Plastid Protein Targeting: Preprotein Recognition and Translocation

P. Chotewutmontri[*,1,2], **K. Holbrook**[†,1,3], **B.D. Bruce**[*,†,4]

[*]Graduate School of Genome Science and Technology, University of Tennessee, Knoxville, TN, United States
[†]University of Tennessee, Knoxville, TN, United States
[4]Corresponding author: e-mail address: bbruce@utk.edu

Contents

Abstract

Eukaryotic organisms are defined by their endomembrane system and various organelles. The membranes that define these organelles require complex protein sorting and molecular machines that selectively mediate the import of proteins from the cytosol to

[1] Contributed equally to the manuscript.
[2] Current address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, United States.
[3] Current address: Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, CA 90095, United States.

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their functional location inside the organelle. The plastid possibly represents the most complex system of protein sorting, requiring many different translocons located in the three membranes found in this organelle. Despite having a small genome of its own, the vast majority of plastid-localized proteins is nuclear encoded and must be posttranslationally imported from the cytosol. These proteins are encoded as a larger molecular weight precursor that contains a special "zip code," a targeting sequence specific to the intended final destination of a given protein. The "zip code" is located at the precursor N-terminus, appropriately called a *transit peptide* (TP). We aim to provide an overview of plastid trafficking with a focus on the mechanism and regulation of the general import pathway, which serves as a central import hub for thousands of proteins that function in the plastid. We extend comparative analysis of plant proteomes to develop a better understanding of the evolution of TPs and differential TP recognition. We also review alternate import pathways, including vesicle-mediated trafficking, dual targeting, and import of signal-anchored and tail-anchored proteins.

1. INTRODUCTION

With rapid advances in genome sequencing, transcriptional profiling, and proteomics, a comprehensive snapshot of how cells differentially express their genome is now possible. In plants, systems biology approaches have led to the generation of a variety of large datasets (Gaudinier and Brady, 2016). These approaches have revealed the complexity of gene expression and moving the field forward will require an understanding of the subcellular localization of the expressed proteins. In plants, these protein dynamics involve a delicate interplay between not only the nuclear genome and the genomes of the semiautonomous organelles, the plastids, and the mitochondria.

Understanding these new challenges comes nearly 40 years after it was first established that some proteins are targeted to the plastid via a post-translation mechanism that required a specific N-terminal targeting sequence (Chua and Schmidt, 1978; Highfield and Ellis, 1978). Despite the functional similarities, the translocation machineries found in the endoplasmic reticulum (ER), mitochondria, and plastids show no similarity in their core components (Schatz and Dobberstein, 1996). Thus, these import processes appear to have evolved independently. Surprisingly, in plants cells, these processes can coexist with little to no missorting. In this review, we provide a detailed update on our understanding of the structure, function, and regulation of plastid import pathways, with a focus on the chloroplast translocons functioning in the two envelope membranes, outer envelope of chloroplasts (TOC) and inner envelope of chloroplasts (TIC). We also

look the evolutionary diversity of these molecular machines across a wide range of phototrophic organisms. We take detailed new look the size and diversity of the transit peptides (TPs) (Bruce, 2000). Finally, we start to build a detailed understanding of how TPs can function without sequence homology yet perform a common universal function (Bruce, 2001).

2. ORIGIN OF PLASTIDS

Over century ago, Mereschkowsky proposed that plastids derived from cyanobacteria (Martin and Kowallik, 1999). It is now widely accepted based on phylogenetic analysis that the primary plastids originated from endosymbiosis of a cyanobacterial ancestor (McFadden, 2014). Interpretation of the fossil record and molecular clock analysis date the endosymbiosis event to over 1.2 billion year ago (Butterfield, 2000; Yoon et al., 2004). The comparisons of genome organization and sequences of the primary plastids from Archaeplastida which includes Viridiplantae (green algae and plants), rhodophytes, and glaucophytes suggest a single endosymbiosis event (Keeling, 2010; Price et al., 2012; Turner et al., 1999). Aside from the advantage of photosynthetic capability, the only other endosymbiosis event resulting in primary plastids was reported in a protist *Paulinella* (Reyes-Prieto et al., 2010). To explain the incredibly rare occurrence of primary plastid endosymbiosis, Ball et al. (2013) showed that the key enzymes required for the host to utilize photosynthetic carbon were derived from another bacterium, the pathogenic *Chlamydiales*. They further proposed that the endosymbiosis occurs only in the infected host where the carbon utilization becomes beneficial. Many attempts to pinpoint the plastid ancestor suggest unicellular nitrogen-fixing cyanobacteria based on 16S RNA, photosynthetic, and metabolic gene sequences (Criscuolo and Gribaldo, 2011; Falcon et al., 2010; Hackenberg et al., 2011; Kern et al., 2011; Pascual et al., 2011). The most comprehensive study using a large-scale comparison of 241 complete genomes including 9 cyanobacteria and 4 photosynthetic eukaryotes indicates that the nitrogen-fixing heterocyst cyanobacteria share the most genes with the photosynthetic eukaryotes (Deusch et al., 2008). Although modern heterocyst cyanobacteria, *Nostoc* sp. PCC7120 and *Anabaena variabilis* ATCC29143 harbor around 5500 genes that encode proteins (Kaneko et al., 2001; Markowitz et al., 2012), plastid genomes of land plants only encode around 80 proteins (Timmis et al., 2004). This reduction of plastid genomes was found to occur mainly by the transferring of plastid DNA to the nuclear genome (Kleine et al., 2009). It was proposed

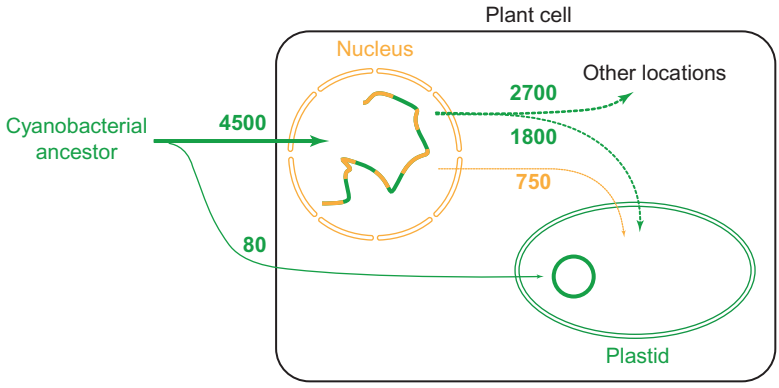

Fig. 1 Endosymbiont gene transfer and protein targeting to the plastid. Bioinformatic analysis of Arabidopsis genome sequence (Martin et al., 2002) indicated that around 4500 nuclear genes are transferred from the cyanobacterial ancestor and only 87 genes remain in the plastid genome. Although the majority of the proteins encoded by the endosymbiont-to-nucleus genes are targeted to other locations in the cell, about 1800 of these proteins are targeted to the plastid. In addition, around 750 proteins encoded by nonendosymbiont nuclear genes are also targeted to the plastid.

that the ability of plastids to import nuclear-encoded proteins enabled the plastid ancestry to transfer its genes to the nucleus without compromising its metabolic capacity (Allen, 2003). During the process of endosymbiosis, the import of proteins encoded by endosymbiont-to-nucleus genes permits the endosymbiont gene copies to undergo pseudogenization and later loss (Martin et al., 1993). In *Arabidopsis*, bioinformatic analysis estimated that around 4500 protein-encoding genes in the nucleus are originated from the endosymbiont as shown in Fig. 1 (Martin et al., 2002). Less than half of these endosymbiont-to-nucleus proteins (about 1800) are targeted to plastids and the rest functions elsewhere in the cell (Martin et al., 2002). Interestingly, nonendosymbiont-derived proteins (about 750) also localize to plastids (Martin et al., 2002) and function in photosynthesis, respiration, and metabolic pathways (Kleine et al., 2009). While plastid protein import was crucial in facilitating the endosymbiosis event in the past, it becomes indispensable to the function of the cells in *Arabidopsis* where over 2500 proteins in *Arabidopsis* must gain access to plastids.

3. PLASTID PROTEIN TARGETING ROUTES

Proteins targeted to plastids can be delivered posttranslationally to six plastid locations: the outer envelope membrane (OM), the intermembrane

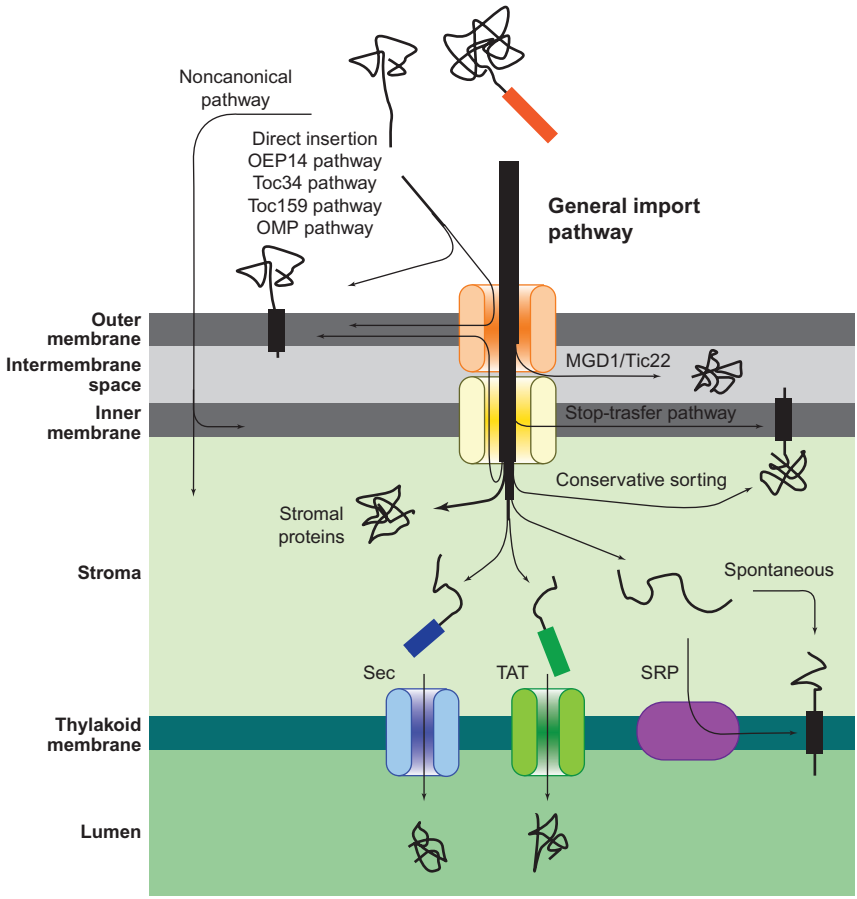

Fig. 2 Plastid protein targeting routes. Proteins targeted to plastids are delivered to six locations: outer envelope, intermembrane space, inner envelope, stroma, thylakoid membrane, and thylakoid lumen. The outer envelope proteins use multiple pathways for targeting while the interior proteins containing TP pass through the envelope(s) via the General Import Pathway. Some of the proteins contain a second targeting signal for thylakoid lumen targeting. In addition, experimental evidence and proteomic analysis reveal that many proteins utilize noncanonical pathway.

space (IMS), the inner envelope membrane (IM), the stroma, the thylakoid membrane, and the thylakoid lumen as shown in Fig. 2 (Keegstra and Cline, 1999; Li and Chiu, 2010). Most of these processes involve protein translocation across the plastid membranes: the outer envelope, the inner envelope, and the thylakoid membranes. In general, protein translocation across or insertion into membrane is mediated by oligomeric membrane complexes termed translocons (Walter and Lingappa, 1986). The majority

of plastid-targeted proteins is synthesized as precursors containing the N-terminal targeting sequences called TPs (Dobberstein et al., 1977; Kleffmann et al., 2004). Similar to the signal hypothesis (Blobel, 1980), TPs act as an intrinsic signal on the precursor protein that is recognized by the targeting receptors associated with the translocons. TPs direct translocation of precursor proteins across the double membranes of plastids via the translocon at the TOC and TIC in a process described as the general import pathway (Cline and Henry, 1996; Schnell et al., 1997; van't Hof and de Kruijff, 1995a). After the precursor is translocated into the stroma, the TP is readily cleaved allowing the mature domain to fold into its native conformation or to be further targeted to the thylakoid (Richter and Lamppa, 2002). So far, the TP has been utilized in targeting precursor proteins to all of the six locations of plastids and is considered to function in the general import pathway. This pathway recognizes diverse TP sequences from various functional groups of proteins. In summary, the general import pathway functions similar to a central transit hub where the majority of plastid-targeted proteins pass through before reaching their final locations.

