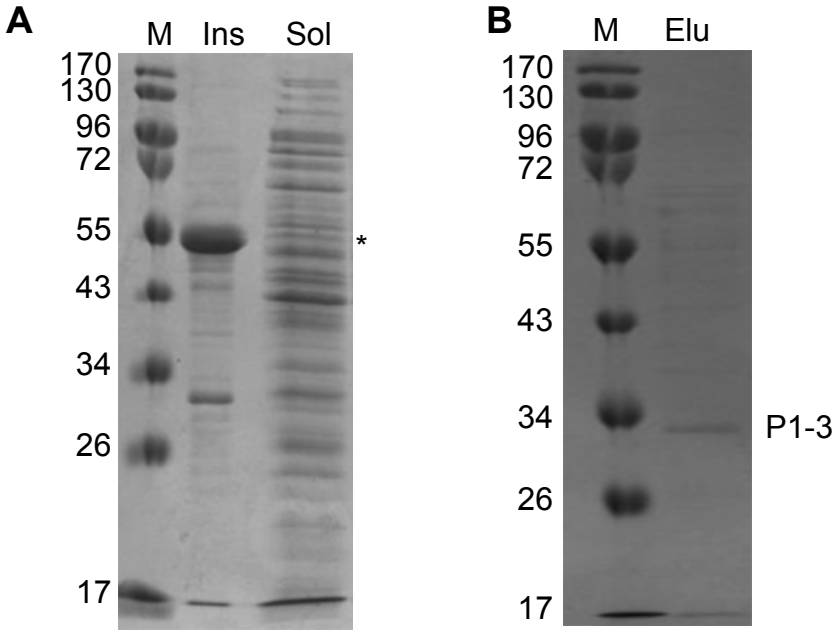


Figure 3.4 POTRA1-3-pTYB2 Purification Failure

Gel A shows insoluble (Ins) and soluble (Sol) fractions of lysed *E. coli* expressing POTRA1-3-pTYB2. Gel B shows the elutions (Elu) of the soluble fraction of gel A. M is EZ-run prestained standards. The P1-3 fusion is represented by the *, and is eluted in gel B.

(varied cell lines: BL21(DE3), BL21(DE3-RIL), ER2566, C41, C43; growth temperature: 10, 15, 20, 25, 30, 37ºC ; [IPTG]: 0.1, 0.2, 0.5, 1.0 mM; growth time: 3, 4, 5, 6, 8, 12, 16 H; media: LB, TB, SV). The insoluble POTRA1-3-pTYB2 could not be refolded before cleavage, and cleavage would not work with unfolded protein. A denatured fusion protein would have neither a chitin-binding domain, which is a conformation dependent interaction, nor an intein domain capable of self-splicing. This led to a decision to change to a new expression system, pET-30(a), a C-terminal 6x-His tag system, in an attempt to make usable amounts of POTRA2 and POTRA1-3. This system may allow refolding of the POTRA domains with less interference of the fusion tag (the intein and chitin tag are 55 kDa, and the 6xHis tag is 1kDa).

3.3 Production of Purified, Folded POTRA Domains

After purification either by Intein cleavage (POTRA1, POTRA3, Figure 3.5) or 6x-His tag (POTRA2, POTRA1-3, Figure 3.6) purified POTRA domains (Figure 3.7 A) were analyzed for intact secondary structure by CD (Figure 3.7 B). Deconvolution of CD spectra showed that all recombinant Toc75 POTRA domains possessed secondary structure, and that most were comparable to structurally solved POTRA domains (Figure 3.8). It is interesting to note that the percentage of alpha helix of Toc75 POTRA1, POTRA3, and POTRA1-3 is lower than the averaged solved POTRA domains and the random coil is higher. The higher ordered structure in solved POTRA domains could be a side effect of crystallization, or just structural differences between plant and bacterial POTRA domains. Initial CD suggests that POTRA2 does have a secondary structure,

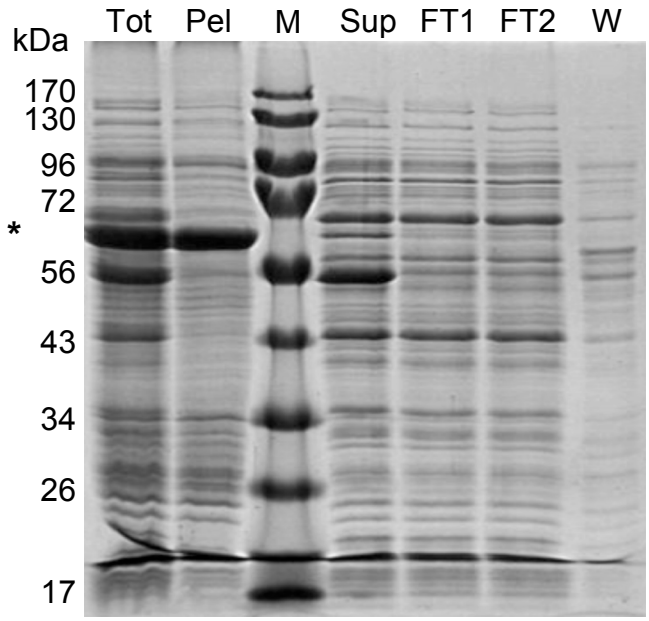


Figure 3.5 POTRA3-pTYB2 Purification Profile

Total lysate (Tot), insoluble fraction (Pel), soluble fraction (Sup), flow-through 1 and 2 (FT1, FT2), and wash (W) of a pOTRA3-pTYB2 prep are run on a 15% tris-glycine SDS-PAGE. M is EZ-run pre-stained marker. The P3-fusion protein is represented by the species with the molecular weight at *.

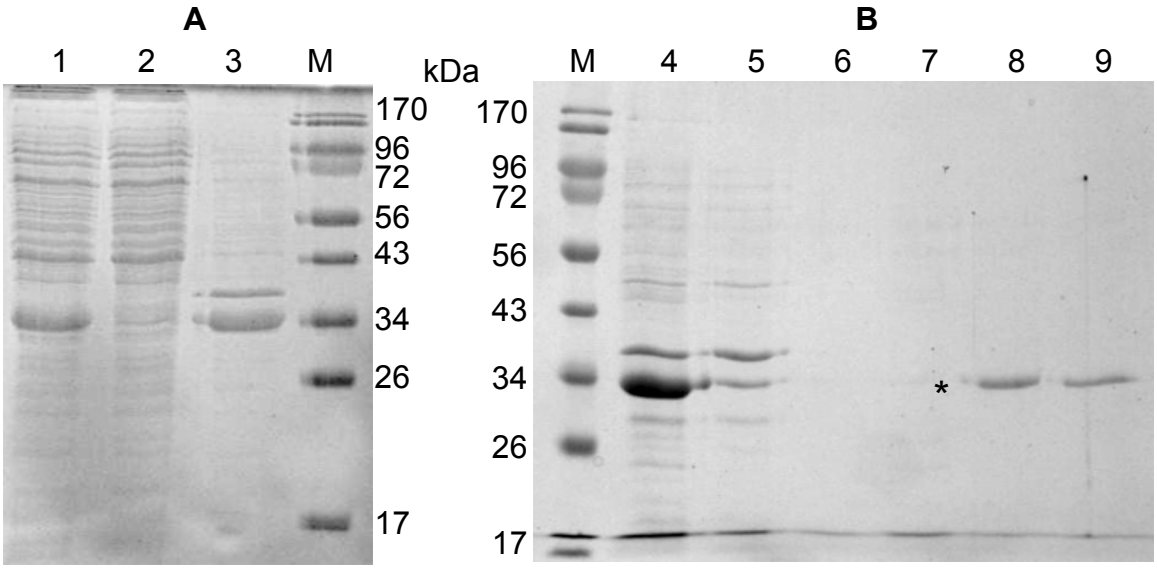


Figure 3.6 POTRA1-3 Purification Profile

Lane 1 is total lysate, lane 2 is supernatant, lane 3 is pellet. M lanes are EZ-run pre-stained standard. Lane 4 is the supernatant after dialysis, lane 5 is the flow through of a his-pure nickel-NTA column, lane 6 is column wash with 0 mM imidazole, lane 7 is column wash with 25 mM imidazole, lane 8 is column wash with 100 mM imidazole, and lane 9 is column wash with 250 mM imidazole. POTRA1-3 is represented by band with the same molecular weight as the *.

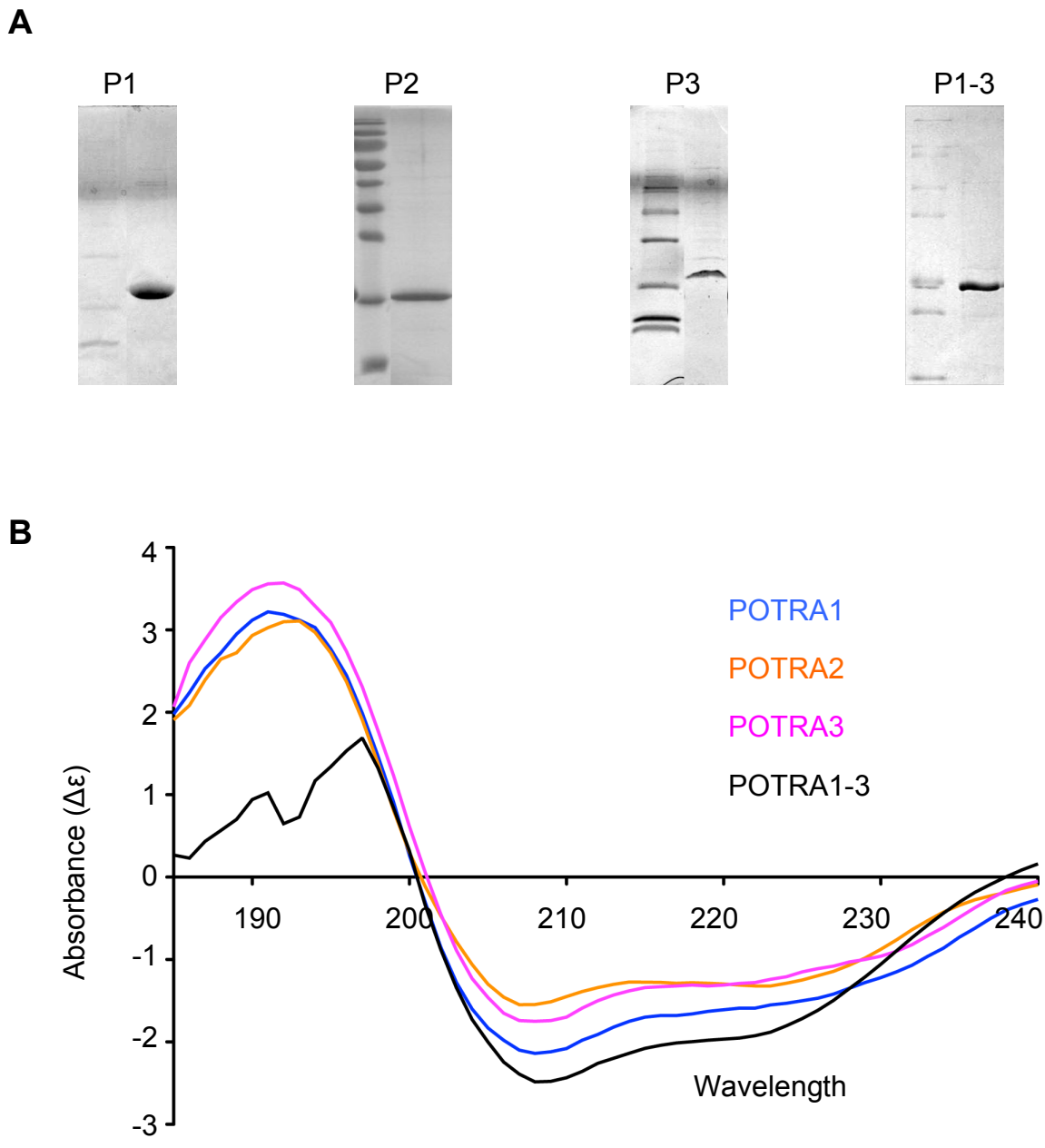


Figure 3.7 SDS-PAGE Confirmation of POTRA Domain Purity and CD Spectra of POTRA Domains

A) SDS-PAGE was performed with purified fractions of POTRA1, POTRA2, POTRA3, and POTRA1-3. B) CD Spectra for individual POTRA domains and POTRA1-3.

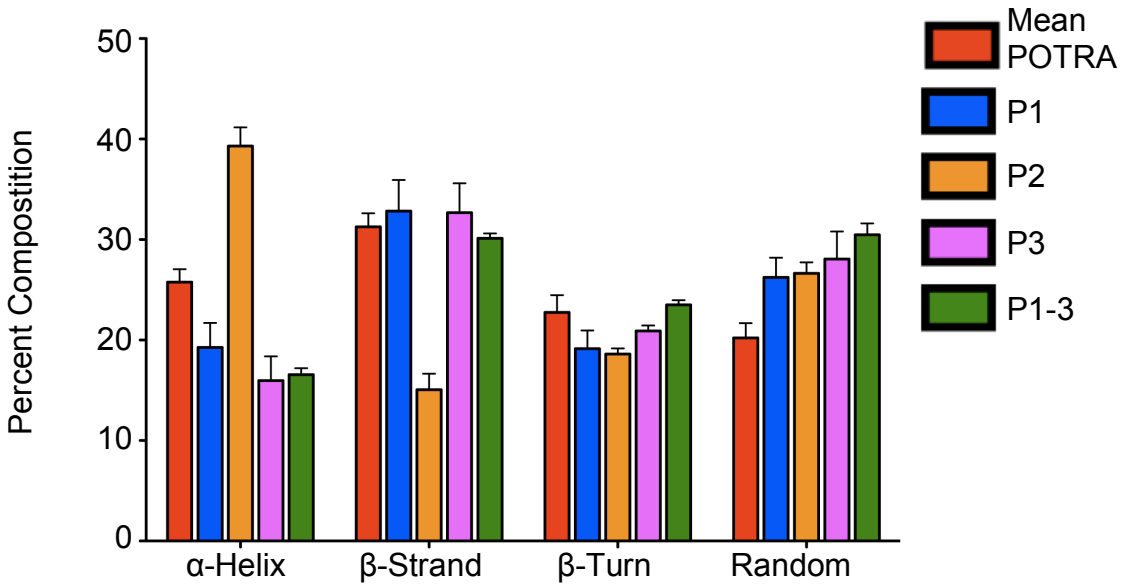


Figure 3.8 Secondary Structure of POTRA Domains

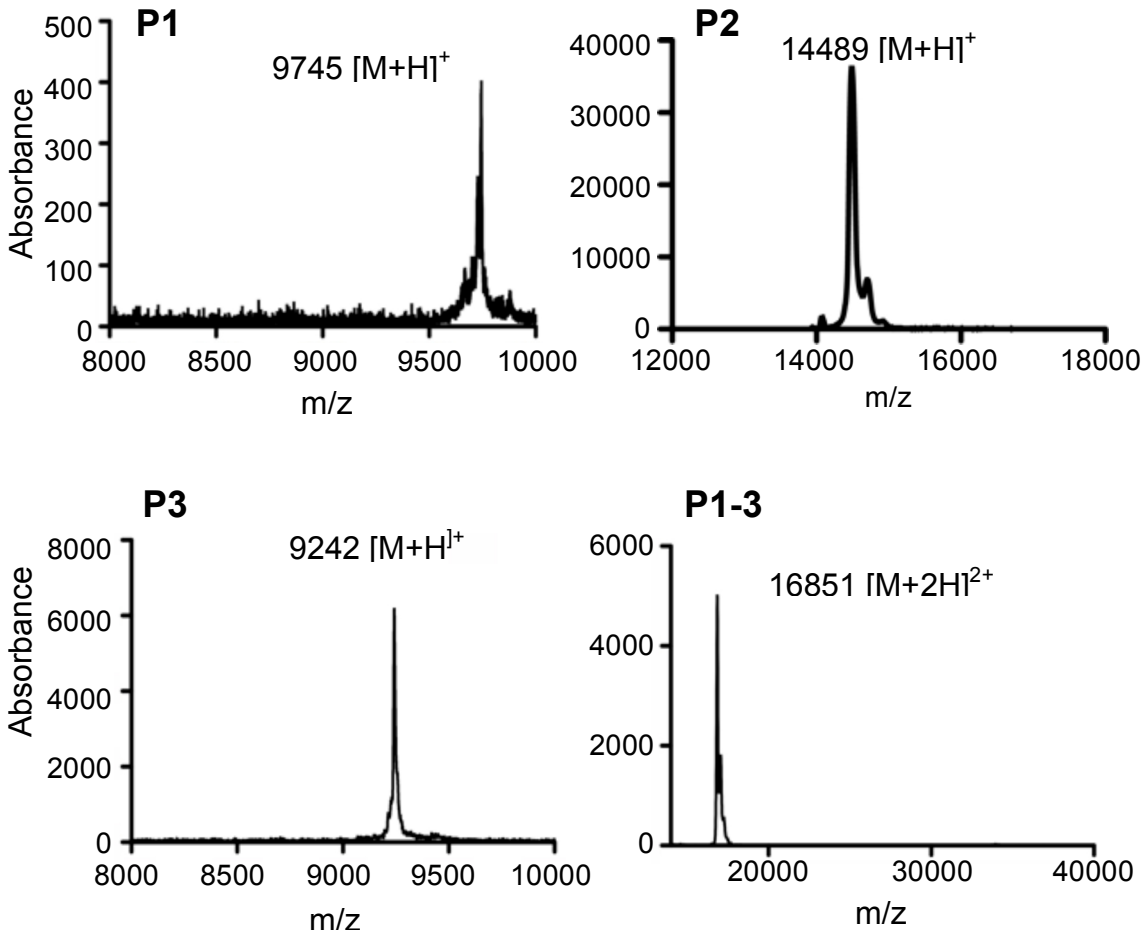
The results for all individual POTRA domains as well as POTRA1-3 are compared to the average secondary composition of sixteen structurally solved POTRA domains.

although it differs greatly from the other Toc75 and other structurally solved POTRA domains, with twice as much alpha-helix and half as much beta-strand as the other POTRA domains. This could be due to the large size of POTRA2, which is 45% larger than the other two POTRA domains.

The purified recombinant POTRA domains were analyzed by MALDI-TOF to confirm their predicted molecular weights (Figure 3.9) of 9667, 14502, 9252, and 33755 Da; for POTRA1, POTRA2, POTRA3, and POTRA1-3. The observed weights of 9745, 14489, 9242, 33702 Da (2 x 16851) corresponded closely to the predicted weights. POTRA1 was 78 Da larger than predicted, which can be explained by an addition of beta-mercaptoethanol (76 Da), a covalent modification common with βME induced fusion cleavage. The predicted and measured masses of POTRA2 and POTRA3 correspond well. The plus-two species of POTRA1-3 is 26.5 Da smaller than predicted mass of the plus-two species, and this error is less that 0.2%

3.4 Oligomerization of POTRA Domains

Because the TOC core complex is a hetero-oligomer (Figure 1.3) of Toc75, Toc34, and Toc159 it is possible that the Toc75 subunits interact with each other to form the complex. A goal of this work is to determine if the POTRA domains, alone, have any ability to oligomerize *in vitro* with itself or with transit peptide. To ascertain whether or not POTRA domains have this ability chemical crosslinking, analytical ultracentrifugation, and pull-down assays are employed.



POTRA Domain	Predicted Mass(Da)	Measured Mass(Da)	Difference
POTRA1	9667	9745	78 Da, 0.81%
POTRA2	14502	14489	13 Da, 0.09%
POTRA3	9252	9242	10 Da, 0.11%
POTRA1-3	33755	33702	53 Da, 0.16%

Figure 3.9 MALDI-TOF Spectra for POTRA Domains

Shown are MALDI-TOF analyses of POTRA1, POTRA2, POTRA3, and POTRA1-3.

The first experiment performed to check *Ps*Toc75 POTRA domain interactions was chemical crosslinking. Glutaraldehyde is a homo-bifunctional, amine-amine, cross-linker whose reaction with proteins is essentially irreversible (Richards and Knowles 1968). The glutaraldehyde cross-linking reaction is non-specific with respect to amines used, but it cross-links peptides that interact with each other at a much higher rate than non-reactive peptides (Richards and Knowles 1968). POTRA1 and POTRA3 were tested for cross-linking alone with glutaraldehyde to check their ability to homo-oligomerize (Figure 3.10). As can be seen from Figure 3.10A, POTRA1 forms homo-oligomers much more readily than POTRA3. In fact, species of homo-dimers (D), trimers (Tri), and tetramers (Tet) are present in the POTRA1-glutaraldehyde reaction (Figure 3.9, P1+), while only a faint homo-dimer (D) is detected in the POTRA3-glutaraldehyde reaction (P3+). The monomer (M) of POTRA1 seems to cross-link to itself more than POTRA3 does as well. The S species is the cleaved fusion protein chitin-binding domain and intein-tag together that seems to cross-link to itself or to other POTRA domains. There is a high level cross-linked oligomer present in P1+ that may be contaminant cross-linked to POTRA1 or a very large POTRA1 oligomer. This experiment has shown that POTRA1 homo-oligomerizes *in vitro* much more easily than POTRA3 does, meaning that it could have a unique role among the Toc75 POTRA domains.

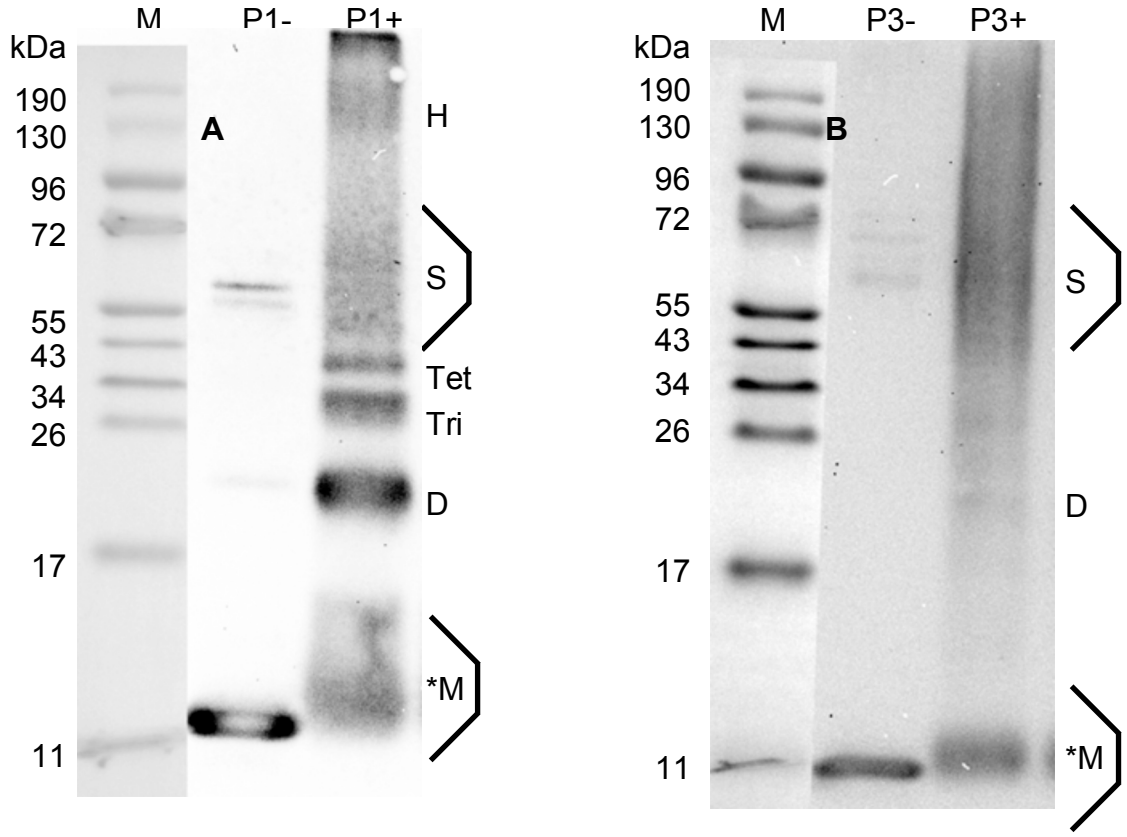


Figure 3.10 Western Blot Analysis of Glutaraldehyde (Ga) Cross-linked POTRA domains

POTRA1 forms discrete homo-oligomers in the presence of Ga (P1+), while POTRA3 does not homo-oligomerize to the same extent (P3+) or in discrete units. A dimer (D), trimer (Tri), and tetramer (Tet) are very clear, with the dimer being the most present species in the crosslinked POTRA1 (P1+). All three classes are much less represented in the crosslinked POTRA3 (P3+), with the trimer and tetramer not even clearly visible and the monomer still the most present species. There is a shift in the monomer (M) of both P1+ and P3+, where the POTRA domain cross-linked to itself. There are also high molecular weight species (S) of intein and chitin binding domain of cleaved fusion protein P1- and P3- and cross-linked in P1+ and P3+. There is present a very high oligomer (H) in the P1+.

3.5 POTRA1 Transit Peptide Crosslinking

After observing the homo-oligomeric interactions of POTRA1 with chemical crosslinking, the same technique was employed to try and illuminate hetero-oligomeric interactions between POTRA1 and transit peptide. Because glutaraldehyde (GA) is a nonspecific cross-linker, it was used to try and elucidate POTRA domain interactions with transit peptide. POTRA1 (Figure 3.11) or POTRA3 (Figure 3.12) were cross-linked alone, or in the presence of SStp*Ps*. POTRA1 is present in all monomeric form at 5 μM without glutaraldehyde present, and it forms homo-oligomers at 5 and 10 μM when cross-linked with glutaraldehyde. A pentamer is detected with the 10 μM POTRA1 + GA alone (lane 7, Pent). However, upon addition of TP, the ordered oligomers are disrupted, and a large smear is present (lanes 5, 6, and 10). At molar ratios of 5:1, a hetero-dimer of POTRA1-SStpPs (lanes 5 and 6, HD) can be detected. TP forms a large oligomer (lane 3, ^) when cross-linked alone, but this species disappears when the concentration of POTRA1 increases. These new interactions could be due to the higher concentration of TP outcompeting POTRA1 self-oligomerization. This experiment has shown that POTRA1 hetero-oligomerizes with transit peptide in vitro, and that this interaction can interfere with the ability of POTRA1 to homo-oligomerize. This could mean a role for substrate interaction for POTRA1.

3.6 POTRA3 Transit Peptide Crosslinking

Although POTRA3 did not homo-oligomerize well, chemical cross-linking was employed to determine whether or not POTRA3 could hetero-oligomerize with transit

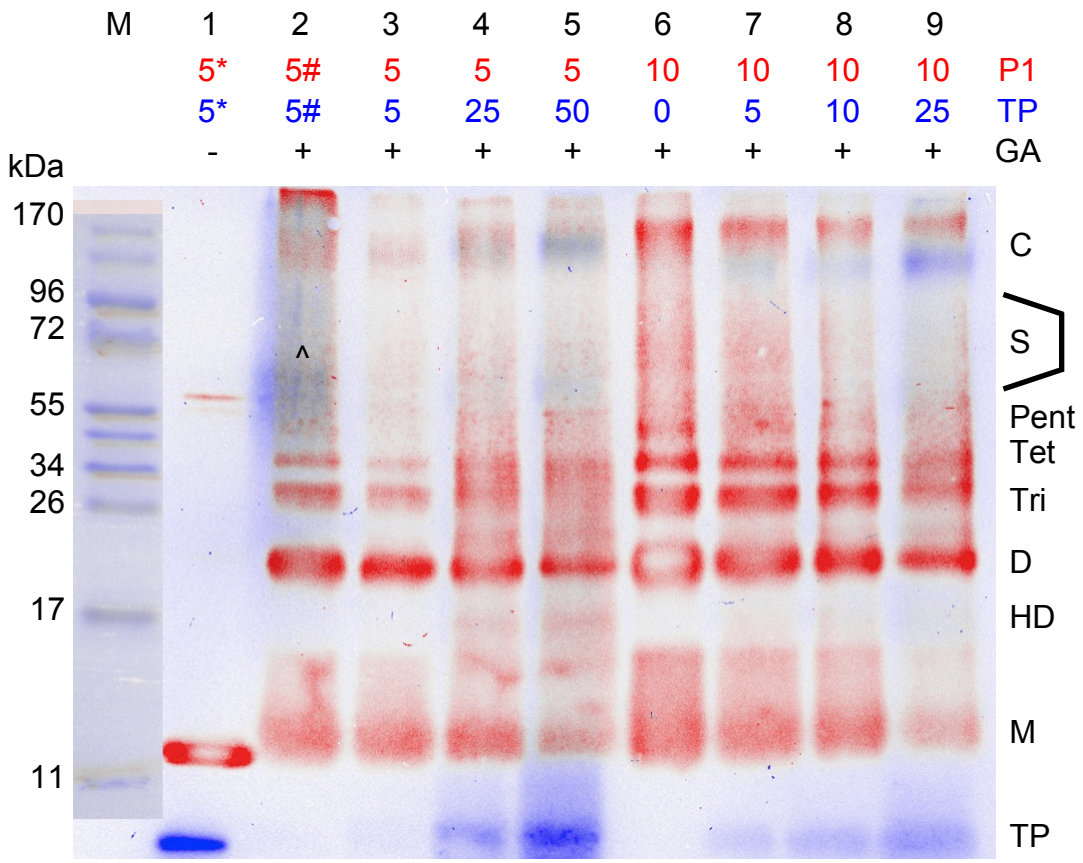


Figure 3.11 Western-Blot Analysis of POTRA1-SStp*Ps* Gluteraldehyde Crosslinking

POTRA1 (red) and SStp*Ps* (TP, blue) both form a monomer (M) alone without Ga (*). P1 forms dimers (D), trimers (Tri), tetramers (Tet), and pentamers (Pent) when cross-linked alone at 5 µM (#) and at 10 µM. TP forms a large oligomer at around 55 kDa (^) when cross-linked alone. S is a large oligomeric smear that seems to have P1 and TP present in it, and C is a large complex consisting of P1 and TP.