3.1 General Import Pathway

The general import pathway is a working model describing a TP-directed translocation of proteins into the stroma of plastids (Bruce, 2000). Fig. 3 illustrates the sequential steps in the pathway that reflect the processes that take place in the cytosol, in both the OM and IM, stromal-localized components, and finally the cleavage and processing of the TP. We have not included steps that are involved in the subsequent targeting to the thylakoid nor do we address the targeting to the OM, IM, or IMS. Fig. 3 represents a somewhat selective model that has consolidated many years of work, but emerging research continues to develop a more complete picture of the import system.

The OM of plastids has been shown to recruit cytosolic precursors using multiple pathways. Work performed using isolated chloroplasts shows that the precursors are able to directly bind to TOC on the surface of chloroplasts (Fig. 3, step 1a). The binding is reversible when lacking ATP/GTP and denoted as energy-independent binding (Jarvis, 2008; Kouranov and Schnell, 1997; Perry and Keegstra, 1994). Addition of GTP also promotes binding (Inoue and Akita, 2008; Young et al., 1999). TP interaction with chloroplast lipids suggests that the precursor can directly bind to the lipid surface (Fig. 3, step 1e) before being transferred to the membrane receptors TOC159 and

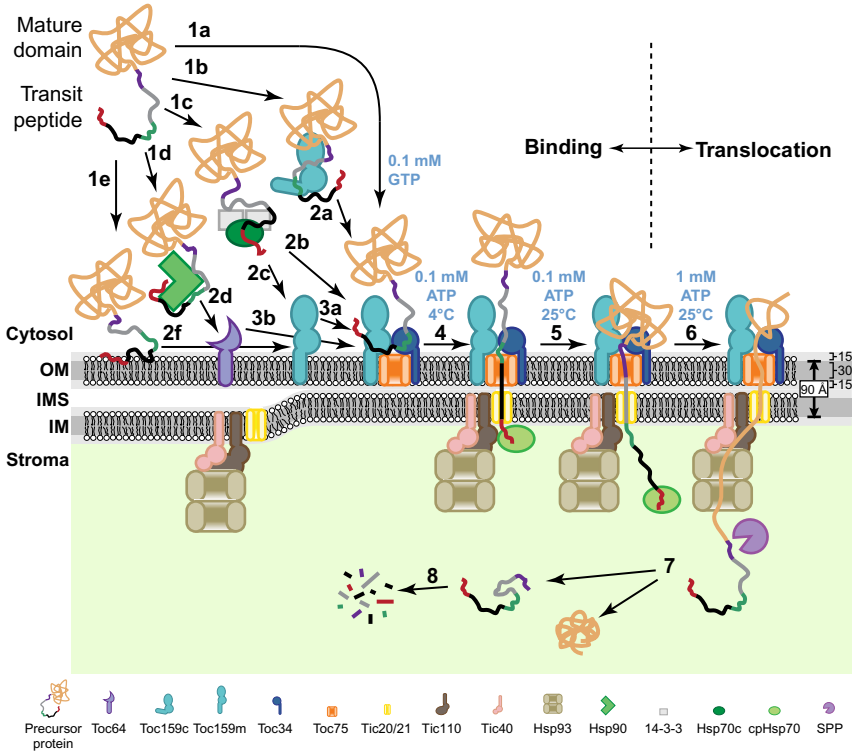

Fig. 3 The general import pathway. Cytosolic precursors can be targeted to plastids directly by interacting with TOC (1a), or by interacting with the lipids (1e) before transferring to membrane Toc159 (2f) and reaching Toc34 (3a). The precursors can also interact with cytosolic Toc159 (1b) before transferring to Toc34 (2a). TPs can interact with Hsp90 (1d) before being targeted to Toc64 (2d) and further transferred to Toc34 (3b). Phosphorylated TPs can interact with the guidance complex (1c) composed of 14-3-3 and Hsp70c in cytosol. The precursors from guidance complexes can be transferred to membrane receptors Toc159 (2c) or Toc34 (2b). The binding state of precursor to the chloroplast can be subdivided based on GTP/ATP level and temperature of the system (4–5). When the ATP level is greater than 1 mM, the translocation process is initiated by Hsp93/cpHsp70 (6). When the precursors emerge into the stroma, stromal processing peptidase (SPP) will cleave TP from precursor proteins releasing the mature domain (7). TP will be further degraded by PreP1/PreP2 peptidases (8).

TOC34 (Fig. 3, steps 2f and 3a) (Pilon et al., 1995; Pinnaduwage and Bruce, 1996). Cytosolic factors were shown to interact and recruit precursors to the membrane receptors. Cytosolic Hsp90 captures precursor (Fig. 3, step 1d) and delivers it to the membrane receptor TOC64 (Fig. 3, step 2d) (Qbadou et al., 2006). Phosphorylated TPs interact with 14-3-3 and form the guidance complex with cytosolic Hsp70 Fig. 3 (step 1c) (May and

Soll, 2000). The guidance complex was proposed to dock to membrane receptors TOC159 (Fig. 3, steps 2c and 3a) or TOC34 (Fig. 3, step 2b) (May and Soll, 2000; Qbadou et al., 2006). Another pathway utilizes the cytosolic TOC159 pool to deliver precursor to the membrane (Fig. 3, steps 1b and 2a) (Hiltbrunner et al., 2001; Smith et al., 2004).

When low levels of ATP (<0.1 mM) are present, with or without GTP, the precursor engages in an irreversible binding state, which is referred to as the early import intermediate (Inoue and Akita, 2008; Kessler et al., 1994; Kouranov and Schnell, 1997; Olsen and Keegstra, 1992; Perry and Keegstra, 1994; Young et al., 1999). The switching from energy-independent to energy-dependent bindings seems to involve TOC75 (Paila et al., 2016). In the presence of 0.1 mM ATP at 4°C, about 110 amino acids are buried in the translocons (Fig. 3, step 4) (Akita and Inoue, 2009; Inoue and Akita, 2008). When the temperature is increased to 25°C, about 130 amino acids are buried (Fig. 3, step 5) (Akita and Inoue, 2009; Inoue and Akita, 2008). At this step, the precursor spans the double membrane via the TOC75 and TIC20/21 channels and TP interacts with TIC110 (Akita et al., 1997; Chen and Li, 2007; Inoue and Akita, 2008; Nielsen et al., 1997) forming the contact site between the double membranes (Schnell and Blobel, 1993). The translocation process is initiated by the ATPase chaperones Hsp93/cpHsp70 when the ATP level is above 1 mM (Fig. 3, step 6) (Shi and Theg, 2010; Su and Li, 2010). When the precursor emerges into the stroma, stromal processing peptidase (SPP) will cleave TP from the precursor, releasing the mature domain (Fig. 3, step 7) (Richter and Lamppa, 1998). TP will be further degraded by presequence peptidases, PreP1/PreP2 (Fig. 3, step 8) (Glaser et al., 2006). The detailed mechanism of processes such as the specificity of TP recognition and threading of precursors into the stroma requires further study.

3.2 Roles of General Import Pathway Components

3.2.1 TPs and Interacting Domains

TPs are N-terminal extensions that are cleaved during or after import. They were first identified 40 years ago by Dobberstein and coworkers (Dobberstein et al., 1977) in *Chlamydomonas* for the small subunit of Rubisco and named TPs by Chua and Schmidt (1978) to be differentiated from the signal peptides. TPs are often encoded by the first exon and represent an addition of new coding information relative to the ancestral gene structure found in cyanobacteria (Kilian and Kroth, 2004; Tonkin et al., 2008). TPs are necessary and sufficient to facilitate protein import into plastids; the

mature domain alone fails to be imported while addition of TP can direct the import of a nonplastid protein into a plastid (Bruce, 2001). Thus, TP contains information governing the import process. The length of TPs varies from 20 to 150 amino acids based on the position of the processing site (Balsera et al., 2009b). However, it has been established that short TPs cannot direct the import (Bionda et al., 2010). Import only occurs when short TPs were extended into their mature domains to reach at least 60 amino acids long (Bionda et al., 2010).

Many attempts have been made to identify the conserved motifs present amid diverse TPs using primary sequence alignment, but the conservation becomes greatly reduced with the increasing numbers of TPs analyzed (Karlin-Neumann and Tobin, 1986; von Heijne et al., 1989). Amino acid composition and organization are also highly divergent (Bruce, 2000). Nevertheless, three regions were loosely defined: (i) N-terminal domain of about 10 residues, lacking charged amino acids, ending with Pro/Gly and preferably having Ala as the second residue, (ii) central domain, lacking acidic amino acids but rich in hydroxylated amino acids, and (iii) C-terminal domain, rich in Arg and possibly forming an amphiphilic β-strand (Bruce, 2001; von Heijne et al., 1989).

A few studies have determined NMR structures of TPs including those of TPs of RuBisCO activase (Krimm et al., 1999) and ferredoxin (Lancelin et al., 1994) from *Chlamydomonas*, as well as the TP of ferredoxin from a higher plant *Silene latifolia* (Wienk et al., 1999). Even though TPs are found to be unstructured in aqueous solution, they were shown to adopt alpha-helical structures in membrane-mimetic environments inferring the possible formation and of an alpha helix in TP recognition (Bruce, 2001). Notably, the most stable alpha helix of the TP present in higher plant ferredoxin contains a semiconserved FGLK motif that was suggested to interact with the translocation apparatus (Schleiff et al., 2002; Wienk et al., 2000). This is an example of the semiconserved physicochemical motifs of TPs that function as interacting domains determining organelle specificity.

Many groups have determined precursor interactions with translocon components in intact chloroplast by crosslinking during different stages of the import process (Akita et al., 1997; Chen and Li, 2007; Inoue and Akita, 2008; Kouranov and Schnell, 1997; Ma et al., 1996; Perry and Keegstra, 1994). These interactions can be confirmed in vitro using purified translocon components and various precursor proteins or TPs. Eleven different precursors (prSSU, prFNR, prOE23, prOE33, prFD, prHsp21, prLHCP, prRBCA, prE1α, prL11, and prPORA) were used in these

experiments. These in vitro assays have uncovered many interacting partners including the guidance complex (May and Soll, 2000), cytosolic Hsp90 (Qbadou et al., 2006), TOC159 (Smith et al., 2004), TOC34 (Schleiff et al., 2002; Sveshnikova et al., 2000b), TOC75 (Hinnah et al., 2002), TIC110 (Inaba et al., 2003), stromal Hsp70 (Ivey et al., 2000; Rial et al., 2000), SPP (Richter and Lamppa, 1999), and presequence peptidases PreP1/2 (Glaser et al., 2006).

While this interaction information confirmed the cross-linking results in identifying the combination of translocon components at each state of the import pathway, it does not provide direct location of the interacting domains on TPs. Only a limited number of studies reported the interacting domains of TPs, and these are summarized in Fig. 4. TPs of the small subunit of RuBisCO (SStp) and ferredoxin (FDtp) are the only TPs with their interacting domains mapped. The mapped domains include lipids, TOC159, TOC34, stromal Hsp70 CSS1, and SPP interacting domains (Becker et al., 2004; Ivey et al., 2000; Lee et al., 2009a; Pilon et al., 1995; Pinnaduwage and Bruce, 1996; Schleiff et al., 2002; Sveshnikova et al., 2000b).