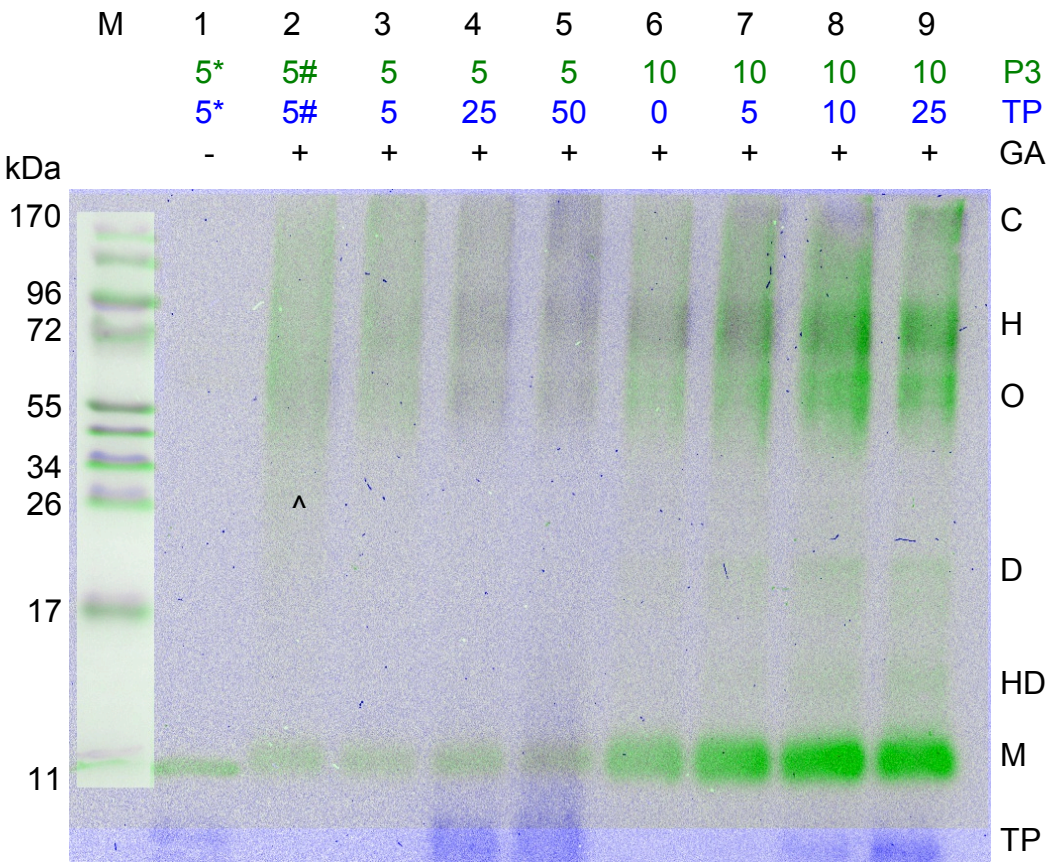


Figure 3.12 Western-Blot Analysis of POTRA3-SStpPs Gluteraldehyde Crosslinking

POTRA3 (green) and SStp*Ps* (TP, blue) both form a monomer (M) alone without Ga (*). P3 aggregates into a large oligomeric smear (O-C) when cross-linked alone (#). P3 forms homo-dimers (D) at 10 µM, in the presence of TP. TP forms a large oligomer at around 55 kDa (^) when cross-linked alone (#). HD is a hetero-dimer of P3 and TP. O, H, and C are large oligomeric complexes that consist of P3 and TP.

peptide. POTRA3 was also cross-linked with GA alone and with TP (Fig 3.13). At 5 μM without GA POTRA3 is all monomer (lane 2), and when GA is added large oligomers (lane 3, O, H) are formed, and the monomer is still present. The large oligomers O and H may be un-cleaved and cleaved (respectively) fusion POTRA3, whatever it is it is recognized by antibodies against TP even when there is no TP in the reaction (lane 7, O, H) Addition of TP does not interfere with the species of POTRA3 present when POTRA3 is at 5 μM (lanes 3-5). It seems that at higher concentrations of POTRA3 10 μM, and TP 10 or 25 μM a homo-dimer of POTRA3 (lanes 9, 10; D) and a hetero-dimer of POTRA3-SStp*Ps* (lanes 9, 10; HD) are formed. This is completely opposite of the effect that TP had on POTRA1 and TP seems to promote homo-dimerization of POTRA3 at higher concentrations. This experiment shows that POTRA3 can interact with transit peptide *in vitro* and that perhaps the role of the POTRA3 domain in plant Toc75 has something to do with substrate interaction.

3.7 POTRA1 Transit Peptide Positional Specific Crosslinking

Because gluteraldehyde can interact with itself forming polymers during the crosslinking process, positional information cannot be determined by this cross-linking method. Bismaleimidohexane (BMH) does not form polymers, and its linker-arm length is set at 13 Å, this means that peptides will only be cross-linked if their reactive cysteines are within 13 angstroms of each other. Both POTRA1 and SStp*Ps* have one cysteine (54C and 57C, respectively) and can be subjected to thiol reactive homo-bifunctional cross-linker BMH. POTRA2 has four cysteines and POTRA3 has none, which makes both peptides poor substrates for BMH crosslinking. BMH has a linker length of ~ 16 Å

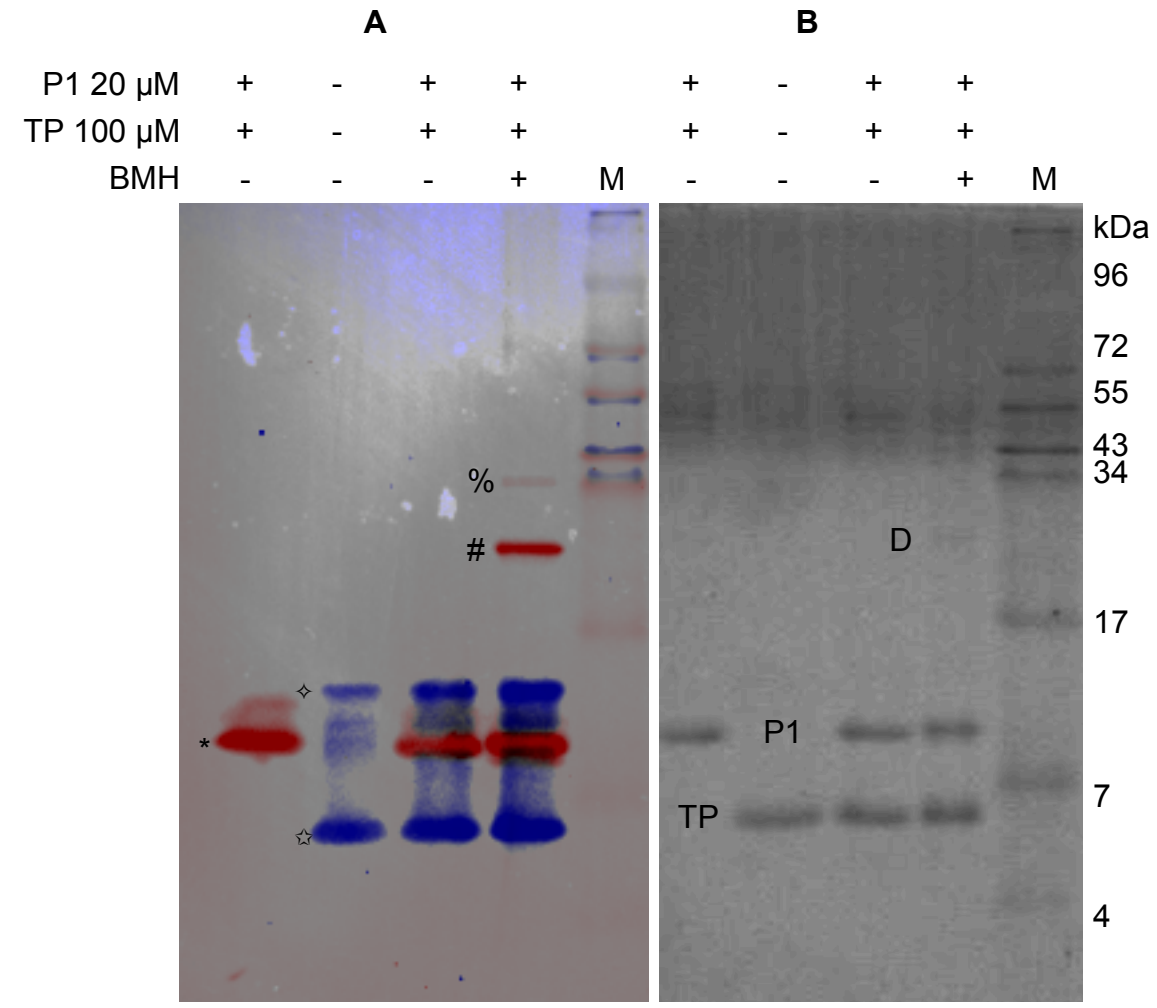


Figure 3.13 Western Blot and Silver Stain Analysis of BMH crosslinking of POTRA1 SStp*Ps*

A) Two identically-loaded and run western blots overlaid on top of each other. POTRA1 (red) is present as a monomer (*) alone (lane 1) and in the presence of five molar excess SStpPs without and with BMH (lane 3, lane 4). SStp*Ps* (blue) is present as a monomer (✩) and dimer (✧) alone (lane 2) and in the presence of POTRA1 (lane 3, lane 4). POTRA1 forms a homodimer (#) in the presence of BMH (lane 4). A unique species (%) is detected in lane 4, and is a possible trimer of POTRA1. There is no POTRA1 and SStp*Ps* heterodimer formed using BMH as a cross-linker. M is see-blue prestained marker. B) is an identically-loaded silver-stained tris-tricine gel. Monomers of POTRA1 (P1) and SStpPs (TP) are visible, as well as a faint POTRA1 dimer (D).

and can chemically cross-link peptides together via two sulfhydryl (-SH) groups, found in reduced cysteine residues. BMH is a more specific cross-linker than glutaraldehyde, and can give positional information on where peptides interact. In the cross-linking reactions performed (Figure 3.12) POTRA1 was all monomer without BMH present (*, Lane 1), while SStp*Ps* homo-dimerized without any BMH present (✧, Lane 2). This auto homo-dimerization of SStp*Ps* could be because the protein is present at a high concentration (100 μM), and SStp*Ps* has shown a tendency to form concentration dependent dimers (data not shown). Lane 3 showed that while the auto-dimer of SStp*Ps* is still present, there is no POTRA1-TP interaction without cross-linker. In lane 4 the addition of BMH yielded a POTRA1 homo-dimer (#), but no POTRA1-SStp*Ps* hetero-dimer. The unique species (%) is odd, the size of the species is that of a POTRA1 trimer, but each POTRA1 domain has only one cysteine to interact with the BMH. Perhaps a single POTRA1 domain is sandwiched between the cross-linked POTRA1 dimer. This experiment shows that the C-terminus of POTRA1 (modeled to be part of the β-strand) can contribute to POTRA1 homo-oligomerization.

3.8 Analytical Ultracentrifugation Supports POTRA1 Homo-Oligomerization

After trapping species of POTRA domains and transit peptide in various homo and hetero-oligomeric states with cross-linking the next step taken was to check whether the POTRA domains could form homo or hetero-oligomers with TP in solution conditions. Analytical ultracentrifugation (AUC) is a technique that investigates weak interacting partners by monitoring sedimentation of different species of oligomers present in the cell. AUC has previously been used in our lab to determine dimerization conditions

for *Ps*Toc34 (Reddick et al. 2007). Analytical ultracentrifugation was used to confirm the presence of POTRA1 homodimers (Figure 3.14 & 3.15) as well as POTRA3-SStp*Ps* heterodimers (Figure 3.16 & 3.17). POTRA1 had a higher sedimentation co-efficient contaminant (Figure 3.14) that was constant across all concentrations of POTRA1. The remaining signal was from either the monomeric or dimeric species of POTRA1. There was a concentration dependent dimerization of POTRA1 above 13 μM (Figure 3.14 and 3.15). The dimer species seemed to stay constant from 17 to 24 μM. This supports the idea that POTRA1 homo-oligomerizes in solution, and is not just trapped in chemically cross-linked oligomeric states.

3.9 Analytical Ultracentrifugation Supports POTRA3 Transit Peptide Interaction

AUC has been attempted on POTRA3 alone at various concentrations to see if an oligomer could be detected, but the results have been inconclusive and are not shown. A different AUC experiment was performed where the concentration of POTRA3 was held constant, and various concentrations of transit peptide were incubated in different cells. Ratios of POTRA3:SStp*Ps* of 1:0, 1:1, and 1:10 (Figure 3.16) were screened to check for concentration dependent hetero-dimerization. At the sedimentation coefficient of ~ 0.5, a TP monomer is present in the 1:1 and 1:10 ratios (Fig 3.16 black and blue lines). The POTRA3 monomer had a sedimentation coefficient of ~ 1.0 in the 1:0 and 1:1 ratios (Fig 3.16 red and black lines). When the molar ratio of P3:TP was 1:10, a hetero-dimer that settled at a sedimentation coefficient of ~ 1.5 was formed (Fig 3.16 blue line). All of the POTRA3 monomer has shifted to this heavier species when incubated with 10 molar excess SStp*Ps*. The percent composition of TP, P3, and P3:TP for each cell in the AUC

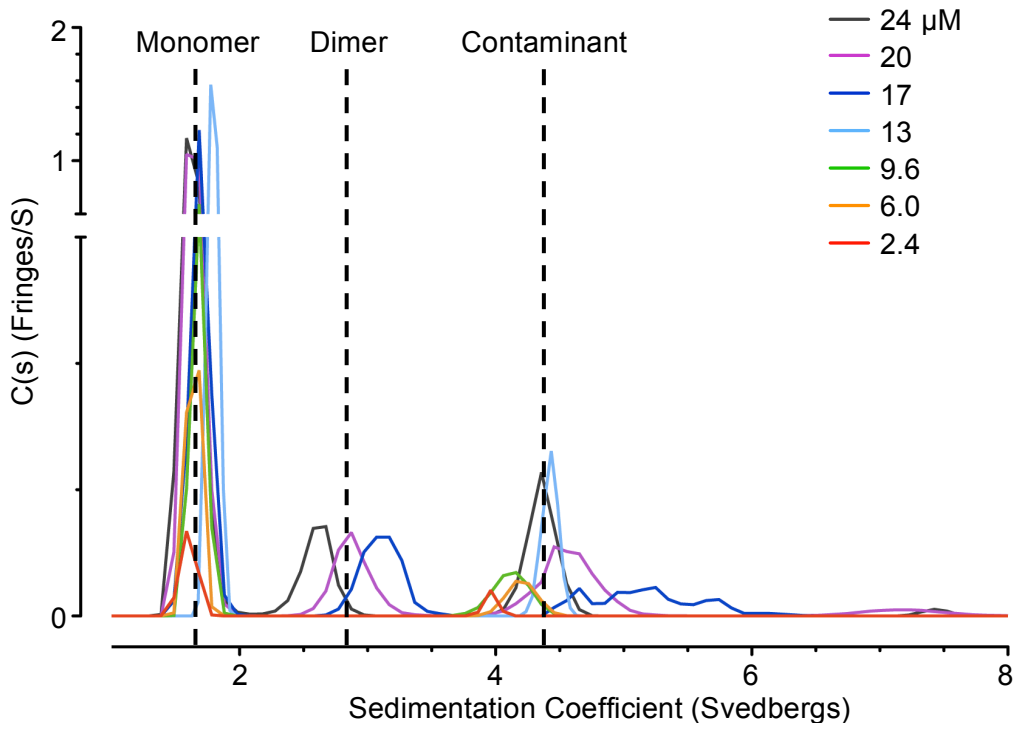


Figure 3.14 Homodimerization of POTRA1 Detected by AUC

Monomer and dimer species of POTRA1 are detected as well as a contaminant at different concentrations of POTRA1 in the AUC cells.

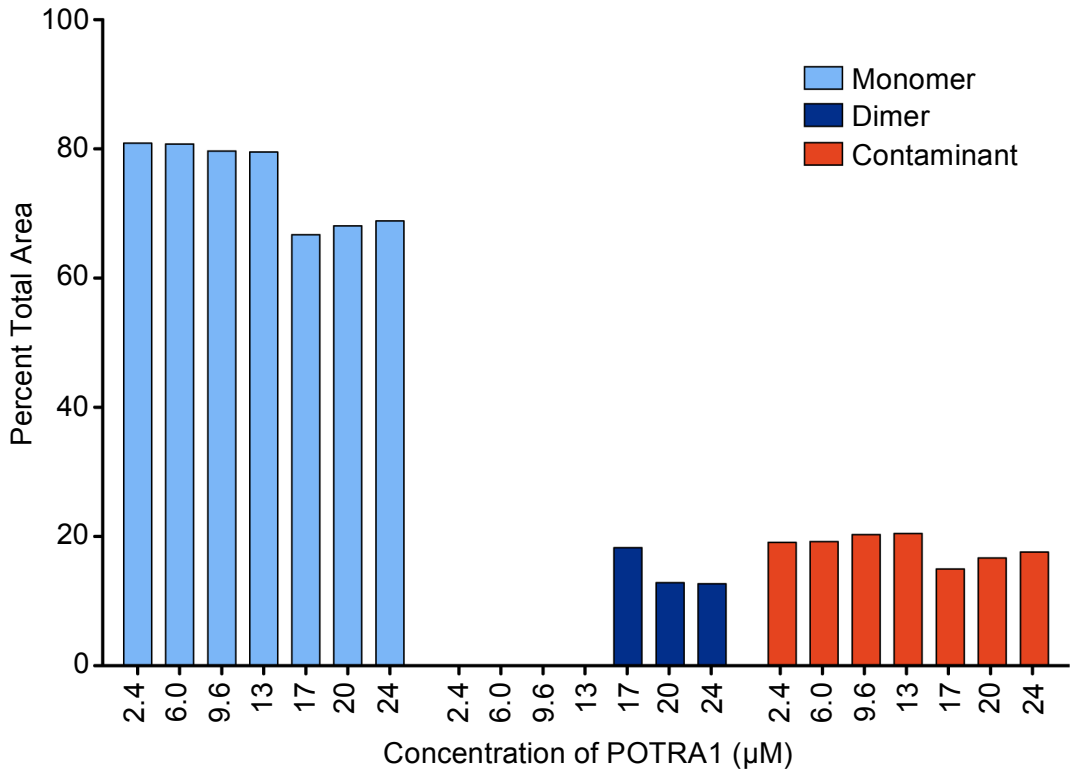


Figure 3.15 POTRA1 Homodimerization via AUC

Quantitation of the monomer and dimer species of POTRA1 detected by AUC. The contaminant is also present.

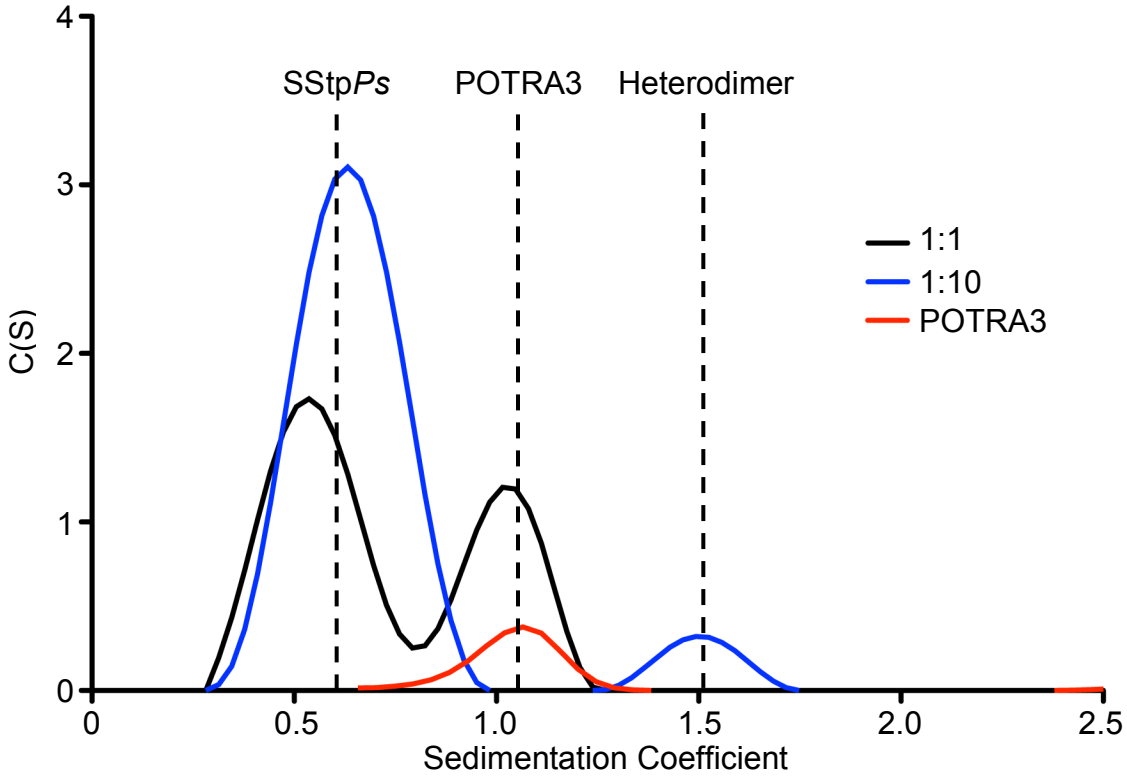


Figure 3.16 POTRA3-SStp*Ps* Heterodimer Supported by AUC

Monomer species of SStp*Ps* and POTRA3 were present in AUC cells with a 1:1 mixture of SStp*Ps* and POTRA3 (black line) and with POTRA3 alone (red line). A SStp*Ps*-POTRA3 hetero-dimer is present (blue line) at a ten fold molar excess of SStp*Ps*.

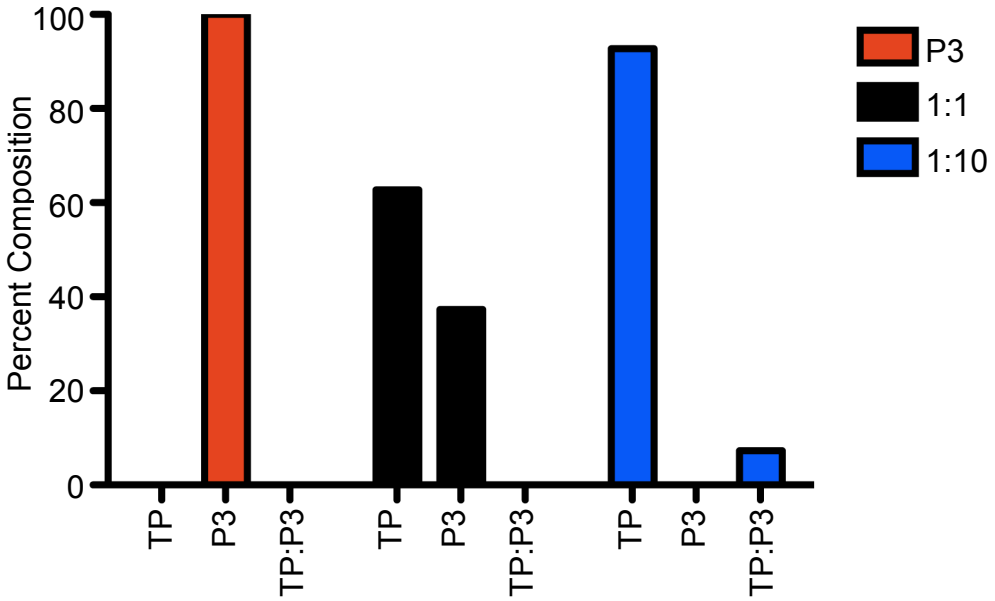


Figure 3.17 AUC of POTRA3-SStp*Ps* Yields Discrete Species

Area under the curve for each AUC cell was calculated, all species (SStpPs monomer, POTRA3 monomer, and SStpPs-POTRA3 heterodimer) contribution was also calculated.

was calculated (Fig 3.17), and supports the shift of POTRA3 to POTRA3:SStp*Ps* in the 1:10 ratio. This heterodimer supports the POTRA3-SStpPs interaction trapped by glutaraldehyde, and demonstrates that the heterodimer can occur in solution as well as being trapped with chemical crosslinking. AUC experiments with POTRA1 and SStp*Ps* yield no clear interactions (data not shown). This could be due to the fact that POTRA1 homo-dimerizes much more easily that POTRA3 as evidenced by crosslinking. It would be difficult to resolve hetero-dimer of P1:TP from homo-dimer P1 using AUC.

3.10 POTRA1 Pull-down Assay Supports Transit Peptide Interaction

With the limitation of AUC in mind a pull-down assay was designed (Figure 3.18) and employed to test the ability of POTRA1 to interact with transit peptide. A chitin column incubated with SStp*Ps*, and a chitin column bound with POTRA1-fusion were used as negative controls. The experiment took advantage of the chitin-binding domain present in the pTYB2 vector to bind POTRA1-fusion to chitin beads, creating a POTRA1 affinity column. The experiment also used the self-cleaving intein tag to elute the POTRA domain to ensure that the protein was interacting with the POTRA domain and not the intein tag or chitin-binding domain.

The experiment bound POTRA1-fusion to a chitin column (Figure 3.18, Flow-through), washed out the excess POTRA1-fusion (Figure 3.18, Wash), incubated the now POTRA1-column with SStp*Ps* (Figure 3.18, Binding), washed out the excess or non specifically interacting SStp*Ps* (Figure 3.18, Wash 2), then cleaved the POTRA1 overnight at 4ºC. The chitin column-SStp*Ps* negative control was done without the binding of POTRA1-fusion, and the chitin column-POTRA1 negative control skipped the

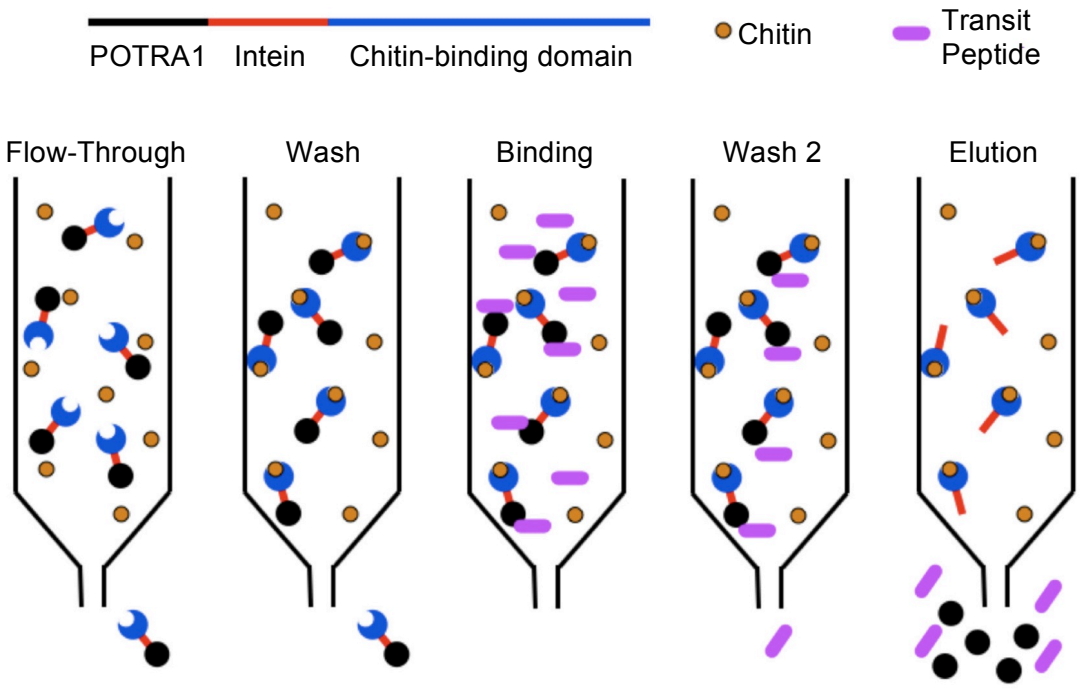


Figure 3.18 Pulldown Assay Scheme

Strategy employed for the pull-down assay. POTRA1 was expressed as an intein-chitin binding domain fusion protein. *E. coli* were lysed and centrifuged to clear the soluble proteins. The supernatant was then passed through a chitin column. The column was then washed with 20 bed volumes of 1xPBS pH 9. Transit peptide (SStp*Ps*) was then loaded onto the fusion protein-bound column and incubated at room temperature for two hours. Unbound SStp*Ps* was then washed out of the column with 5 bed volumes of buffer. The column was incubated with 1xPBS pH 9 containing 50 mM β-Me overnight and eluted. A column containing chitin beads but not the POTRA fusion protein was used as negative control.

incubation with SStp*Ps*. Elutions from each of the columns were taken after incubation with 50 μM β-Me overnight to induce intein self-cleavage (Figure 3.18, Elution). In the experimental column when POTRA1 was eluted SStp*Ps* co-eluted with it, as confirmed via Western blotting (Figure 3.19, lane 9, P1 and TP). The transit peptide was not present in either of the other columns used as negative control. This experiment supported the POTRA1-SStp*Ps* hetero-dimer formation that was demonstrated in the chemical crosslinking experiment. It showed that there is some specific interaction of POTRA1 with SStp*Ps* that allows interaction while on the column, and co-elution off of the column as well.