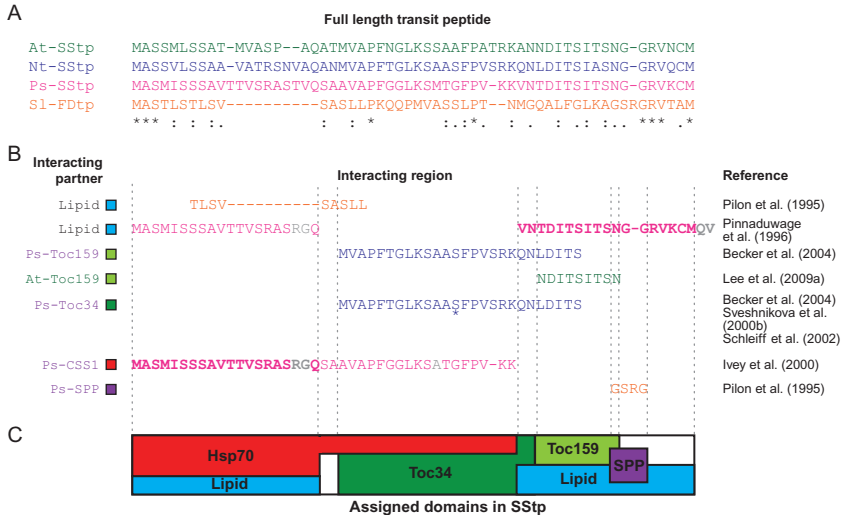

Fig. 4 Interacting domains of transit peptides. (A) Amino acid sequences of full-length TPs. SStp, TP of small subunit of Rubisco; FDtp, TP of ferredoxin. (B) Interacting domains in TPs. For lipid and Ps–CSS1 interaction, the sequences shown in bold have stronger interactions than sequences with regular characters. *Asterisk* indicates phosphorylated Ser. (C) All the interacting domains found on SStp. At, *A. thaliana*; Nt, *N. tabacum*; Ps, *P. sativum*; Sl, *S. latifolia*.

Mutagenesis (Holbrook et al., 2016; Lee et al., 2002, 2009a; Pilon et al., 1995), deletion (Holbrook et al., 2016; Kindle, 1998; Kindle and Lawrence, 1998; Rensink et al., 1998, 2000), Ala scanning (Lee et al., 2002, 2006, 2008, 2009a), domain swapping (Chotewutmontri and Bruce, 2015; Chotewutmontri et al., 2012; de Castro Silva Filho et al., 1996; Lee et al., 2009a; Smeekens et al., 1986), bioinformatics analysis (Chotewutmontri and Bruce, 2015; Chotewutmontri et al., 2012; Lee et al., 2008; von Heijne et al., 1989), and the use of synthetic peptides (Perry et al., 1991; Pinnaduwage and Bruce, 1996; Schnell et al., 1991) have identified a large number of critical regions in TPs containing information for the import process. However, the functions of most of these regions are still unknown. More importantly, these regions seem to be unique sequences; the same exact sequence could not be found in any two TPs. This makes it difficult to elucidate their function. These identified regions also undermine the hypothesis that translocon component recognition of TPs is based on highly conserved sequence motifs. Instead of exact sequence motifs, a few import critical regions of TPs can be identified using algorithms that quantify amino acid composition. These regions are the N-terminal highly uncharged domain (von Heijne et al., 1989), the Hsp70-interacting domain (Chotewutmontri and Bruce, 2015; Ivey et al., 2000), and the FGLK domain (Chotewutmontri et al., 2012; Pilon et al., 1995). Using quantitative in vitro and in vivo analyses, we established that the location of a N-terminal Hsp70 interaction domain is required to facilitate interaction with the plastid-localized molecular motors (Chotewutmontri and Bruce, 2015). This motif is followed by a second region that interacts with the TOC receptor proteins, which appear to promote TP binding, but cannot support import alone. This region is termed "FGLK," which is defined as having the following: an aromatic residue; a turn-inducing or helix-breaking residue, a small nonpolar residue, a basic residue, and lacking any negatively charged residue (Chotewutmontri et al., 2012). Even though the FGLK motif is semiconserved in TP sequences, its global importance to the recognition and import of preproteins remains unclear. Many steps in the general import pathway may recognize TP by physicochemical properties, which would also explain the lack of conserved motifs in TPs.

3.2.2 Cytosolic and Lipid Modulators of Plastid Targeting

Precursors are proposed to maintain import competency by interacting with cytosolic chaperones (Jackson-Constan et al., 2001). Multiple studies have investigated this in both mitochondria and chloroplasts. One of the common

proteins identified in both systems are members of the hsp70 class of chaperones. In many cases, this work has relied on in vitro studies that show a direct interaction. Unfortunately, hsp70s are essential cellular proteins and cannot be deleted or completely downregulated. Although multiple Hsp70 binding sites have been shown in the majority of TPs (Ivey et al., 2000; Rial et al., 2000; Zhang and Glaser, 2002) their role both in vitro and in vivo is not clear. For example, while cytosolic Hsp70 is essential for the in vitro import of a membrane protein, the precursor of the light harvesting chlorophyll a/b-binding protein (Waegemann et al., 1990), it is not necessary for the in vitro import of precursors of soluble proteins such as ferredoxin (prFD) (Pilon et al., 1990) and the small subunit of RuBisCO (prSSU) (Dabney-Smith et al., 1999). Clearly more work needs to be done to elucidate the role of cytocolic hsp70 in plastid protein targeting.

Another biochemically identified cytosolic factor, the guidance complex composed of 14-3-3 and Hsp70 proteins, improves the efficiency of import (May and Soll, 2000) 14-3-3 recognizes the phosphorylated TPs (May and Soll, 2000). This guidance complex was proposed to deliver the precursor to membrane receptors TOC64, TOC159, or TOC34 (May and Soll, 2000). Later experiments showed that the complex associates with TOC34 but not with TOC64 (Qbadou et al., 2006). Furthermore, some precursors associate with another cytosolic chaperone, Hsp90. This chaperone delivers precursors to TOC64 through the interaction between tetratricopeptide repeats of TOC64 and Hsp90 (Qbadou et al., 2006). The precursor is subsequently transferred to TOC34 (Qbadou et al., 2006). In addition, TP receptor TOC159 (Ma et al., 1996; Perry and Keegstra, 1994) has been suggested to distribute both in soluble cytosolic and membrane-bound forms (Hiltbrunner et al., 2001) and it has been proposed that cytosolic TOC159 interacts with precursor proteins and shuttle them to the translocon.

Although cytosolic factors were shown to associate with some precursors and in certain cases increase import efficiencies, these factors were found to be nonessential both *in organello* (Dabney-Smith et al., 1999; Pilon et al., 1990) and in vivo (Aronsson et al., 2007; Hofmann and Theg, 2005c; Nakrieko et al., 2004). The rate of *in organello* protein import without cytosolic factors was also suggested to be sufficient to sustain chloroplast development (Pilon et al., 1992b). Holbrook et al. (2016) combined detailed analysis of TP import using *in organello* and in vivo assays. These analyses are summarized in Fig. 5 and provide a highly quantitative and resolved biochemical insight while confirming a clear physiological role in living cells. However, it is clear that there are differential results between the *in organello*

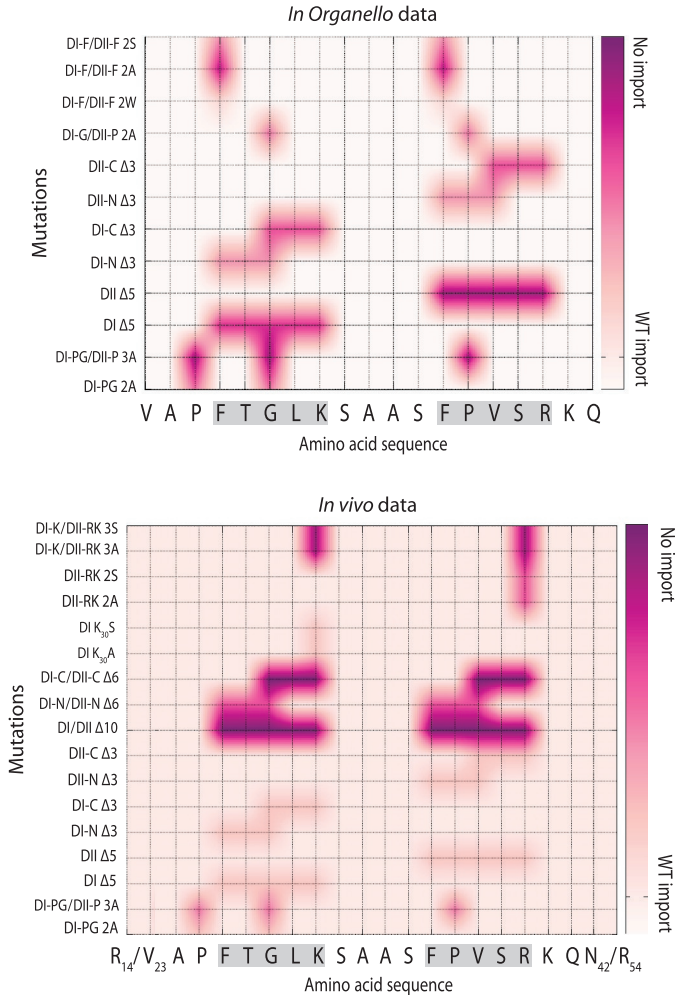

Fig. 5 *In organello* and in vivo TP import results. *Top* (*in organello*) and *bottom* (in vivo) heat maps summarize import results using mutants of RuBisCo small subunit TP, removing one or both FGLK physicochemical domains as indicated. *Magenta color* corresponds to a stronger abrogation of import in the tested mutants.

and in vivo results. Although mutagenesis of a single FGLK physicochemical domain shows a clear import defect in in vitro assays, mutagenesis of both domains is required to abrogate in vivo import. This effect is likely due to the presence of cytosolic factors in the in vivo experiments that can partially compensate for loss of a TP physicochemical domain.

Plastid outer envelope lipids have been implicated to have a role in precursor targeting and recognition. The composition of lipids making up plastid envelope membranes resembles that of cyanobacteria (Joyard et al., 1991). These membranes contain high levels of glycerolipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfolipid (SL), and low levels of phospholipids, phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylinositol (PI). The composition of the lipids in plastids seems to be maintained over all plastid forms (Joyard et al., 1991). The presence of galactolipids, MGDG, and DGDG make the outer membrane of the plastids unique among the cytosolically exposed membranes (Bruce, 1998).

The roles of lipids in the general import pathway have been analyzed mainly through the interactions with TPs (Pilon et al., 1995; Pinnaduwage and Bruce, 1996; van't Hof and de Kruijff, 1995b; van't Hof et al., 1991, 1993). TPs interact specifically with MGDG containing membranes (Pilon et al., 1995; Pinnaduwage and Bruce, 1996), which has been proposed to be mediated by hydroxylated amino acids (Pilon et al., 1995). A study using an *Arabidopsis dgd1* mutant containing less than 10% wild-type DGDG levels showed that the mutant chloroplasts import proteins to the stroma slower than the wild-type chloroplasts (Chen and Li, 1998). This import rate correlated with the reduction of the early import intermediate suggests that the increased proportion of MGDG in *dgd1* mutant envelope might trap the precursor at the energy-independent binding state (Chen and Li, 1998). Another study using an *Arabidopsis* MGDG synthase mutant (*mgd1-1*) producing 42% wild-type MGDG levels showed that protein import was not affected, which may be due to the high levels of remaining MGDG (Aronsson, 2008). However, how TP–lipid interactions function in the import process remains unclear. However, the ability of TPs to reorient MGDG containing bilayers has been hypothesized to be involved (Bruce, 1998; Chupin et al., 1994). Another possibility is based on the observations that TPs only adopt secondary structures upon binding to lipids. It was proposed that the TP-chloroplast membrane interaction triggers the folding of a specific recognition element for protein import (Bruce, 1998).