3.11 Synergism of POTRA1-3 in *in vitro* Chloroplast Protein Import

To see if all of the *in vitro* interactions detected so far could occur during actual protein import into the chloroplast, an assay was used to determine the effect of recombinant POTRA domains on chloroplast protein import *in vitro*. Purified, intact chloroplasts retain the ability to import proteins. An assay has been developed (Figure 3.20) that allows measurement of radiolabeled precursor protein either bound to or imported into the purified chloroplasts. Binding and import of precursor proteins into the chloroplast can be separated by varying energetic conditions (Figure 3.20 A). Fixed amounts of radio-labeled precursor to the small subunit to rubisco (^{35}S-prSSU) is incubated with a fixed amount of purified pea chloroplasts with various amounts of POTRA1-3 for 15 min at room temperature (Figure 3.20 B). The chloroplasts, now radiolabeled with imported mature protein or bound with radiolabeled precursor protein, are re-isolated, adjusted to the same protein concentration (Figure 3.20 C), run via SDS-

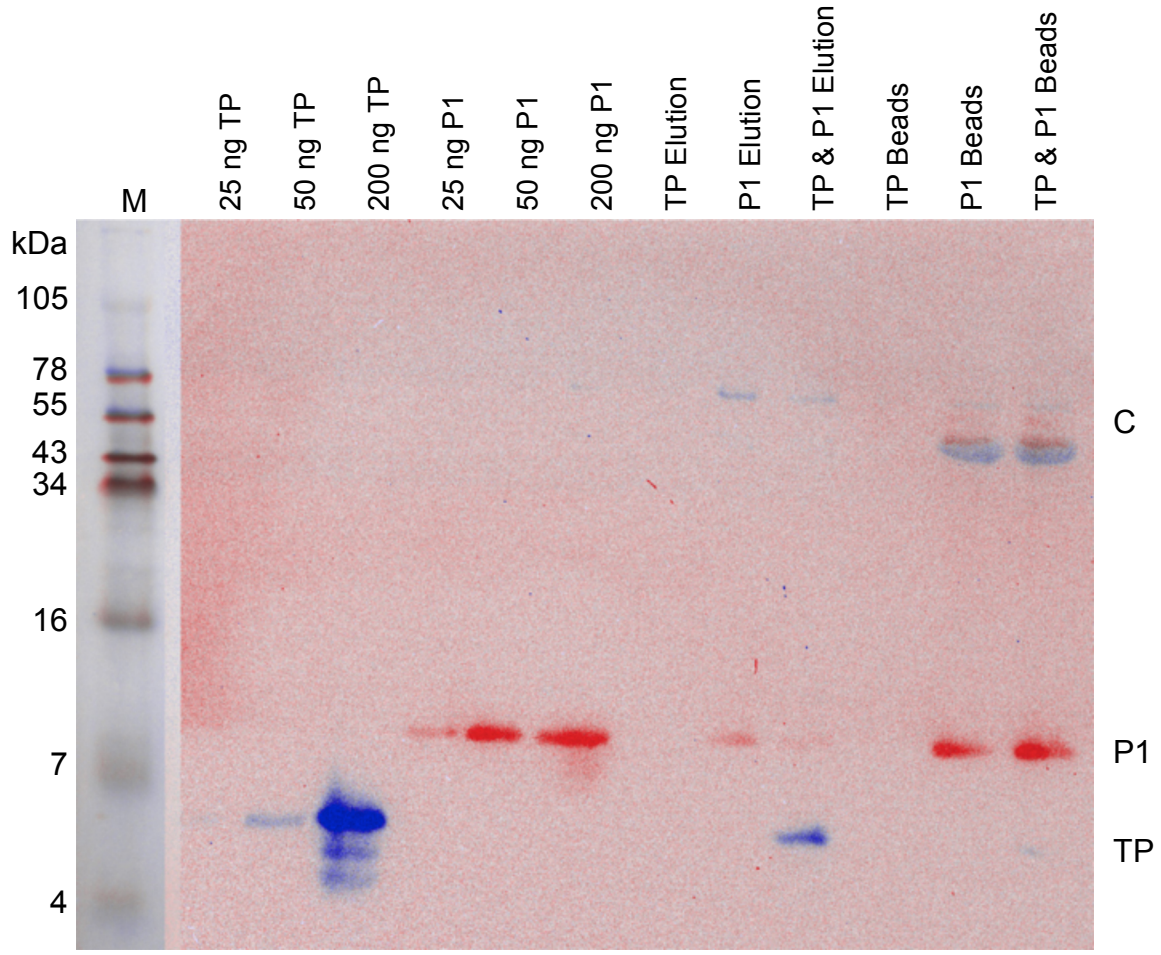


Figure 3.19 Pulldown Assay Elutions Western Blot

Increasing concentrations of SStpPs and POTRA1 were loaded in lanes 1-3 and 4-6. Overnight cleavages and subsequent elutions of columns from figure 3.13 were run and blotted in lanes 7-9. Samples of the chitin columns were run in lanes 10-12. C is cleaved intein and chitin binding domain.

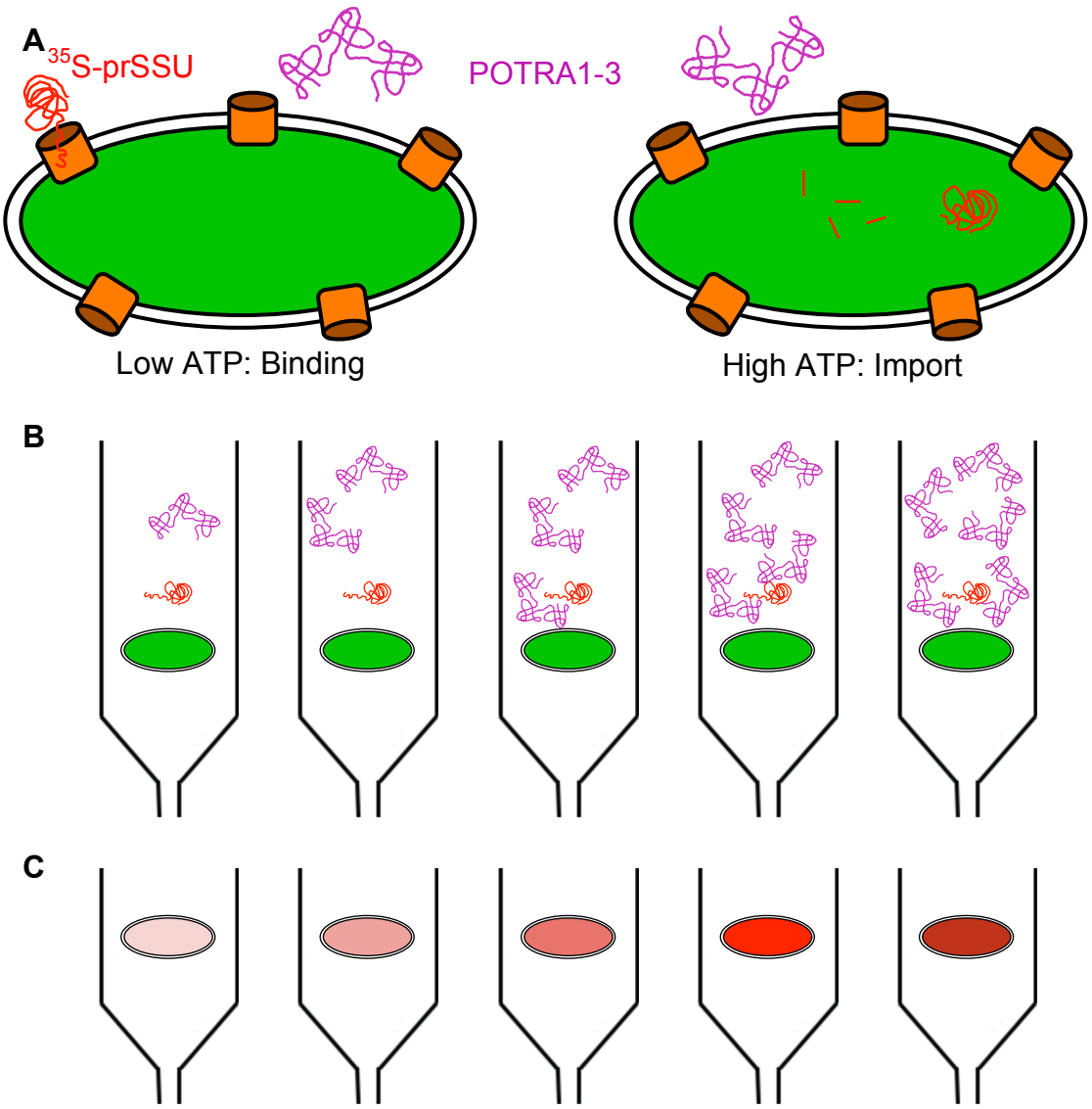


Figure 3.20 *In vitro* Import Competition Assays

Binding and import of precursor proteins into the chloroplast can be separated by varying energetic conditions (A). Fixed amounts of 35S-prSSU is incubated with a fixed amount of purified pea chloroplasts with various amounts of POTRA1-3 for 15 min at room temperature (B). The chloroplasts are then washed, re-isolated, adjusted to an equal protein concentration (C) and later run via SDS-PAGE and quantitated by autoradiography and/or scintillation counting.

PAGE (Figure 3.21 D), and quantitated by autoradiography. Effectors are mature small subunit of rubisco (mSSU) for negative control, it should not interact with the translocon because it does not have a transit peptide; prSSU which acts as a positive control, it should interact with the translocon and compete for import. Both the positive and negative controls compete with the radiolabeled prSSU for import through the general import pathway, the POTRA1-3 could interact with the radiolabeled prSSU in solution, preventing the prSSU from making its way into the chloroplast. The results are then graphed versus the amount of competitor for the bound (Figure 3.21 A), intermediate (Figure 3.21 B), and imported Figure 3.21 C) intensities for each competitor mSSU, prSSU, and POTRA1-3. These results showed that at all concentrations POTRA1-3 increased the amount of radiolabeled precursor bound, imported, and an intermediated species when compared against the negative control (Figure 3.21). This means that the recombinant POTRA1-3 has a stimulatory effect on chloroplast protein import.

3.12 Conclusions

*Ps*Toc75 POTRA domains have been modeled after crystal structures and chosen by sequence alignment. POTRA1 and POTRA3 can be recombinantly expressed and purified as part of a fusion protein in the pTYB2 vector. POTRA2 and POTRA1-3 can be expressed, refolded, and purified with a 6xHis tag in the pET-30(a) vector. The various POTRA domains have been purified and subjected to MALDI-TOF to confirm purity and that each POTRA domain was close to its expected size. CD was also performed on all POTRA domains to determine whether or not there was secondary structure in the natively purified and refolded domains. The secondary structure deconvoluted from the

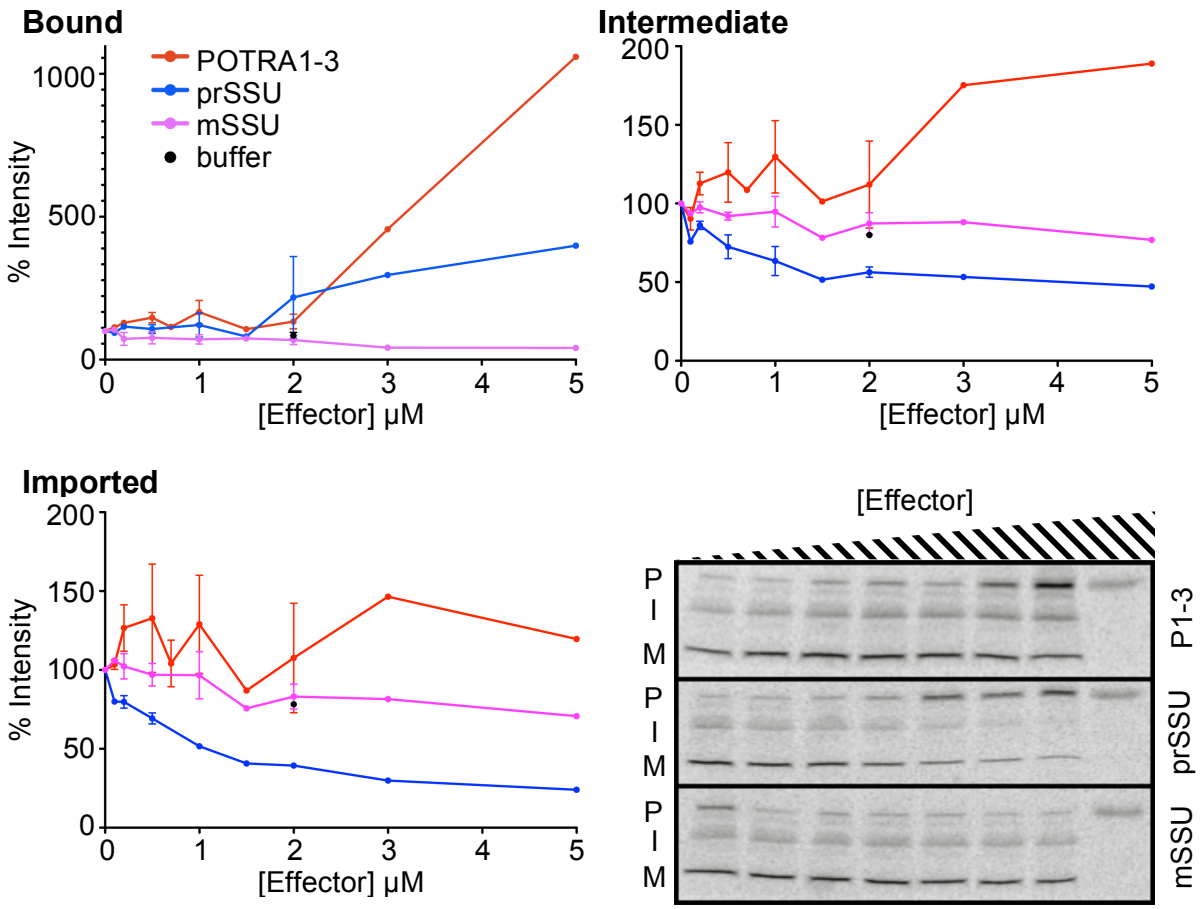


Figure 3.21 Stimulation of Protein Import by POTRA1-3

The graphs show the amount of bound (A), intermediate (B), and imported (C) ^{35}S-SStp*Ps* into chloroplasts determined by scanning the autoradiogram (D). Bound graph is the intensity of the P (precursor) bands in the autoradiogram, which are bound to the chloroplast but not imported. Intermediate graph is the intensity of the I (intermediate) bands in the autoradiogram, which are imported but only partially cleaved or mis-cleaved. Imported graph is the intensity of the M (mature) bands in the autoradiogram, which are fully imported into the stroma of the chloroplast and correctly cleaved.