3.2.3 TOC Apparatus

3.2.3.1 Core Complex

The TOC-TP recognition event is the first step in the general import pathway in chloroplasts. The TOC core complex is composed of three subunits, TOC159, TOC75, and TOC34, and forms a heterooligomeric complex

ranging from 500 to 1000 kDa (Chen and Li, 2007; Kikuchi et al., 2006; Schleiff et al., 2003b). The subunits are named based on their predicted molecular weights in kiloDalton (Schnell et al., 1997). The stoichiometry of these subunits is still debatable. Ratios of 4:4:1 and 6:6:2 for TOC34:TOC75:TOC159 have been proposed (Kikuchi et al., 2006; Schleiff et al., 2003b). This complex size difference also indicates the possibility of dynamic complex forming higher order structure (Jarvis, 2008). Both TOC159 and TOC34 contain a GTPase domain (Kessler et al., 1994) while TOC75 is a beta-barrel protein (Hinnah et al., 1997). The size and components of the active TOC core complex are dynamic. Through the course of plastid differentiation, plastids can increase in size greater than 100-fold and proteins, lipids, and cofactors necessary for photosynthesis accumulate (Mullet, 1988). The chloroplast import apparatus must therefore be able to accommodate the increase in the expression of key photosynthetic proteins. The protein import capability of chloroplasts decreases over the course of development and has been experimentally determined to decline as much as 20-fold in in vitro import assays (Dahlin and Cline, 1991). Thus, the core complex can be modified and its size depends on the metabolic and developmental state of the cell. Two-dimensional electrophoresis, utilizing BN-PAGE in the first dimension and SDS-PAGE in the second dimension, has been shown to be a useful system for the determination of the oligomeric status of membrane protein complexes (Schagger et al., 1994). We used this system to investigate the oligomeric states of core complex members TOC159, TOC75, and TOC34 in developing pea plants. Fig. 6A–F summarizes our analyses. Chloroplasts isolated from young pea plants have more TOC159 associated with the TOC/TIC supercomplex and more intact TOC159 participating in complexes in the 1400–630 kDa range (Fig. 6A and D). The distribution of TOC75 is unchanged throughout the age ranges (Fig. 6B and D), but young plants have more TOC34 associated with complexes in the 1400–880 kDa range (Fig. 6C and E). This increased association of receptors with large complexes suggests that the TOC complexes may be importing precursors with greater efficiency due to increased binding. These experiments compare the ratio of TOC components in a particular size range to the total amount observed, which only allows the comparison of the proportions of TOC components associated with complexes of differing sizes, but not an overall change in the number of complexes with respect to age. However, it is clear that the core complex is dynamic and this modulation represents another level of regulation on the general import pathway.

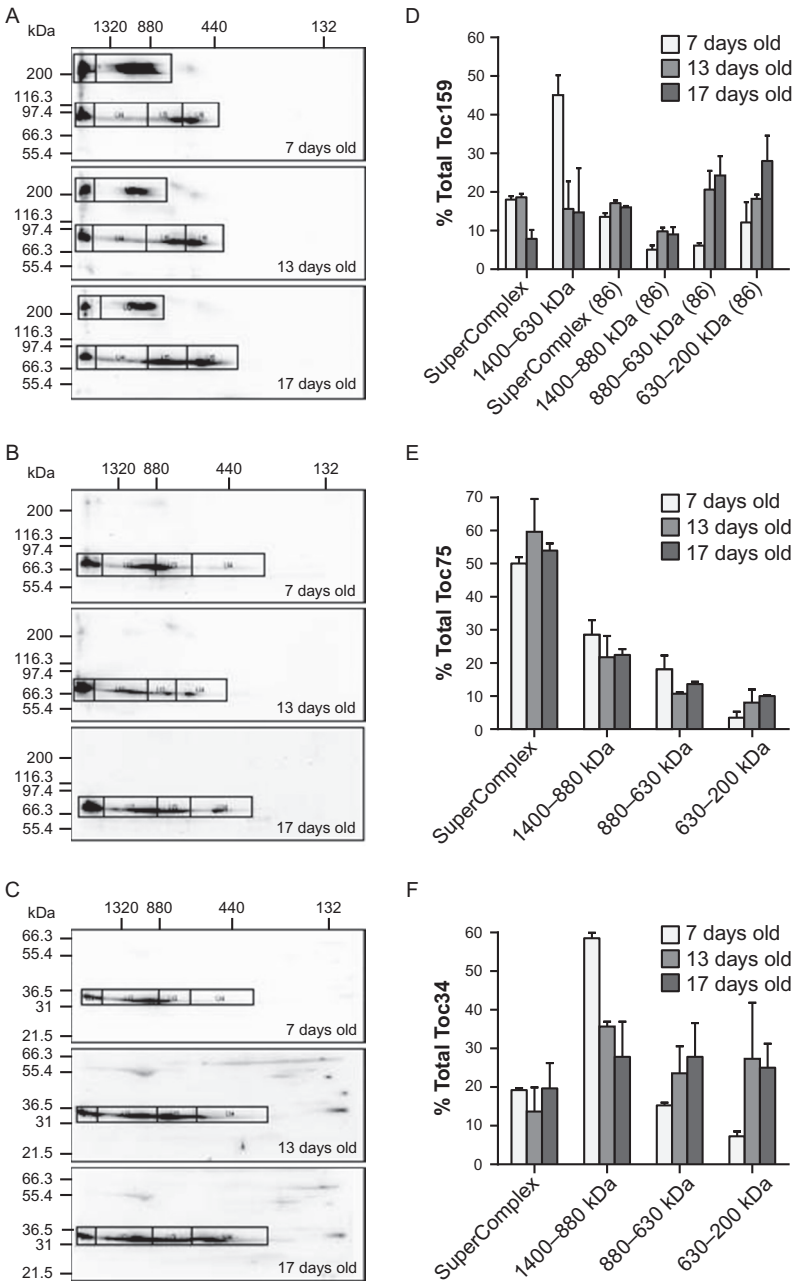

Fig. 6 Dynamics of the TOC core complex. (A)–(C) show representative 2D analysis (blue-native electrophoresis and SDS-PAGE) of complexes from chloroplasts isolated from pea plants at 7, 13, and 17 days old. Blots were probed with antibodies against TOC159 (A), TOC75 (B), or TOC34 (C). (D)–(F) show graphical quantification of the % signal for each complex species at 7, 13, or 17 days old. Quantification was generated by measuring the pixel intensity of each species using QuantOne software and GraphPad Prism 5.0 was used to generate the graphs.

3.2.3.2 Identification of TOC Core Components

TOC86, the proteolytic form of TOC159, was identified as a major interacting partner of prSSU during binding (Ma et al., 1996; Perry and Keegstra, 1994). Also, antibodies against TOC86 were found to inhibit prSSU binding to chloroplasts (Hirsch et al., 1994). These findings support a role of TOC159 as a primary receptor for precursor proteins (Hirsch et al., 1994; Kessler et al., 1994). Yet, chloroplasts lacking TOC159-binding activity are able to import prSSU with lower efficiency (Chen et al., 2000). The intact form of TOC86 and TOC159 was discovered after the *Arabidopsis* genome became available (Bolter et al., 1998; Chen et al., 2000). TOC159 contains three domains, the N-terminal acidic or "A domain," the central GTPase or "G domain," and the C-terminal membrane anchor or "M domain" (Chen et al., 2000). The A domain is intrinsically disordered (Richardson et al., 2009) and provides selectivity for precursor protein binding (Inoue et al., 2010). The G domain is required for insertion of TOC159 into the outer envelope (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003). Recently, the M domain was found to function as a reverse TP. Unlike TP, which is located at the N-terminus of the proteins, the M domain is located at the C-terminus of TOC159. It has been shown that the reverse sequence from C- to N-termini of the M domain fused to the N-terminal of GFP functioned as TP and was able to direct GFP into the chloroplast stroma (Lung and Chuong, 2012).

TOC34 was identified from isolated translocons in complex with a precursor protein (Schnell et al., 1994) and was shown to contain two domains, the N-terminal GTPase domain (G domain) and the C-terminal membrane anchor domain (M domain) (Kessler et al., 1994; Koenig et al., 2008a; Seedorf et al., 1995; Sun et al., 2002). The crystal structures of the G domain of TOC34 (TOC34G) are related to those of the small GTPases of the Ras family (Koenig et al., 2008a; Sun et al., 2002). TOC34G also formed dimers in the crystal where the Arg133 was proposed to function similarly to the Arg finger of GTPase-activating proteins (GAPs) of Ras GTPases (Kessler and Schnell, 2002).

TOC75 was discovered at the same time as TOC86 (Perry and Keegstra, 1994). It was shown to be an integral membrane protein (Schnell et al., 1994) forming 16- or 18 beta-stranded barrel structures (Hinnah et al., 2002; Inoue and Potter, 2004; Schleiff et al., 2003a; Sveshnikova et al., 2000a). Electrophysiological measurements show that TOC75 has an intrinsic ability to specifically recognize TP and has a pore with a diameter at the restriction zone of about 14 Å (Hinnah et al., 2002). The mature TOC75 harbors three repeats

of polypeptide transport-associated (POTRA) domains followed by the C-terminal beta-barrel domain (Reddick et al., 2008; Sanchez-Pulido et al., 2003). Although a study showed that the N-terminal POTRA domains are localized in the cytosol (Sommer et al., 2011), susceptibility of POTRA domains to proteolysis, and the specific interaction with an IMS component TIC22 suggest that POTRA domains are in the IMS (Paila et al., 2016). POTRA domains are shown to have multiple essential roles in TOC75 function by interacting with TP, TIC22, and the assembly of the TOC complex (Paila et al., 2016).

3.2.3.3 GTPase Cycle of TOC Receptors: TOC34 and TOC159

GTPases exert their control by changing between an inactive GDP-bound state and an active GTP-bound state, thereby acting as molecular switches (Gasper et al., 2009). The common denominator of GTPases is the highly conserved guanine nucleotide-binding (G) domain that is responsible for binding and hydrolysis of guanine nucleotides. Both TOC34 and TOC159 belong to the paraseptin group of GTPases, which is most closely related to the septin GTPase group (Weirich et al., 2008). Similar to other paraseptin members, TOC GTPases contain the five motifs (referred to as G1–G5) characteristic for GTP/GDP-binding proteins (Sun et al., 2002). In addition, paraseptin GTPases contain multiple expansion segments including additional helices at N- and C-termini, insertions between helix 4 and strand 6 and between strand 6 and helix 5 as shown in Fig. 7 (Sun et al., 2002; Weirich et al., 2008). The paraseptin and septin GTPase group together form the septin family which are oligomer-forming GTPases (Weirich et al., 2008). The dimerization of TOC34 has already been shown to influence chloroplast protein import (Aronsson et al., 2010; Lee et al., 2009b). A large number of researchers have focused on elucidating the GTPase cycles of TOC receptors to further understand the protein import process, however, some of the results were shown to be conflicting.

TOC34 was crystallized nearly 15 years ago (Sun et al., 2002). Structurally, the overall fold of the TOC34 monomer is divided into a globular GTP-binding domain (G domain) and a small C-term alpha helix. TOC34 is remarkably similar in structure to the well-studied small GTPase p21RAS. Structural superposition of Ras-GppNp and TOC34-GDP reveals that TOC34 has six sequence inserts I1–I6 (Fig. 7), which except for I5, have implications in dimerization and GPTase activity (Pai et al., 1989; Sun et al., 2002). Despite this high-resolution structural data, many aspects of TOC34 function, as well as the physiological function of GTP

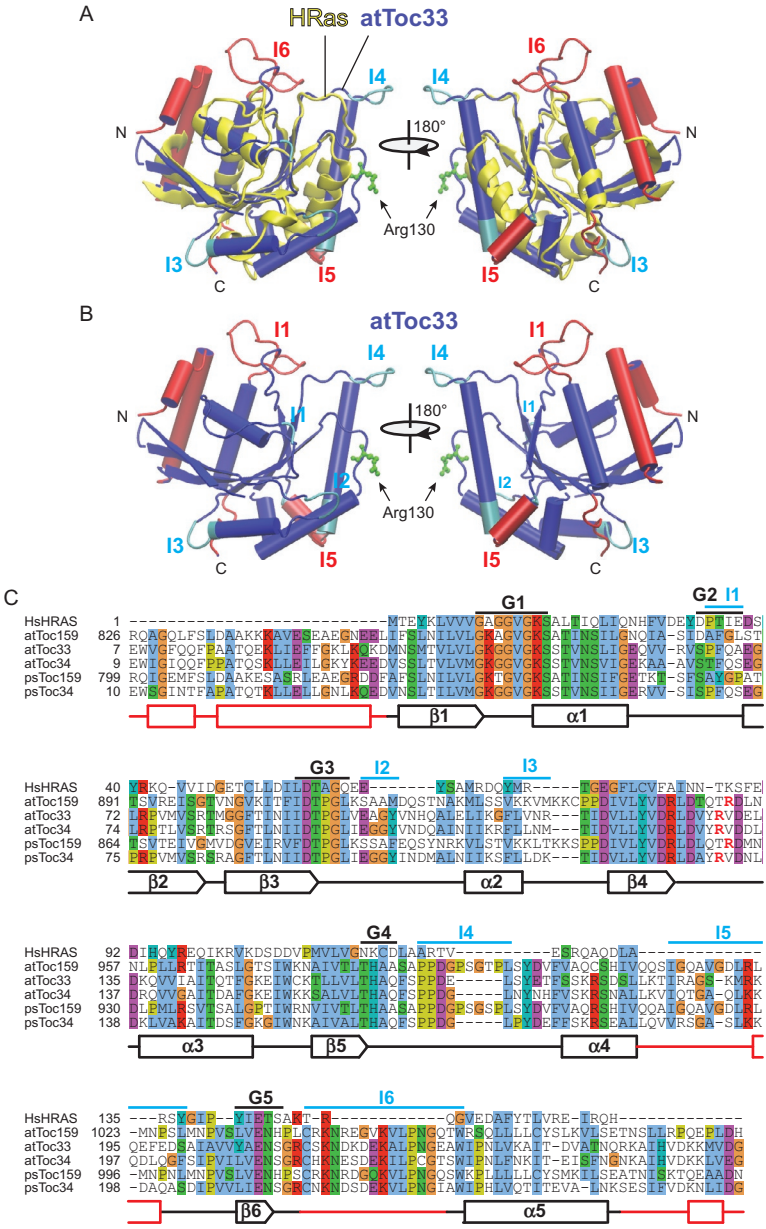

Fig. 7 TOC GTPase domains and Ras alignment. (A) Structural alignment of atToc33 (PDB 3BB4) and human p21Ras (HRas; PDB 5B2X) using jFATCAT tool on PDB server. Paraseptin GTPase atToc33 shows expansion segments (in *red* and *cyan*) in comparison to Septin GTPase HRas (in *yellow*). I1–5, sequence inserts; G1–5, GTP/GDP-binding protein motifs. (B) Structure of atToc33 as in panel A but without HRas. (C) Alignment of TOC GTPases with HRas. *Top label* indicates features of TOC GTPases as in panel A. The secondary structure depiction at the bottom was derived from atToc33 (PDB 3BB4), psToc34 (PDB 3BB1), and HRas (PDB 5B2X).

hydrolysis on protein import, remain unclear. Homodimerization of TOC receptors are common; atTOC33, psTOC34, and psTOC159 have been shown to dimerize (Koenig et al., 2008a,b; Oreb et al., 2011; Reddick et al., 2007; Sun et al., 2002; Yeh et al., 2007). Heterodimerizations between atTOC33 and atTOC159 and between psTOC34 and psTOC159 were also observed (Bauer et al., 2002; Becker et al., 2004; Hiltbrunner et al., 2001; Rahim et al., 2009; Smith et al., 2002). Dimerization of atTOC33 was determined to be involved in the transition of precursor proteins from binding to translocation state of the import (Lee et al., 2009b).

It is clear that at least three factors have been found to affect dimerization in vitro. (i) The first factor contributing to dimerization is the protein concentration. Monomer–dimer equilibrium of atTOC33 and psTOC34 depends on their concentrations with the dissociation constants (K_d) around 400 and 50 μM, respectively (Koenig et al., 2008a; Oreb et al., 2011; Reddick et al., 2007). Based on the closed proximity of TOC34 in the core TOC complex (Schleiff et al., 2003b), TOC34 is likely to favor a dimer form in the complex (Lumme et al., 2014). (ii) Nucleotide loading state is a second factor that affects dimerization. In both atTOC33 and psTOC34 cases, the GDP-bound forms produce more dimers than the GTP-bound forms (Koenig et al., 2008a; Lumme et al., 2014). The same effect was also reported in the heterodimerization between atTOC159 and atTOC33 (Smith et al., 2002). Surprisingly, the addition of a GTP transition state analog, aluminum fluoride, shifted the equilibrium of both atTOC33 and psTOC34 to exclusively become dimers (Koenig et al., 2008b). These findings and recent work using FRET interaction analysis indicate that the dimerization is likely triggered by GTP hydrolysis (Lumme et al., 2014). Lastly, (iii) the effect of TP on TOC receptor dimerization and function has been the subject of many studies. GTP-bound forms of atTOC33 (Gutensohn et al., 2000), psTOC34 (Jelic et al., 2002; Schleiff et al., 2002), and psTOC159 (Becker et al., 2004; Kouranov and Schnell, 1997) have higher affinities for TP than the GDP-bound forms. TP is well known to stimulate TOC receptor GTP hydrolysis (Becker et al., 2004; Jelic et al., 2003; Oreb et al., 2011; Reddick et al., 2007). Two separate roles of TP in GTP hydrolysis have been reported. First, it was shown that TP stimulated GTP hydrolysis of psTOC34 while maintaining the same nucleotide exchange rate suggesting a role of TP as a GAP but not guanine nucleotide exchange factors (GEF) (Reddick et al., 2007, 2008). Since GAP and GEF can both increase GTP hydrolysis, but GAP lowers the transition state energy (Scheffzek et al., 1997) while GEF stimulates GDP exchange (Bos et al., 2007). Interestingly, another study found that

TP stimulated GTP hydrolysis of atTOC33 only when atTOC33 was in the dimeric form but not in the monomeric form (Oreb et al., 2011). Further analysis found that TP stimulated atTOC33 GTP hydrolysis through interacting with the dimer and increases the nucleotide exchange rate, which suggests the role of TP as a GDP-dissociation inhibitor displacement factor, where each atTOC33 in the dimer acts as GDP-dissociation inhibitor and TP disrupts the dimer (Oreb et al., 2011). Although the TOC GTPase cycle has been studied in detail in vitro, less is understood about how the system works in concert to contribute to precursor import.

3.2.3.4 Models of TOC Receptor Function

How TOC receptors function together in protein import is at an early stage of understanding. There have been several models developed to explain how these GTPases mediate preprotein recognition and targeting (Bedard and Jarvis, 2005; Kessler and Schnell, 2004; Reddick, 2010; Smith, 2006). The "targeting model" (Keegstra and Froehlich, 1999) implicates cytosolic TOC159 in the capture of precursor proteins in the cytoplasm (step 1b of Figs. 1–3) and their targeting to the plastid surface (Bauer et al., 2002; Hiltbrunner et al., 2001; Lee et al., 2003; Smith et al., 2002). TOC159-precursor complex is proposed to interact with TOC34 (Fig. 3, step 2a) through the G domains (Bauer et al., 2002). This process is likely controlled by GTPase cycling, which results in the transfer of precursor to the TOC75 channel to initiate translocation (Smith et al., 2002). In the "motor model" (Becker et al., 2004), TOC34 on the outer envelope is proposed to act as the primary receptor (Fig. 3, step 1a). TOC159 in this case is proposed to function as a GTPase motor pushing precursor proteins through the TOC75 channel (Soll and Schleiff, 2004). The "TOC Clock model" (Reddick, 2010) was developed through analysis of TPs, psTOC159, and psTOC34 interactions together with GTP hydrolysis. It was proposed that in the inactive state, both TOC159 and TOC34 are in GDP-bound forms. Differential interactions with GTP and TP are hypothesized to modulate the affinity of the TOC receptors for TP. In this model, TP acts as GAP stimulates TOC34 GTP hydrolysis, and high to low affinity interactions between TOC34 and TP allows the precursor protein to be imported into the plastid. Recent studies have classified TOC34 as a member of the GAD GTPase family, a subclass of small GTPases that are activated by nucleotide-dependent dimerization (Gasper et al., 2009; Lumme et al., 2014). Putative GAD members have a role in a wide variety of cellular processes, including membrane fusion and fission (atlastin) (Bian et al., 2011), vesicle trafficking (dynamin)

(Chappie et al., 2010), and cytokinesis (septin) (Sirajuddin et al., 2009). GADs possess all the structural elements of the classic "switch" mechanism of small GTPases, but reciprocally activate their catalytic sites in the dimeric state, rendering classic GEFs and GAPs unnecessary for activity (Gasper et al., 2009). The proposed GAP function of TPs is not at odds with a GAD mechanism for TOC34. Based on FRET and PELDOR analysis, Lumme et al. (2014) suggest that TOC34 can interact with TPs in a dimeric GDP-bound state, and that GTP alters TOC34 conformation to a "looser" structure that would allow for precursor protein import through the TOC75 beta-barrel channel. Thus, TP GAP may function as a regulatory switch to ensure the specificity of plastid import. Ultimately, more detailed analysis is required to understand how the TOC GTP hydrolysis cycle contributes to the mechanism of TP recognition and precursor import. Because both TOC34 and TOC159 hydrolyze GTP, the ability to distinguish the catalytic events of one TOC GTPase protein over the other would aid in understanding the overall import process. It is likely that the detailed mechanism of import involves a combination of several of these models, and import regulation may depend on the metabolic and developmental states of the plant.

3.2.3.5 Preprotein Recognition by TOC GTPases

How diverse TPs are selectively recognized and processed by the TOC translocon complex? This is still a paradox, since several 1000 different TPs exist in a given plant species, yet show very little similarity with each other—however, they seem to function in a common pathway that involves the recognition of one or more TOC GTPases, which are proposed to function as "gatekeepers." Analysis of plant genomes has revealed that ~2100 proteins in *Arabidopsis thaliana*, and up to ~4800 proteins in *Oryza sativa* (rice), are imported from the cytosol to their functional location in the plastid (Richly and Leister, 2004). We propose a bimodal model for TP recognition (Fig. 8A), where TPs are recognized at the plastid surface through interactions between the TOC receptors and the semiconserved physicochemical FGLK motif (Holbrook et al., 2016). After GTP hydrolysis, the TP can access the stroma, where the second recognition event occurs, interaction between the N-terminal Hsp70 interaction domain and the stromal motors (Fig. 8A and B). This two-part interaction model would explain how the TOC translocon is able to manage such a wide variety of TP sequences (Fig. 8B). Careful analysis of each component of the TOC core complex is necessary to understand how the complex can balance import specificity while recognizing a diverse range of TPs.

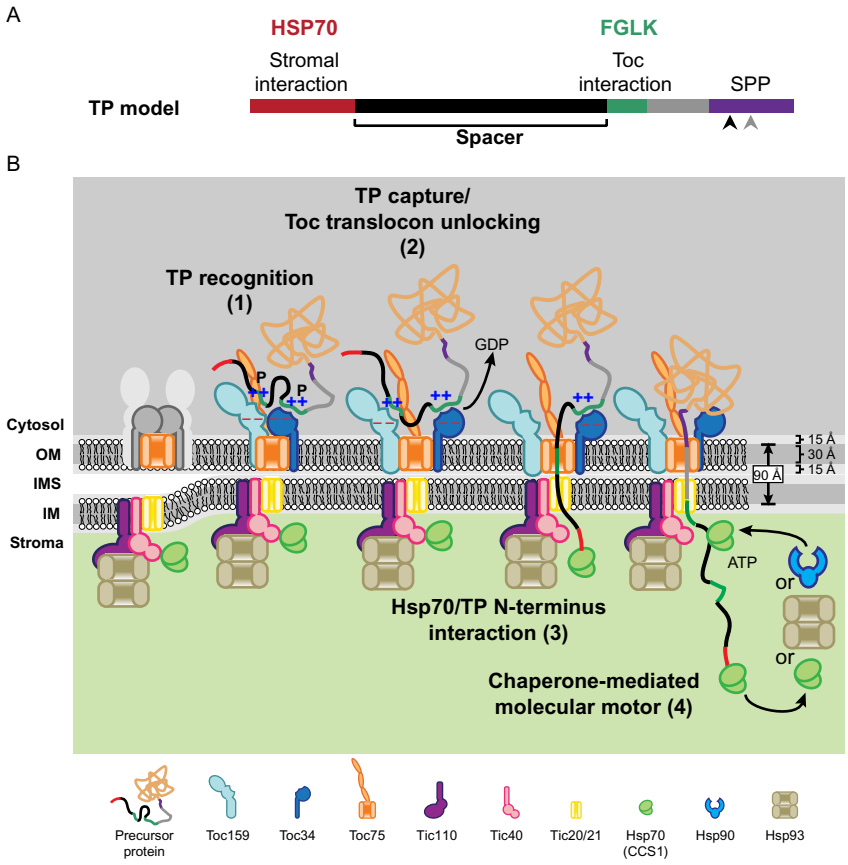

Fig. 8 Bimodal interaction model for transit peptide recognition. (A) The majority of TPs contains an N-terminal Hsp70-interacting domain (HSP70) linked to a FGLK motif (FGLK). SPP, the stromal processing peptidase. (B) We proposed the bimodal interaction model where TP is captured by Toc receptor via the FGLK motif. An optimal length spacer allows the stromal molecular motor such as Hsp70 to trap/pull the N-terminal Hsp70-interacting domain to initiate the translocation within a rapid timeframe.