CD spectra was compared to a set of POTRA domains that were structurally solved to help determine if the recombinant POTRA domains were correctly folded. The POTRA domains were tested for an ability to interact with each other and transit peptide, the substrate of Toc75. The majority of the testing was done with POTRA1 and POTRA3 because POTRA2 and POTRA1-3 were produced recently. POTRA1 has shown the ability to homo-oligomerize. Glutaraldehyde (GA) cross-linking showed that oligomers up to pentamers of POTRA1 can be identified. BMH cross-linking demonstrated that homo-dimers of POTRA1 can be isolated, and that the proteins interact near their C-terminus. This homo-oligomerization is not an artifact of cross-linking either, as AUC shows that POTRA1 can also form homo-dimers in a concentration dependent manner in solution. GA cross-linking has also shown that the homo-oligomerization of POTRA1 can be interrupted by addition of transit peptide. The pull-down assay supports the POTRA1 transit peptide interaction by showing that transit peptide interacts with and co-elutes with POTRA1. These two discoveries are interesting because they suggest a dual role for POTRA1. They could help with TOC core complex formation, and still direct transit peptides into the translocation pore. Perhaps POTRA1 could interact with other members of the TOC core complex to help with assembly.

While POTRA1 homo-oligomerization is supported by cross-linking and AUC, neither of those experiments show that POTRA3 homo-oligomerizes to nearly the same extent as POTRA1. The glutaraldehyde crosslinking showed that the majority of POTRA3 was a monomer, and that discrete higher molecular weight oligomers were not formed. BMH crosslinking was not performed because the POTRA3 does not contain a cysteine. AUC done on POTRA3 alone was inconclusive, although when AUC was

performed on POTRA3 and transit peptide, a heterodimer was detected at high concentrations of transit peptide. This information could mean that the role of POTRA3 is not in assembly of the TOC core complex, but it could function in funneling precursor proteins into the translocation pore of the Toc75.

The full length POTRA domain seems to have a stimulatory effect on protein import into the chloroplast during *in vitro* import assays. This means that the recombinant POTRA1-3 helps the general import pathway during chloroplast protein import. A model is discussed that would accomplish this in the discussion section. It is interesting to note that the roles and possible interactions of the POTRA domains could be so diverse.

Chapter 4 Discussion

4.1 Introduction

POTRA domains have been found on proteins on the outer membranes of gram-negative bacteria, chloroplasts, mitochondria, and cyanobacteria. They are involved in protein translocation either into or through membranes. Their roles have been suggested in protein translocation machinery assembly and interactions with substrates. This work has found some possible interaction partners of *Ps*Toc75 POTRA domains *in vitro*. This has been accomplished with two types of chemical cross-linking, AUC, pull-down assays, and a purified chloroplast protein import assay. It is interesting to look for roles common for specific POTRA domains and to try and determine how POTRA domains of different species and different organelles differ and how they relate to each other.

4.2 Conservation of POTRA Domains

Between one and seven POTRA domains reside in the N-terminal portions of proteins in the Omp85/TPS superfamily (Arnold et al. 2010; Gentle et al. 2004; Gentle et al. 2005; Sanchez-Pulido et al. 2003). The Omp85s of Photosynthetic prokaryotes possess three POTRA domains (Arnold et al. 2010; Koenig et al. 2010), equivalent to the number of POTRA domains in Toc75 in chloroplasts (Sanchez-Pulido et al. 2003). POTRA domains tend to cluster according to their position. In a cluster analysis of POTRA domains the clusters of the N-terminal and C-terminal POTRA domains of Toc75, Omp85, and cyanobacterial Omp85 are all in close proximity (Figure 4.1, Arnold et al. 2010). The

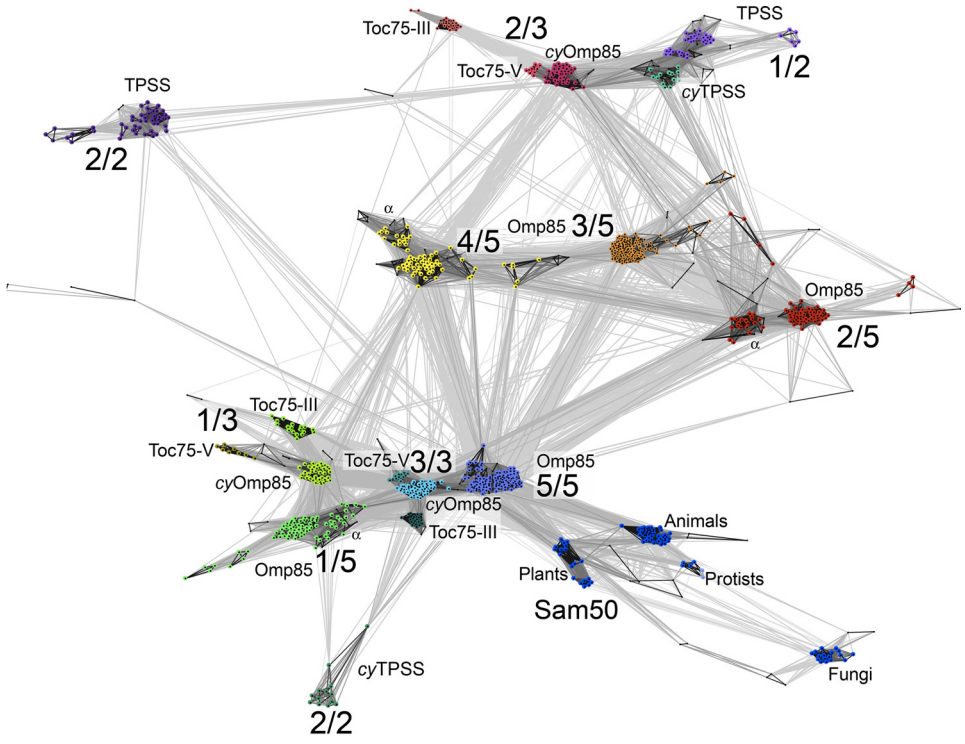


Figure 4.1 Cluster Analysis of POTRA Domains

POTRA Domains from Sam50, Toc75-III, Toc75-V, *cy*Omp85, proteobacterial Omp85, cyanobacterial (*cy*TPSS), and proteobacterial TPSS. Each dot is a single sequence, and the intensity of each connecting line is the quality of BLAST p-value light gray ($1x10^{-2}$) to black ($1x10^{-20}$). Figure used from Arnold (2010).

POTRA2 of cyanobacterial Omp85s and Toc75 aligns well with POTRA2 and POTRA4 of Omp85.

The C-terminal POTRA domains have the tightest cluster, meaning that their function is probably the most conserved and the most important. Data for the importance of the most C-terminal POTRA domain is around in the literature as the C-terminal POTRA domain knock-out has been shown to be lethal in Omp85 in *Neisseria meningitidis* (Bos et al. 2007). This suggests that all C-terminal POTRA domains (especially of Toc75, Omp85, and cyanobacterial Omp85) may be necessary for organism or plastid survival. POTRA3 of *ana*Omp85 has a loop between the first beta-strand and first alpha-helix, that is conserved in chloroplast and cyanobacteria but not mitochondria or proteobacteria (Figure 4.2).

The large size of POTRA2 in Toc75 is not conserved across POTRA2 domains in cyanobacteria, and there has yet to be an algal or plant POTRA domain structurally solved. POTRA2 in *Ana*Omp85 was determined to be 80 residues (Koenig et al. 2010) and POTRA2 in *Te*Omp85 is 70 residues (Arnold et al. 2010). The helix α1 and the β-strand β1 of POTRA2 in *Ana*Omp85 (Koenig et al. 2010) are both interrupted resulting in a β-bulge, which is also present in the third POTRA domain of BamA (Kim et al. 2007). Also conserved is a stable orientation for the POTRA2 and POTRA3 domains and more flexibility between the POTRA2 and POTRA1 (Koenig et al. 2010), this mirrors the flexibility between POTRA2 and POTRA 3 of BamA (Gatzeva-Topalova et al. 2008; Kim et al. 2007). POTRA1 of *ana*Omp85 has a small two-stranded β-sheet near its N-terminus, which form a β-cap (Koenig et al. 2010) . The first β-strand of the cap is

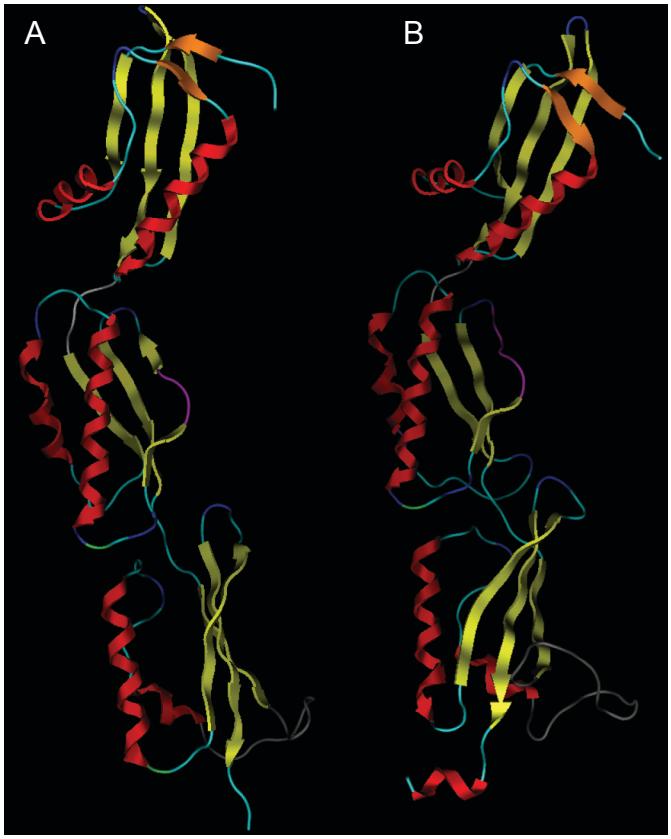


Figure 4.2 Crystal Structure of Cyanobacterial Omp85 POTRA Domains

Crystal structures of *Te*Omp85 (A, pdb id. 2X8X) and *Ana*Omp85 (B, pdb id. 3MC8) modeled in M.O.E. are pictured side by side. The β-cap of POTRA1 is colored in orange, the flexible linker between POTRA1 and POTRA2 is colored in light gray, the β-bulge in POTRA2 is colored in magenta, the L1 loop of POTRA3 is colored in dark gray.

N-terminal to the β1 of POTRA1, and the second β-strand of the cap if between α1 and α2 of POTRA1. All of these unique portions on the POTRA domains could be the biological explanation for the unique roles that POTRA domains seem to have in their various Omp85 and TPS proteins. It will be interesting to see if the unique features of each POTRA domain are responsible for the roles of the POTRA domains.

4.3 Role and Model of Action for Individual POTRA Domains

The β-sheet of POTRA1, more specifically β2, can interact with a β-strand or β-sheet via β-augmentation (Harrison 1996). The ability of POTRA1 to participate in β-augmentation has been noted in in BamA (Gatzeva-Topalova et al. 2008; Kim et al. 2007) and *ana*Omp85 (Koenig et al. 2010). This β-augmentation has been shown to be able to occur in both a parallel (Kim et al. 2007; Koenig et al. 2010) and an anti-parallel manner (Gatzeva-Topalova et al. 2008). Transit peptides have a region towards their C-terminus that has been predicted to form an amphiphilic β-strand (von Heijne et al. 1989(von Heijne et al. 1989), this means that POTRA domains and TP could interact via β-augmentation.

Data collected in this work supports a role for POTRA1 in substrate recognition (interactions with SStp*Ps*) and formation of a protein translocation complex (self-dimerization). In an attempt to explain this proposed dimerization could facilitate the TOC core complex assembly a model was proposed (Figure 4.3). The hinge region between POTRA2 and POTRA1 could allow POTRA1 the large degree of freedom needed to make adjacent interactions. POTRA1 could move around until it encountered

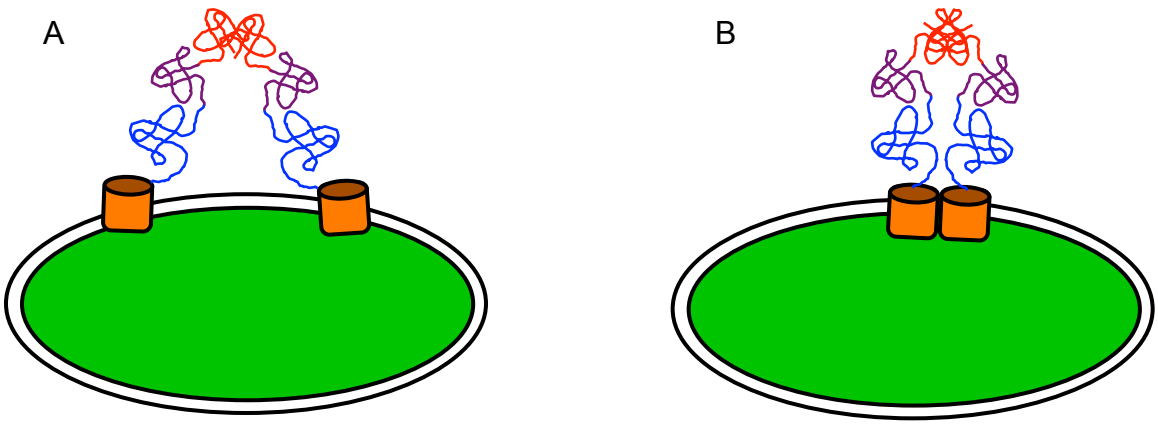


Figure 4.3 Model of POTRA1 Mitigating TOC Complex Oligomerization

POTRA1, POTRA2, POTRA3 of Toc75. A) POTRA1 domains interacting, helping to bring a pair of Toc75s into contact with each other B)

another POTRA1 at which point the ability of POTRA1 to self-dimerize would allow time for the β-barrel pores of the two Toc75s to come into contact with each other. Alternatively this interaction could compete for an in-plug formation that could occlude the protein conducting channel. The outer edge of the β-barrels could interact with each other, perhaps via β-augmentation, and this would help to form the 4:4:1 stoichiometry (Agne et al. 2009) of Toc75:Toc34:Toc159 that is present in the TOC core complex.

POTRA1 has been shown to interact with substrates of the Omp85 or TPS that it belongs to. POTRA1 of BamA in *E. coli* has been shown to interact with nascent LptD, LamB, and OmpF (all OMPs destined for the outer membrane in *E. coli*) and has even been implicated in proper insertion of BamA itself into the outer membrane (Bennion et al. 2010; Knowles et al. 2008). POTRA domains belonging to TpsB transporters interact with their TpsA partner (Clantin et al. 2007; Hodak et al. 2006). In particular POTRA1 of FhaC was shown to interact with FHA, although not as well as the entire periplasmic domain of FhaC does (Delattre et al. 2011). This means that POTRA2 of FhaC must interact with the FHA substrate as well. POTRA2 of Toc75 is very large and may contain a β-bulge like POTRA2 of *ana*Omp85 or POTRA3 of BamA. If this is the case Toc75 POTRA2 would have more space to interact with transit peptides or other members of the TOC machinery.