3.2.3.6 Protein Conducting Channel: TOC75

Most models of chloroplast protein import suggest that the protein conducing channel across the outer envelope is a beta-barrel protein that is characteristic of bacterial outer membranes and the outer membrane of chloroplasts and mitochondria. The participation of a beta-barrel in protein translocation is a distinction from the IM, the thylakoid, the ER, and the both membranes of the mitochondria, which utilize only alpha-helical bundles in their translocons. It has been shown that the pore of the Toc complex in the plastid outer

membrane is formed by the TOC75 protein, a member of the OMP85/TPS (outer membrane protein of 85 kDa/two partner secretion) superfamily (Andrès et al., 2010; Simmerman et al., 2014). This superfamily is found in outer membranes of gram-negative bacteria, mitochondria, and chloroplasts, consistent with their evolutionary origins. TOC75 is comprised of the typical Omp85/TPS 2-domain architecture, with a N-terminal domain containing soluble (POTRA) domains and a C-terminal beta-barrel channel formed by beta strands (Sanchez-Pulido et al., 2003; Simmerman et al., 2014).

TOC75 has been shown to oligomerize with itself and other members of the TOC core complex (Reddick et al., 2007; Seedorf et al., 1995; Simmerman et al., 2014). TOC75 is the only member of the TOC core complex that contains an N-terminal cleavable TP, however, it is a bipartite sequence (Inoue and Keegstra, 2003). A C-terminal polyglycine stretch appears to be necessary for retention at the envelope. The N-terminal region containing the TP reaches the chloroplast stroma where it is cleaved by signal processing peptidase. The C-terminal portion of the targeting sequence spans the IMS and is cleaved by an envelope bound signal peptidase (Inoue and Keegstra, 2003). Mature TOC75 consists of three soluble N-terminal POTRA domains and a C-terminal channel comprised of up to 16-beta strands (Sanchez-Pulido et al., 2003).

In vitro experiments have established that TOC75 interacts with precursors during import and is associated with envelope bound import intermediates (Hinnah et al., 2002; Ma et al., 1996; Perry and Keegstra, 1994; Schnell et al., 1994). Furthermore, antibodies against TOC75 block protein import (Tranel and Keegstra, 1996). Patch clamp analysis has established that reconstituted pea TOC75 forms a voltage gated ion channel estimated to be 14 Å at its narrowest point (Hinnah et al., 1997). The N-terminal POTRA domains are proposed to function in TOC complex assembly, precursor recognition, or chaperone activity (Simmerman et al., 2014). Recent findings confirmed the multifunctional roles of POTRA domains in TOC assembly, TP recognition, and recruitment of TIC22 (Paila et al., 2016).

3.2.3.7 TOC Complex Assembly

The exact sequence of events in TOC complex assembly remains unclear. However, studies have established that levels of TOC75 protein are significantly higher than both TOC34 and TOC159 at early stages of development during plastid biogenesis (Kouranov et al., 1998). Therefore, it is likely that "free" TOC75 is able to nucleate the assembly of new TOC complexes. The TOC75 channel could facilitate interaction between the

transmembrane helices of the TOC core complex proteins and the lipid bilayer (Hofmann and Theg, 2005b; Richardson et al., 2014). Analysis of atTOC75 revealed that POTRA domains recruit atTOC33 and atTOC159 and the deletions of POTRA domains altered TOC complex assembly (Paila et al., 2016), supporting the nucleation role of TOC75. Reconstitution of the complex from individual components has established that TOC159 insertion is dependent on both TOC75 and TOC34, suggesting that TOC159 is the final element of the core complex assembly (Wallas et al., 2003). Interactions between the G domains of the TOC34 and TOC159 receptors likely maintain the stability and stoichiometry of the complex. This assembly mechanism would suggest that the receptor integration determines the rate of formation of new TOC complexes (Richardson et al., 2014). However, analysis of TOC complexes suggests that core complexes can be structurally distinct based on developmental stage and metabolic conditions (Fig. 6A–F). However, TOC75 is the common element in TOC complexes. Although the measured size of TOC complexes are heterogeneous, most complexes are found at over ~800 kDa, suggesting that the core complex contains two molecules of TOC159 and six molecules each of TOC34 and TOC75 (Chen and Li, 2007; Kikuchi et al., 2006; Schleiff et al., 2003b) (Fig. 6A–F). Excess TOC75 could provide a pool of nucleating protein for rapid assembly of distinct complexes, with varying integration of TOC34/TOC159 family members. Additional analysis of the targeting of the individual TOC core components plastid targeting and expression is required to fully understand the factors that control complex assembly.

3.2.4 TIC Apparatus

The TIC complex was identified to be composed of TIC20, TIC21, TIC22, TIC32, TIC40, TIC55, TIC62, and TIC110 (Bedard and Jarvis, 2005; Kessler and Schnell, 2006; Smith, 2006; Soll and Schleiff, 2004). Their functions in protein import range from facilitating precursor translocation through IMS, TOC–TIC formation, TIC channel formation, stromal motor function, and regulation of import. Many TIC subunits have been hypothesized to form the translocation channel of the inner envelope. However, the first proposed TIC channel was identified via electrophysiological measurement, called protein import-related anion channel (PIRAC) (van den Wijngaard and Vredenberg, 1999). PIRAC opening is regulated by TP (Dabney-Smith et al., 1999; van den Wijngaard et al., 1999) and it was found to associate with TIC110 (van den Wijngaard and Vredenberg, 1999). The other electrophysiological experiment identified

TP regulated cation channel TIC110 as a TIC channel (Heins et al., 2002). This result excludes PIRAC as functioning directly as the TIC channel since all other protein translocation channels are cation selective (Heins et al., 2002). It was later found that TIC110 contains six transmembrane helices and the opening was regulated by Ca^{2+} (Balsera et al., 2009a). TIC110 is also essential for TIC complex formation (Inaba et al., 2005). The other potential TIC channels are TIC20 and TIC21, which are related to the pore-forming subunits of the mitochondria translocon in the inner envelope (Reumann and Keegstra, 1999; Teng et al., 2006). The *attic20* RNAi knockdown and *attic21* knockout plants showed defective translocation across inner membranes (Chen et al., 2002; Teng et al., 2006). While TIC20 is expressed mainly in early development, TIC21 is expressed at a later stage indicating a shared function of these proteins (Teng et al., 2006). Components of the TIC system still require additional research to fully understand their role in the import cycle.

3.2.5 Stromal Motors

It is well established that internal ATP hydrolysis is the driving force for plastid protein translocation (Theg et al., 1989). Since the identification of Hsp70 family of proteins involved in protein import into ER and mitochondria (Chirico et al., 1988; Deshaies et al., 1988; Murakami et al., 1988; Zimmermann et al., 1988), ATPase chaperones have been proposed to drive the translocation of precursor proteins into plastids (Keegstra and Cline, 1999; Marshall et al., 1990). Two classes of chaperones, Hsp70 and Hsp100, have now been established to associate and facilitate the protein translocation. Three Hsp70 proteins associated with chloroplasts were first identified from pea OM and stroma (Marshall et al., 1990). Later, a Hsp70 was identified in the pea chloroplast OM as an import intermediate-associated protein (IAP) (Schnell et al., 1994). This IAP can be detected by antibodies against mammalian cytosolic Hsp70, mAb SPA-820 (Schnell et al., 1994). Because the Hsp70 IAP was protected from an OM impermeable protease thermolysin, it was proposed to localize in the IMS and interact with incoming precursor proteins translocated through TOC75 (Schnell et al., 1994). The gene corresponding to the Hsp70 IAP has not yet been identified. A later study, however, found that the protein recognized by mAb SPA-820 is located in the stroma (Ratnayake et al., 2008). The other outer membrane Hsp70s from pea and spinach, the thermolysin-sensitive Com70s, were shown to associate with various precursors (Ko et al., 1992; Kourtz and Ko, 1997). Com70 also has higher sequence similarity with mammalian cognate Hsp70s than *Escherichia coli*

Hsp70, DnaK (Ko et al., 1992). It was later suggested that Com70 is the cytosolic Hsp70-1 (Guy and Li, 1998; Li et al., 1994). A stromal Hsp70 was later shown to associate with the translocation complexes (Nielsen et al., 1997).

In *Arabidopsis*, the only two Hsp70s predicted to harbor TP were shown to localize in the stroma (Ratnayake et al., 2008; Su and Li, 2008; Sung et al., 2001). These stromal Hsp70s are closely related to the bacterial Hsp70, DnaK (Ratnayake et al., 2008) and were named atcpHsc70-1 and atcpHsc70-2 (Su and Li, 2008). These cpHsc70s were shown to be directly involved in protein import. The *atcphsc70-1 atcphsc70-2* double knockout is lethal and the single knockout plants showed reduced protein import efficiencies (Su and Li, 2010). Biochemical analysis also found atcpHsc70s stably associate with the translocons and the precursors (Su and Li, 2010). The stromal Hsp70, ppHsp70-2 involved in protein import was concurrently identified in moss *Physcomitrella patens* (Shi and Theg, 2010).

The stromal Hsp93 (ClpC), a member of the Hsp100 chaperone family, was first identified to be stably associated with the translocons (Nielsen et al., 1997). Two homologs were found in *Arabidopsis*, atHsp93-V and atHsp93-III (Kovacheva et al., 2005). Crosslinking data suggested that Hsp93, TIC110, and TIC40 function in concert during protein import (Chou et al., 2003), consistent with genetic analysis results (Kovacheva et al., 2005). The molecular interactions between these proteins have been studied. The binding of TP to TIC110 triggers the association of TIC110 with TIC40, which in turn induces the release of TP from TIC110 (Chou et al., 2006). TIC40 was also shown to stimulate Hsp93 ATP hydrolysis. Because it has lower affinity to ADP-bound Hsp93, TIC40 was suggested to act as an ATPase-activating protein of Hsp93 (Chou et al., 2006). Nonetheless, a mutant of Hsp93 that obviates its ability to interact with Clp proteolytic core demonstrates the role of Hsp93 in quality control (Flores-Pérez et al., 2016).

Both Hsp70 and Hsp100 systems are essential for viability of plants (Shi and Theg, 2011). Knockout of each system are lethal such as those observed in *atcphsc70-1 atcphsc70-2* double knockout (Su and Li, 2008), *athsp93-V athsp93-III-2* double knockout (Kovacheva et al., 2007), and *pphsp70-2* knockout (Shi and Theg, 2010). However, these results may reflect not only their roles in protein import but also other roles in plant development (Constan et al., 2004; Lee et al., 2007; Su and Li, 2008). In minimally invasive cases, the knockout lines of a single homolog of each system, such as *atcphsp70-1*, *atcphsp70-2*, and *athsp93-V* single mutants, import efficiencies dropped to about 40–60% (Su and Li, 2010) indicating important roles in protein import of each system. When the functions of both systems were reduced by knocking

out a single homolog from each system such as the *atcphsp70-1 athsp93-V* double knockout plant, import was further reduced to 30% (Su and Li, 2010). This additional reduction compared to the effect of each single mutant, together with the coimmunoprecipitation of Hsp70 and Hsp93 suggested that both systems act in concert in the same complex (Shi and Theg, 2011).

Interesting, quantitative analysis to determine the energetics of preprotein import have shown it to be an energy intensive process. Using two different precursors prepared by three distinct techniques, Shi and Theg have shown that the import of a precursor protein into chloroplasts is accompanied by the hydrolysis of ~650 ATP molecules. This translates to a ΔG protein transport of some 27,300 kJ/mol protein imported. When the cost of preprotein import is expanded to include the complete biogenesis of the organelle, they estimate that protein import across the plastid envelope membranes consumes ~0.6% of the total light-saturated energy output of the organelle (Shi and Theg, 2013b).

3.2.6 Peptidases

The processing of prSSU was discovered nearly 40 years ago (Dobberstein et al., 1977). This finding led to the identification of two SPPs from pea (Oblong and Lamppa, 1992; VanderVere et al., 1995) and one in *Arabidopsis* (Richter and Lamppa, 1998; Zhong et al., 2003). SPP is classified as a member of the metalloprotease M16B subfamily with a zinc-binding motif (Aleshin et al., 2009; Richter and Lamppa, 1998; Richter et al., 2005; VanderVere et al., 1995). It was shown that SPP is the general processing enzyme of plastid protein imports by its ability to proteolyze various precursors (Richter and Lamppa, 1998). SPP specifically binds to the C-terminal 12 residues of TP of prFD (Richter and Lamppa, 1999, 2002, 2003). In vitro, the TP directs precursor binding to SPP (Richter and Lamppa, 1999). The mature domain is released immediately after cleavage of TP while TP remains attached (Richter and Lamppa, 1999). TP is further fragmented before release from SPP (Richter and Lamppa, 1999). The SPP recognition sites at the C-terminus of TP seem to share a weak-binding motif (Emanuelsson et al., 1999; Gavel and von Heijne, 1990). Later, the physicochemical properties at specific residues were proposed to form a SPP-binding motif (Richter and Lamppa, 2002). In fact, the regions at the C-termini of TPs show a positive net charge and are conserved at the position −1 for basic residue and positions −4, −3, −2, and +1 for uncharged residues related to the SPP cleavage sites (Richter and Lamppa, 2002).

Free cleaved TPs in the stroma are potentially harmful (Glaser et al., 2006) and are degraded by presequence peptidases (PrePs) (Bhushan et al., 2005). PrePs were originally identified from the mitochondrial matrix as mitochondrial presequence degradation enzymes (Stahl et al., 2002). They are classified as members of the metalloprotease M16C subfamily (Glaser et al., 2006). *Arabidopsis* has two PrePs, atPreP1 and atPreP2. Both proteins were found to have dual localizations in both chloroplasts and mitochondria (Bhushan et al., 2003, 2005; Moberg et al., 2003; Stahl et al., 2002). In addition, these proteins have different tissue-specific expression patterns; atPreP1 is expressed highly in flowers and can be detected in siliques while atPreP2 is expressed in leaves, shoots, and roots (Bhushan et al., 2005).

3.3 Noncanonical Trafficking

Although the general import pathway directs the transport of thousands of plastid-targeted proteins, noncanonical pathways are critical for organelle function. The chloroplast proteomic data suggest that around 20% of chloroplast proteins lack any predictable targeting sequence (Kleffmann et al., 2004) and around 1–8% of chloroplast-targeted precursors contain an ER signal peptide (Kleffmann et al., 2004; Zybailov et al., 2008). Thus, a small fraction of protein lacking TPs are still able to target to the plastids. In recent years, several alternate targeting pathways have been reported (Fig. 9). Noncanonical transport pathways include vesicle-mediated trafficking, import of tail-anchored (TA)/signal-anchored (SA) membrane proteins, and import of proteins with dual targeting signals to the mitochondria and plastid. In this section, we summarize the current understanding of noncanonical transport, although many aspects of these pathways remain unclear and the focus of ongoing research. Developing a clearer picture of the integration of noncanonical transport with the general import pathway will provide insight into plastid biogenesis and organelle maintenance.

3.3.1 Vesicle-Mediated Trafficking

Organelles cannot synthesize every molecule they require, so a variety of metabolites, lipids, and proteins must be exchanged via the secretory pathway, which is well studied between the ER, Golgi, and plasma membrane. Accumulating evidence suggests that both a cytosol to plastid and intraplastid vesicle delivery system exist for both lipids and proteins (Karim and Aronsson, 2014; Khan et al., 2013; Lu, 2016). In agreement, *Arabidopsis* genome analysis, bioinformatics, and web-based localization prediction tools have confirmed

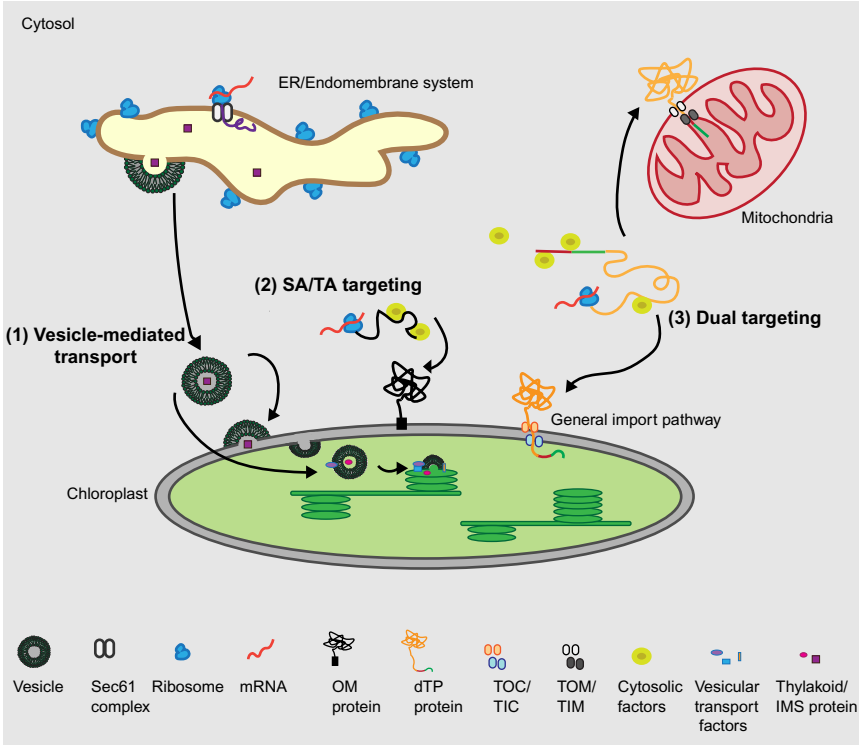

Fig. 9 Noncanonical trafficking pathways. Schematic depicts noncanonical plastid trafficking pathways: (1) vesicle trafficking of a protein from the ER/endomembrane system to the plastid; vesicle trafficking of a protein/lipids from the IM to the thylakoid; (2) SA/TA protein cytosolic translation and insertion into the plastid OM; and (3) dual targeting of a protein to the mitochondria and plastid through the TOM/TIM (mitochondria) and TOC/TIC complexes.

that there are plastid-localized proteins with high sequence similarity to the components of the cytosolic vesicular system (Khan et al., 2013). Analyses have identified eight COPII-related proteins, as well as proteins related to SNAREs and RABs (Andersson and Sandelius, 2004; Brandizzi, 2011). Furthermore, active GTPase homologs to SAR1 and dynamin were identified (Khan et al., 2013). Thus, intraplastid vesicular trafficking appears to use a system analogous to COPII-mediated transport. However, even though putative components of the intraplastid vesicular trafficking mechanism have been identified, substantial research is required to understand their specific roles in transport.

The evolutionary basis for plastid vesicle trafficking has been the focus of several studies. Interestingly, several of the proteins implicated in plastid vesicular trafficking are conserved in cyanobacteria (Keller and Schneider, 2013;

Khan et al., 2013), implying that vesicle trafficking may have developed from endosymbiosis. However, there are also clear similarities with the cytosolic system, suggesting possible transfer of secretory pathway components to the plastid via divergent evolution (Vothknecht and Soll, 2005). Analysis of organisms from diverse lineages suggests a late evolutionary development of plastid vesicular trafficking (Westphal et al., 2003). However, new phylogenetic analyses of *A. thaliana* and *O. sativa* suggests that vesicular trafficked proteins are of eukaryotic (not cyanobacterial) origin (Gagat et al., 2013). The early eukaryotic host-derived proteins were already targeted to the endomembrane system by their signal peptide and preadapted to be targeted to plastids via the same system. Furthermore, endomembrane transport was the only viable plastid import route for several eukaryotic proteins that require posttranslational modifications or dual targeting to the plastid and cell wall. The phylogenetic results are consistent with a later development of endomembrane trafficking to plastids (Gagat et al., 2013). It is likely that the system developed in response to selective pressure to maintain a complex thylakoid membrane (Karim and Aronsson, 2014), which is spatially separated from the chloroplast envelope by the aqueous stroma in higher plants.

The lipids that comprise the thylakoid are synthesized through a pathway that involves exchange of lipid precursors from the ER to the IM; the final lipid products are produced in the IM but must reach their destination in the thylakoid (Douce and Joyard, 1990). Although there are several hypotheses for lipid transfer, both ultrastructural and biochemical evidence supports a vesicular-mediated trafficking system for lipid movement to the thylakoid (Karim and Aronsson, 2014). Although vesicular lipid movement has been characterized, protein targeting through vesicles is less defined. Furthermore, it remains unclear if vesicles only play a role in intraplastid localization of proteins, or if proteins can be targeted from the cytosol to the plastid via vesicles. Two such proteins that utilize vesicle trafficking from the cytosol contain signal peptide for ER targeting, carbonic anhydrase 1 (Villarejo et al., 2005), and nucleotide pyrophosphatase/phosphordiesterase 1 (Nanjo et al., 2006), were shown to localize to plastids. Additionally, a recent study searched for nuclear-encoded proteins that may be trafficked to the thylakoid via vesicles. Bioinformatic analysis identified homologs to thylakoid-localized proteins with a COPII selection motif and found 21 transmembrane and 12 soluble candidates (Khan et al., 2013). About 45% of these candidates are linked to photosynthesis, implying that a subset of nuclear-encoded proteins are trafficked to the thylakoid via vesicles (Khan et al., 2013). Furthermore, RNAi knockdown of a candidate SAR1

homolog GTPase (*cspar1*) resulted in downregulation of several nuclear-encoded photosynthetic proteins, suggesting they are potentially trafficked via vesicles (Bang et al., 2012). Thus, both bioinformatics and experimental data support a role for vesicles in both intraplastid delivery and cytosol to plastid-mediated protein transport. Developing a better understanding of this system could have important implications in plastid biogenesis, thylakoid maintenance, and assembly of the photosynthetic apparatus (Lu, 2016).

3.3.2 SA and TA Proteins

Outer membrane proteins play a key role in intracellular communication, including exchange of metabolites and lipid synthesis. At least six pathways mediate protein targeting to the outer envelope of plastids (Hofmann and Theg, 2005a; Jarvis, 2008; Richardson et al., 2014) (Fig. 2). Furthermore, correct localization of plastid outer membrane proteins is critical for the function of the general import pathway. Outer membrane proteins are a group of diverse proteins that can be divided into alpha-helical transmembrane domain (TMD) containing proteins and beta barrels consisting of multiple transmembrane beta strands (Inoue, 2015). TMD containing proteins can be further classified based on the number and location(s) of their TMD sequence (N-terminal, middle, or C-terminal) (Lee et al., 2014). These proteins are nuclear encoded and synthesized on cytosolic ribosomes; they have distinct, specific requirements for post-translationally targeting to their functional location. In plastids both SA and TA proteins play an important role in outer membrane communication. Although both SA and TA proteins have hydrophobic TMD domains that direct insertion into the plastid OM, the secondary signals that mediate organelle specificity are not well understood. Physicochemical domains near the TMD, such as positively charged residues, seem to play a role in trafficking (Lee et al., 2014). Cytosolic factors likely play a role in the posttranslational insertion of SA/TA proteins. Some OM proteins also contain a specialized TP sequence. For example, TOC75 utilizes a bipartite targeting sequence composed of stromal TP and envelope targeting signal (Inoue and Keegstra, 2003; Tranel and Keegstra, 1996). The insertions of OEP14, TOC159, and TOC75 also require some components of the general import pathway. Other outer envelope targeting pathways have been shown but are not well characterized (Hofmann and Theg, 2005a).