This work has shown the ability of excess transit peptide to interfere with POTRA1 homo-oligomerization (Fig 3.12). Perhaps when precursor proteins are present at the outer membrane of the chloroplast they can interrupt this homo-oligomerization of POTRA1 (Figure 4.4) and begin to begin a journey down the POTRA domains into the pore (Figure 4.5). We have shown that POTRA1 interacts with itself in the formation of

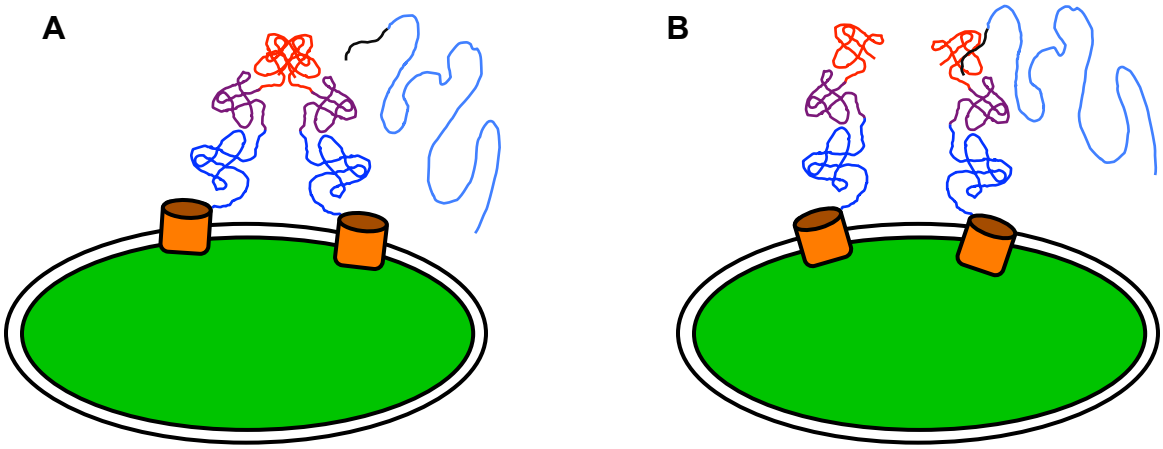


Figure 4.4 Transit Peptide Disrupts POTRA1 Oligomers

(A) POTRA1 homo-dimers interrupted by (B) heterodimerization of POTRA1 with transit peptide of a precursor protein.

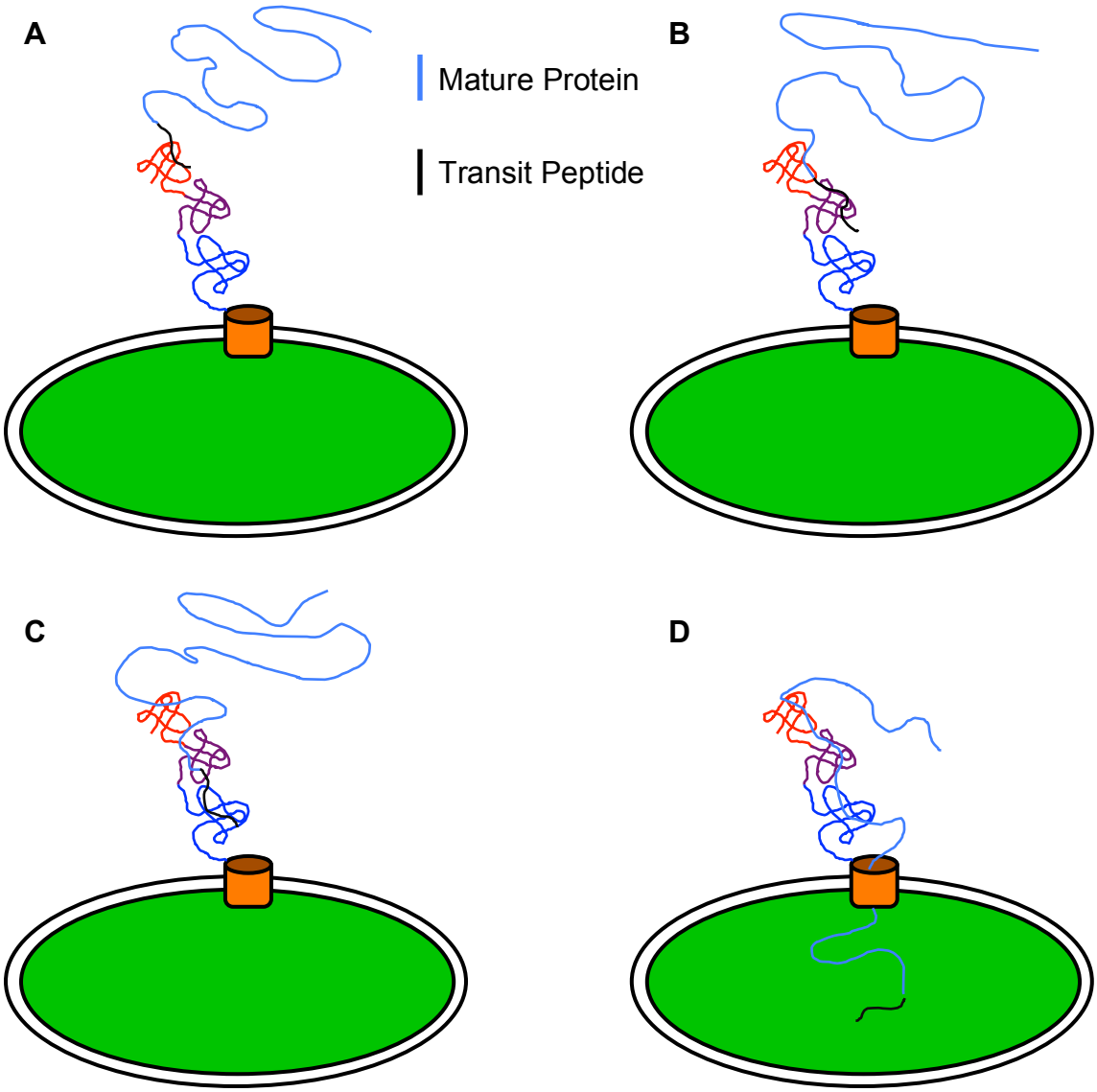


Figure 4.5 Proposed Role of POTRA in Protein Import

Transit peptides interact with the N-terminus of the Toc75 (Ertel, 2005). Above is the proposed interaction order of TP with Toc75, with the TP of a precursor protein interacting with POTRA1 (A), POTRA2 (B), POTRA3 (C), before import via the C-terminal pore of Toc75 (D).

homo-oligomers, and with transit peptide by the formation of heterodimers. In fact, we have shown that elevated levels of transit peptide can disrupt POTRA1 homo-oligomers *in vitro*. If POTRA1 domains homo-oligomerize *in vivo* then the ability of transit peptides to disrupt these POTRA1 oligomers would benefit protein import by allowing the precursor proteins access to all POTRA domains and to the pore domain of the Toc75.

It has been shown that in *Ana*Omp85 the POTRA domains gate the pore, as constructs without the POTRA containing N-terminus of *Ana*Omp85 allow sucrose to diffuse freely into the liposomes they are reconstituted in (Ertel et al. 2005; Koenig et al. 2010). More specifically the most C-terminal POTRA domain, POTRA3, of *Ana*Omp85 contains a loop deemed L1 loop between the first beta-strand and first alpha-helix, that is conserved in chloroplast and cyanobacteria but not mitochondria or proteobacteria, that has been shown to have a gating effect on the pore of *Ana*Omp85 (Koenig et al. 2010). Another possibility for the observed gating effect on the pore of the translocon is that there are β-strands in the pore that are unstable, and they have to interact with something to stabilize them, and the stabilizing proteins are called "in-plug" domains (Naveed et al. 2009). In unpublished collaboration with Dr. Jie Liang some β-strands of the *Ps*Toc75 barrel have been predicted to be weakly stable. A possibility is that part of the most C-terminal POTRA domain is responsible for acting as this in-plug and helps stabilize the pore in an open conformation after the POTRA domain has interacted with the substrate.

4.4 Models of POTRA Interaction with Peptide Substrates

Class I major histocompatibility complex (MHC) molecules carry short peptides from proteins degraded in the cytoplasm of nucleated vertebrate cells to the cell surface

via secretory vesicles (Falk et al. 1991). T cells have receptors (TCR) that can recognize the antigens presented by these MHC molecules (Townsend and Bodmer 1989) on the surface of cells. If the peptide presented is part of a foreign protein the cell will be marked for destruction by cytotoxic T cells (Khan et al. 2000). The peptide-binding portion of MHC molecules is made up of an eight β-stranded sheet with a binding groove between two long α-helices (Bjorkman et al. 1987a; Bjorkman et al. 1987b; Saper et al. 1991). The tertiary structure of this MHC is strikingly similar to a POTRA domain (Figure 4.6). However, POTRA domains cannot interact specifically with transit peptides, because there are thousands of different transit peptides (Bruce 2000), and only three POTRA domains in chloroplasts. There is a difference in the way that the two different domains behave with respect to interacting with their respective substrates. Class I MHC molecules bind peptides very strongly *in vitro* with fast association rates (Springer et al. 1998) and slow dissociation rates (Buus et al. 1986). In fact class I molecules are capable of forming complexes with half-lives lasting tens of hours with many different peptide substrates (Khan et al. 2000). This differs from POTRA domains because POTRA domains do not form stable complexes with their substrates. If the binding affinity of POTRA domains was too high, it would severely hamper the ability of their proteins to export/import substrates or to insert proteins into the outer membranes. Another way the mode of interaction of POTRA domains and MHC molecules differ is that MHC interactions are specific. Side chains of peptides in the binding groove of MHC molecules reside in specific pockets, showing a sequence-dependence on peptide binding (Garrett et al. 1989; Guo et al. 1993; Saper et al. 1991).

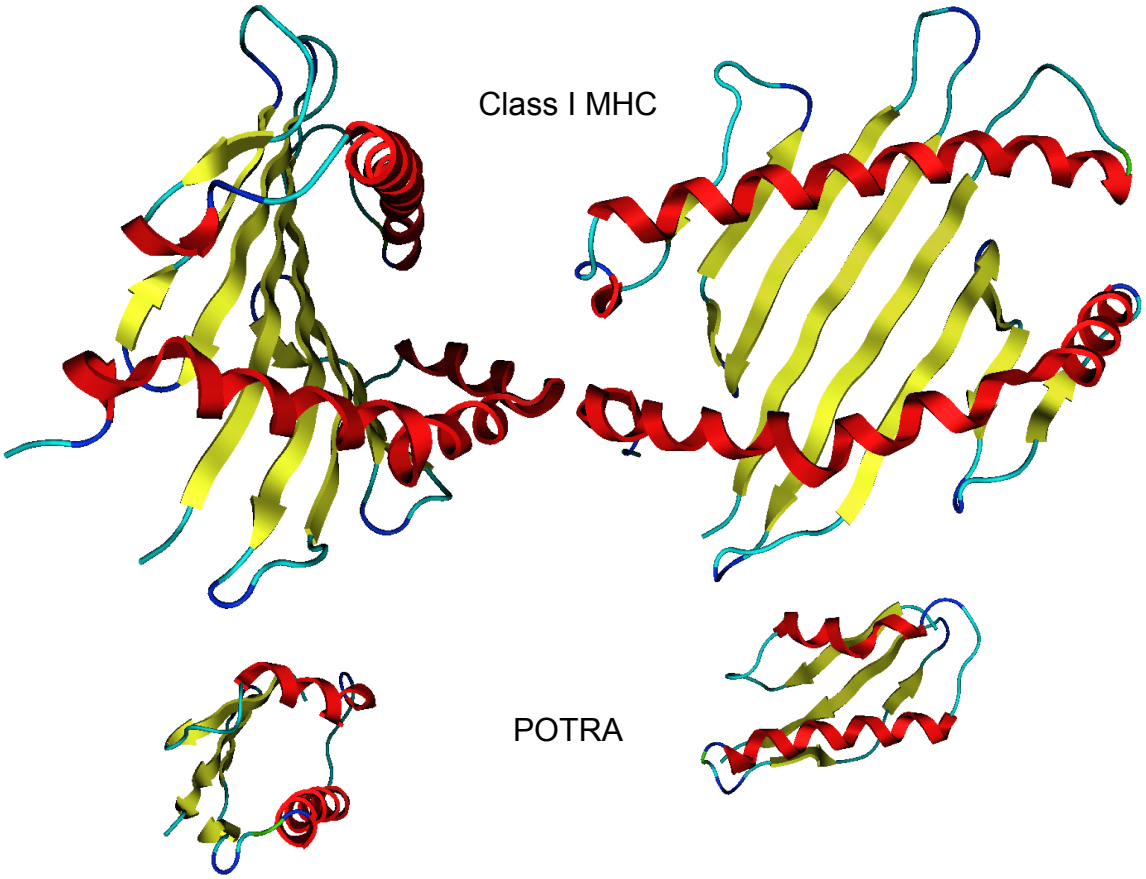


Figure 4.6 Tertiary Structural Similarity between POTRA Domain and Class I MHC

Class I MHC (pdb id: 1DUZ, top) shares similar structural identity with POTRA Domains (POTRA2 pdb id: 2X8X, bottom)

Another set of molecules that use β-strands to bind their substrates are the molecular chaperones of the 70-kDa heat shock protein (hsp70) family, and they are present in the cells of all organisms (Zhu et al. 1996). These proteins have constitutive and stress-induced functions (Saito and Uchida 1978) and are involved in events such as translocation and nascent chain folding as well as anti-aggregation functions (Rothman 1989). There are ATP-binding and substrate binding domains present on hsp70s (Welch and Feramisco 1985). The crystallized structure of the substrate binding domain of DnaK (the hsp70 with DNA replication functions in E. coli (Saito and Uchida 1978)) has a sandwich of a dimer of four β-strands followed by five alpha helices (Zhu et al. 1996). The peptides that interact with the substrate binding domain form hydrogen bonds with the β-sandwich domain of DnaK. Hsp70-peptide complexes, like class I MHC interactions, have long half-lives (Schmid et al. 1994). DnaK, uniquely, binds peptides in extended conformations, some of the hydrogen bonds formed between the peptide and the DnaK are generic and between the peptide backbone with a few key specificity determining pockets (Zhu et al. 1996).

Another group of surface domain proteins use β-strands to interact with peptides. PDZ domains are common domains of 80-110 residues (Boxus et al. 2008) present in the C-terminal portions of signaling proteins of bacteria, yeast, plants (Ponting 1997), and animals (Cho et al. 1992). PDZ domains derived their name from the first three different proteins that they were noticed in: post synaptic density protein of 95 kDa (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (Zo-1) (Kennedy 1995). PDZ domains have six β-sheets and two alpha helices (Figure 4.7). The β-augmentation can be seen in the crystal structure in figure 4.7, an exposed β-strand of

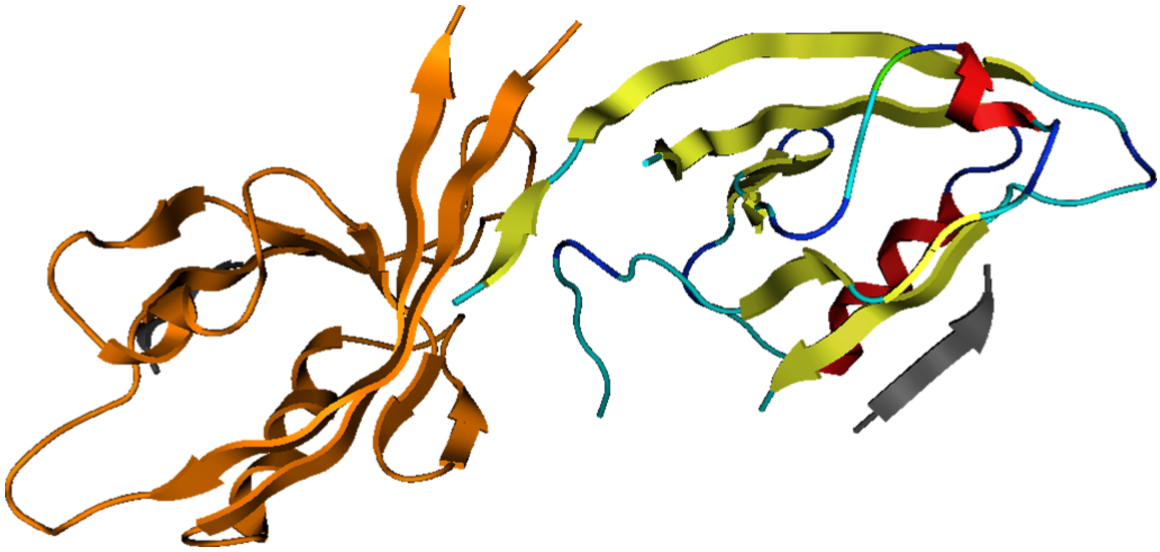


Figure 4.7 PDZ Domain Dimer Interacting via Beta Augmentation

PDB id 3QJM SH3 and multiple ankyrin repeat domains dimer from *Rattus norvegicus* a PDZ domain forms a dimer (1 monomer in orange, 1 colored according to secondary structure) in a crystal lattice and also interacts with a synthetic peptide (gray).

one PDZ interacts with a different exposed β-strand on the other PDZ. β-augmentation also occurs between an exposed β-strand of the PDZ domain and the synthetic peptide used in the crystallization experiment (Lee et al. 2011). Interestingly, the structure of PDZ domains and POTRA domains is also highly similar, with both domains being much more similar in size than POTRA domains are to MHC domains. Both β-augmentation interactions in this crystal structure are anti-parallel, but parallel interactions can occur (Kim et al. 2007; Koenig et al. 2010). PTB domains, regions of 100-150 residues present in insulin receptor substrates 1 and 2 also form hydrogen bonds with β-stranded peptides near the exposed edge of a β-sheet (Harrison 1996). Furthermore, this ability to interact with two or more substrates at once could explain why there was a gradual decrease in POTRA1 homo-oligomer with an increase in concentration of transit peptide, perhaps the POTRA1 homo-oligomer could accommodate some transit peptide and only at higher concentrations did the transit peptide start to out compete all of the homo-oligomeric interactions.

4.5 Synergism of Recombinant POTRA1-3 in Chloroplast Protein Import

We tried to take advantage of the interactions of POTRA domains, whether they occur in an MHC-like fashion or by β-augmentaion, with transit peptide in the *in vitro* import competition assay. The results (Fig 3.21) from the experiment described in Fig. 3.20 were unexpected. Recombinant POTRA1-3 was expected to interact with the precursor proteins in solution, competing with the POTRA1-3 present on the outer envelope of the chloroplasts. If this competition would have occurred the increase in concentration of recombinant POTRA1-3 would have resulted in an inverse relationship

of radiolabeled precursor protein imported into the chloroplast and concentration of recombinant POTRA1-3. The experiment supported the opposite, and a direct correlation of radiolabeled precursor with POTRA1-3 concentration was seen (Fig. 3.21 C).

The ability of POTRA domains to homo-oligomerize was not taken into account in the hypothesis for this experiment. An alternative model (Figure 4.8) is put forth that takes the homo-oligomerization of POTRA domains, more specifically POTRA1, into account. The cartoon on the left of the model shows a chloroplast with the POTRA1 domain of a Toc75 molecule interacting with a precursor protein, beginning the proposed journey down the POTRA domains through the pore of Toc75. The cartoon on the right side of the model depicts recombinant POTRA1-3 (dashed lines) in the reaction with a chloroplast and precursor proteins. The POTRA1 of the recombinant POTRA1-3 is interacting with POTRA1 of the Toc75 in the outer envelope of the chloroplast. This allows a longer working POTRA domain repeat when compared to the three POTRA domains present in the left of the cartoon. This additional length of the POTRA domain in the right cartoon could allow the recombinant POTRA1-3 bound Toc75 to interact with and eventually funnel more precursor proteins into the chloroplast it belongs to. The recombinant POTRA domains could increase the effective concentration of transit peptide, allowing the pore to stay open longer if the in-plug model is correct and would also allow faster import of the protein through other Toc75 channels. This would help to explain why there was high binding and import in the competition assay when the concentration of recombinant POTRA1-3 was increased.

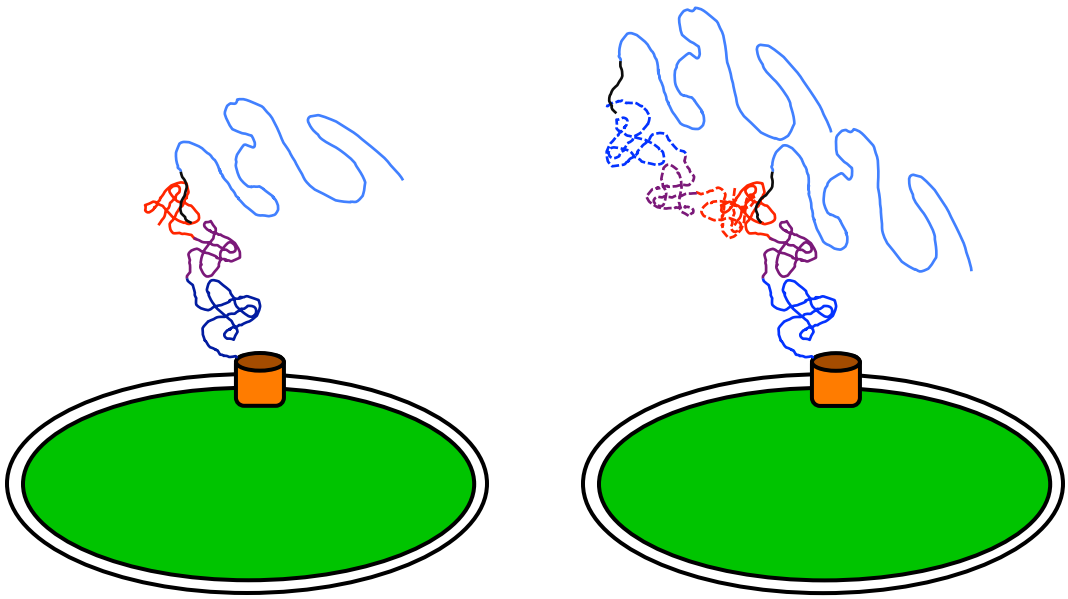


Figure 4.8 Proposed POTRA Domain Synergism

POTRA1 of Toc75 in purified chloroplasts could homodimerize with POTRA1 of recombinant POTRA1-3 (dashed lines) in the competition assay. This would result in a Toc75 with a POTRA reach approximately 2/3 longer than chloroplasts without recombinant POTRA1-3 present. This synergism would help explain the stimulation in precursor import reported in figure 3.15.

4.6 Conclusions

It is interesting to see the unique interactions of the POTRA domains of Toc75 with transit peptides and in homo-oligomerization. These interactions mimic the unique roles of POTRA domains found in the literature. It will be interesting to see whether or not the differences in the interactions of the POTRA domains are due to a difference in the secondary structure. That is, does the β-cap of POTRA1 seen in the cyanobacterial Omp85s allow the increase in homo-oligomerization seen in Toc75 POTRA1? Are the structural similarities that are shared between the cyanobacterial Omp85 POTRA domains even conserved in higher plant Toc75 POTRA domains? Perhaps the β-bulge in POTRA2 serves a purpose, and the L1 loop in POTRA3 is some sort of an in plug that gates the translocation pore until POTRA3 interacts with transit peptide.

This work has shown what some of the POTRA domains of Toc75 can interact with. That POTRA domains can homo-oligomerize, interact with transit-peptide containing substrates, and stimulate protein import is clear, but what is not clear is the method of interaction. It will be of great interest to see which method or methods of interaction the POTRA domains use. To see if they interact with their substrates in a manner more like class I MHC binding, whether they are more like PDZ β-augmentation, or could they form β-sandwiches that interact with peptides like the hsp70s that bring precursor proteins to the TOC assembly in the first place will be very exciting.

Chapter 5 Future Directions

There are experiments that will add to the knowledge of the roles of the individual POTRA domains that need to be performed. Glutaraldehyde cross-linking experiments can be run on POTRA2 and POTRA1-3 (currently underway) now that those domains can be produced. Cross-linking reactions will be carried out with the domains alone and also in the presence of transit peptide. This will allow us to map each POTRA domain's contribution to homo and hetero-oligomerization. AUC needs to be performed on these domains alone to help determine the extent of homo-oligomerization. AUC also needs to be repeated on POTRA3 alone because both previous trials were unsuccessful. AUC should be repeated with POTRA1 and transit peptide to see the extent that these two domains can hetero-dimerize. AUC could help determine binding affinities for the various dimer species. The pTYB2 pull-down assay should be repeated using POTRA3 as the anchor to support the POTRA3 transit peptide interaction visualized in chemical crosslinking and AUC. Perhaps a Ni-NTA column could be bound with P2-His or P1-3-His to perform a pull-down assay with those POTRA domains. Finally the import competition assay could be performed with each POTRA domain individually to possibly see the contribution each POTRA has to import synergism. All of the above experiments are to test POTRA interactions with themselves and with transit peptide, it would be interesting to replace transit peptide with the GTPase members of the TOC core complex. This would allow us insight into the assembly of the whole complex and possibly a model for import of precursor protein into the chloroplast.

To determine the method of POTRA interaction, solving the POTRA1-3 structure would be of tremendous value. This would also allow us to know whether or not our modeling was correct and possibly remake individual POTRA domains if our predictions were incorrect. A large amount of POTRA1-3 can be produced, although it is not stable very long once it is refolded. If the protein can be concentrated, conditions that led to solved crystal structures of cyanobacterial Omp85s can be screened around.

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Appendix

Appendix 1 POTRA1-3 PCR Recipe

10x ExTaq Buffer	5 µl
10 mM dNTP mix (2.5 mM each)	4 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Template DNA (200 ng/µl)	0.4 µl
ExTaq (5 Units/µl)	0.25 µl
ddH_2O	39.45 µl
Total Reaction Volume	50 µl

Appendix 2 PCR Cycling Conditions for POTRA1-3-His

1. 94ºC	5 min.
2. 94ºC	30 sec.
3. 55ºC	30 sec.
4. 72ºC	1 min.
5. Repeat steps 2-4 34 times	
6. 72ºC	5 min.
7. 4ºC	Hold

Appendix 3 Ligation of POTRA1-3 into pGEM-T Easy

2x Rapid Ligation Buffer	5 µl
pGEM-T Easy Vector (100 ng/µl)	1 µl
PCR Product (22.4 ng/µl)	3 µl
T4 DNA Ligase (3 Units/µl)	1 µl
Total Reaction Volume	10 µl

Appendix 4 Colony PCR Screen Recipe

5x GoTaq Buffer	5 µl
dNTP mix (2.5 mM each)	2 µl
M 13 Forward Primer (10 µM)	0.5 µl
M 13 Reverse Primer (10 µM)	0.5 µl
GoTaq Polymerase (5 Units/µl)	0.1 µl
30% Triton X-100	0.2 µl
ddH_2O*	16.7 µl
Total Reaction Volume	25 µl

* *E. coli* colony is added directly to the ddH_2O in the PCR tube before adding remaining ingredients

Appendix 5 PCR Cycling Conditions for Colony PCR of POTRA1-3-His

1. 94ºC	5 min.
2. 94ºC	30 sec.
3. 50ºC	30 sec.
4. 72ºC	1.5 min.
5. Repeat steps 2-4 34 times	
6. 72ºC	7 min.
7. 4ºC	Hold

Appendix 6 Ligation of POTRA1-3 into pET-30(a)

2x Rapid Ligation Buffer	5 µl
pET-30(a) (48.6 ng/µl)	1 µl
POTRA1-3 (22.5 ng/µl)	3 µl
T4 DNA Ligase (3 Units/ µl)	1 µl
Total Reaction Volume	10 µl

Appendix 7 Colony PCR Screen Recipe

5x GoTaq Buffer	5 µl
dNTP mix (2.5 mM each)	2 µl
T7 Promoter F (10 µM)	0.5 µl
T7 Terminator R (10 µM)	0.5 µl
GoTaq Polymerase (5 Units/µl)	0.1 µl
30% Triton X-100	0.2 µl
ddH_2O*	16.7 µl
Total Reaction Volume	25 µl

* *E. coli* colony is added directly to the ddH_2O in the PCR tube before adding remaining ingredients

Appendix 8 PCR Cycling Conditions for Colony PCR of POTRA1-3-His

1. 94ºC	5 min.
2. 94ºC	30 sec.
3. 50ºC	30 sec.
4. 72ºC	1.5 min.
5. Repeat steps 2-4 34 times	
6. 72ºC	7 min.
7. 4ºC	Hold

Appendix 9 Tris-Tricine Gel Recipe

	Stacking (µl)	Separating (µl)	Resolving (µl)
49.5% acrylamide - 3% bis-acrylamide	120	305	0
49.5% acrylamide - 6% bis-acrylamide	0	0	1000
3 M Tris-HCl pH 8.4, 0.3% SDS	375	500	1000
80% Glycerol	0	0	375
ddH_2O	1000	700	600
APS	24	20	20
TEMED	10	5	5

Vita

Richard Franklin Simmerman was born in Knoxville, Tennessee on May 6, 1982. He attended primary through high school at Farragut High School in Farragut, Tennessee. He graduated from Farragut High School, in Farragut, Tennesse in 2000. He obrained his Bachelor's Degree in Biochemistry, Cellular, and Molecular Biology from the University of Tennessee, Knoxville in 2005. He worked for Dr. Chris Dealwis and later for Dr. Alexandre at the University of Tennessee after graduation, and joined the graduate program in Biochemistry, Cellular, and Molecular Biology in the fall of 2006. He joined the lab of Barry Bruce in the fall of 2008 to conduct research on protein import into chloroplasts, focusing on determining the interaction partners of Toc75 and more specifically its POTRA domains. After completing his Master's Degree in August of 2011, he plans to pursue his PhD in the field of alternative energy in the Biochemistry, Cellular, and Molecular Biology.