The most studied pathway for OM proteins requires an N-terminal TMD targeting signal anchor, which is found in proteins such as the outer envelope protein of 14 kDa (OEP14) and the TOC subunit of 64 kDa (TOC64) (Hofmann and Theg, 2005b; Lee et al., 2001). SA proteins are

a class of OEPs with a single TMD that acts as both the localization signal and membrane anchor (Rapoport, 2007). SA proteins lack a cleavable TP; a moderately hydrophobic TMD acts as the primary targeting signal. Another required motif for targeting of SA proteins appears to be a C-terminal positively charged domain (Rapaport, 2003). However, the distinguishing the similar localization signals for plastids vs mitochondria has proven challenging (Lee et al., 2011, 2014). Another pathway involves a C-terminal "tail anchor" TMD targeting sequence such as those present in TOC34 and TOC159 (Smith et al., 2002; Tsai et al., 1999). TA proteins contain a single C-terminal TMD and basic residues in a C-terminal sequence. Similar to SA proteins, TA proteins lack a cleavable TP. Plastid TA sequences do not share any obvious conserved sequence motifs, but they have significantly higher hydrophobicity index vs mitochondrial TA proteins (Lee et al., 2014).

Only a few plastid TA proteins have been studied in depth, including TOC complex receptors TOC33 and TOC34. The C-terminal TMD domain of these proteins is necessary but not sufficient for OM localization; the GTPase domain of these proteins is required as well (Dhanoa et al., 2010). However, the exact targeting mechanism is unclear, because a truncated form of the TOC159 GTPase domain lacking a TA domain still correctly localizes to the OM (Smith et al., 2002). Thus it appears that the TA sequence is not always sufficient and other sequence context is necessary for correct localization. The mechanisms that regulate SA/TA protein organelle specificity and insertion will be the key to understanding organelle biogenesis and plant development in depth.

3.4 Dual Targeting to Plastids and Mitochondria

Analogous to plastids, mitochondria import more than 1000 proteins that are nuclear encoded and synthesized in the cytosol as precursor proteins (Murcha et al., 2014). The mitochondrial protein import complex is a set of multisubunit protein complexes that recognize and transport mitochondrial proteins to their functional location (Schulz et al., 2015). The majority of mitochondrial proteins destined for the mitochondrial matrix contain a cleavable N-terminal presequence that is necessary and sufficient for import (Murcha et al., 2014; Schulz et al., 2015). Mitochondrial presequence amino acid composition is remarkably similar to chloroplast TPs. Mitochondrial presequences exhibit a high concentration of hydrophobic and positively charged residues, with an abundance of both proline and glycine, and an absence of negative charge (Bhushan et al., 2006; Huang et al., 2009a).

Comparison of TPs and presequences suggests that specificity is likely determined by precise structural and physicochemical motifs that are required for explicit recognition and translocation (Bhushan et al., 2006; Huang et al., 2009b). However, a subset of proteins functions in both mitochondria and chloroplasts. Mechanisms for dual import must balance both organelle selectivity but maintain the ability to target either organelle.

Organelle targeting is normally highly specific. Interestingly, in in vitro studies, mistargeting of chloroplast proteins into mitochondria has been reported, but mislocalization of mitochondria proteins into chloroplasts has never been observed (Bhushan et al., 2006; Cleary et al., 2002; Huang et al., 2009b; Lister et al., 2001; Murcha et al., 2014). The lack of in vivo evidence for chloroplast to mitochondria mistargeting implies that there are several factors that provide organelle specificity. Cytosolic factors have been implicated in providing an extra level of targeting control (Rudhe et al., 2002). The OM lipids of mitochondria and chloroplasts also have a proposed role in protein recognition and organelle specificity. Additionally, both mitochondrial presequences and chloroplast TPs contain intrinsically disordered regions in aqueous medium that form secondary structure in membrane-mimetic environments, which may aid in organelle selection. The mature protein itself appears to contain signals that assist correct organelle localization, possibly through interaction with the protein import machinery on the outer organelle envelope (Dabney-Smith et al., 1999). Furthermore, mitochondrial presequences appear to contain a positively charged chloroplast import avoidance signal near the N-terminus (Ge et al., 2014). Thus, it is likely that specificity is driven by a combination of several physicochemical factors. However, exactly how a dual targeting signal can be recognized by distinct receptors is still unclear.

Dual-targeted transit sequences have been divided into two major classes: twin presequences or an ambiguous dual targeting peptide (dTP) (Murcha et al., 2014; Peeters and Small, 2001; Silva-Filho, 2003). Twin presequences comprise two specific targeting domains that are positioned in tandem. Protein expression produces two proteins with alternate targeting signals because of two alternate in frame translation initiation codons (Danpure, 1995; Silva-Filho, 2003). Alternatively, dTPs involve a single ambiguous targeting sequence. Most dTPs contain overlapping targeting signals for both chloroplast and mitochondrial import (Carrie and Small, 2013). Thus, the dTP contains weak targeting signals for both organelles, and subtle sequence and physicochemical properties are essential for the fidelity of targeting. However, how a protein containing weak targeting signals for both organelles "selects" an organelle remains unclear. This is likely regulated by both the

metabolic state and developmental stage of the cell. dTP containing proteins include proteins involved in translation, DNA synthesis and maintenance, cellular protein folding, turnover and maintenance, and energy production (Carrie and Small, 2013; Carrie and Whelan, 2013; Carrie et al., 2009). It is not surprising that many of these proteins are trafficked to both organelles because similar processes are required for the function of both chloroplasts and mitochondria. Interestingly, bioinformatics and experimental analysis suggests that there is a larger proportion of dual-targeted proteins than expected; over 500 *Arabidopsis* proteins are predicted to be dual targeted (Baudisch et al., 2014; Mitschke et al., 2009). Recent analysis suggests that dual-targeted proteins are trafficked in the same pathways as organelle-specific precursors (Baudisch et al., 2014; Langner et al., 2014). Therefore, understanding the signals that allow a dTP to be recognized by distinct translocons may also provide insight into canonical targeting pathways.

4. REGULATION OF PLASTID PROTEIN IMPORT

4.1 Organism-Specific Recognition of TPs

4.1.1 Evolution of Translocon Subunits

The evolution of translocon subunits from algae to higher plants has been well documented and reexamined when additional genomes became available (Kalanon and McFadden, 2008; Reumann et al., 2005; Shi and Theg, 2013a). However, only a few higher plant genomes were included and subclass information of each subunit is lacking. By utilizing the plant comparative genome resource, Gramene (Tello-Ruiz et al., 2016), the number of each translocon subunits in 39 plant species from algae to higher plants, were determined based on the retrieved Gene Tree of the *Arabidopsis* subunit genes. Although the trees are not manually curated and included pseudogenes, these phylogenetic trees were constructed to recognize gene duplication event (Vilella et al., 2009), which allows classification of a subclass of the subunits. The results are summarized in Table 1. In addition, the numbers of subunits from eight additional genomes were determined from BlastP results retrieved from GenBank (*C. tobin*, *P. glauca*, *N. nucifera*), UniProt (*V. carteri*, *B. prasinos*, *C. crispus*), or Phytozome (*S. polyrhiza*, *Z. marina*) after aligned to the representative subunit sequences from *Arabidopsis*.

Fig. 10 shows phylogentic trees of the subunits that form multiple subclasses. The TOC159 and TOC34 GTPase receptors are related and each undergo duplication forming other subclasses (Reumann et al., 2005).

Table 1 Survey of the Number of Translocon Subunits in Algal and Plant Genomes

			Dicots													Monocots		
Group	**Subunit**	**Query**	*Arabidopsis thaliana*	*Arabidopsis lyrata*	*Brassica rapa*	*Brassica oleracea*	*Theobroma cacao*	*Populus trichocarpa*	*Prunus persica*	*Medicago truncatula*	*Glycine max*	*Vitis vinifera*	*Solanum lycopersicum*	*Solanum tuberosum*	*Nelumbo nucifera*[b]	*Sorghum bicolor*	*Zea mays*	*Setaria italica*
Toc159	Toc159	AT4G02510, AT5G05000	1	3	2	2	2	4	1	2	3	2	2	2	0	2	4	3
	Toc132/ Toc120		2	2	2	3	1	3	1	1	2	1	2	2	0	1	2	1
	Toc100		1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	Toc90		1	1	1	1	1	2	1	1	2	1	1	1	0	1	1	1
	Unclassified Toc159		0	0	0	0	0	0	0	1	1	0	0	0	9	0	0	0
Toc34	Toc33		1	6	2	2	1	2	0	0	0	0	2	2	0	0	0	0
	Toc34		1	1	0	1	0	0	1	2	2	2	0	0	0	0	0	0
	Unclassified Toc34		0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	1
Toc75	Toc75	AT3G46740	2	3	5	3	1	2	1	3	2	1	1	1	1	2	3	2
OEP80	OEP80		1	1	1	1	1	2	1	1	2	1	2	1	2	1	2	1
	OEP80tr		2	1	1	1	1	2	1	2	3	1	1	1	2	1	1	1
	Unclassified OEP80		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

			Monocots															
Group	**Subunit**	**Query**	*Leersia perrieri*	*Oryza barthii*	*Oryza brachyantha*	*Oryza glaberrima*	*Oryza glumaepatula*	*Oryza longistaminata*	*Oryza meridionalis*	*Oryza nivara*	*Oryza punctata*	*Oryza rufipogon*	*Oryza sativa Indica*	*Oryza sativa japonica*	*Brachypodium distachyon*	*Triticum aestivum*	*Triticum urartu*	*Aegilops tauschii*
Toc159	Toc159	AT4G02510, AT5G05000	2	3	3	2	3	4	3	3	3	3	3	3	2	11	2	2
	Toc132/ Toc120		0	1	1	1	0	0	1	0	1	1	1	1	1	5	1	1
	Toc100		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Toc90		2	1	1	1	1	1	1	1	1	1	1	1	1	4	1	1
	Unclassified Toc159		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toc34	Toc33		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Toc34		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Unclassified Toc34		1	1	1	1	1	1	1	1	1	1	1	1	1	4	2	1
Toc75	Toc75	AT3G46740	3	2	1	3	3	3	3	2	3	3	4	3	2	6	2	2
OEP80	OEP80		1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1
	OEP80tr		1	1	1	2	1	1	1	1	1	1	1	1	1	3	1	1
	Unclassified OEP80		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

			Monocots				Flowering plant	Club-moss	Moss	Green algae				Red algae			Haptophyte
Group	**Subunit**	**Query**	*Hordeum vulgare*	*Musa acuminata*	*Spirodela polyrhiza*[b]	*Zostera marina*[b]	*Amborella trichopoda*	*Selaginella moellendorffii*	*Physcomitrella patens*	*Chlamydomonas reinhardtii*[c]	*Ostreococcus lucimarinus*	*Volvox carteri*[b]	*Bathycoccus prasinos*[b]	*Cyanidioschyzon merolae*	*Galdieria sulphuraria*[b]	*Chondrus crispus*[b]	*Chrysochromulina tobin*[b]
Toc159	Toc159	AT4G02510, AT5G05000	2	3	0	0	1	0	0	0	0	0	0	0	0	0	0
	Toc132/ Toc120		1	2	0	0	1	4	4	0	0	0	0	0	0	0	0
	Toc100		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Toc90		1	1	0	0	2	0	0	0	0	0	0	0	0	0	0
	Unclassified Toc159		0	0	5	4	0	0	0	0	1	3	5	1	1	0	0
Toc34	Toc33		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Toc34		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Unclassified Toc34		1	2	2	1	1	2	3	1	1	3	1	1	0	0	0
Toc75	Toc75	AT3G46740	1	4	0	0	2	5	4	1	1	1	1	0	0	0	0
OEP80	OEP80		2	1	0	0	1	2	1	0	0	0	0	0	0	0	0
	OEP80tr		1	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	Unclassified OEP80		0	1	0	0	0	1	0	1	1	0	0	0	1	0	0

Toc64	Toc64/ OEP64 clade A	AT3G17970	1	0	2	3	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
	Toc64/ OEP64 clade B		1	1	0	1	1	2	1	1	3	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4	1	2	1	1	1	1	0	0	3	0	0	0	0	0	0	0	0
Tic22	Tic22-III	AT4G33350	1	1	1	1	2	1	1	1	2	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	3	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
	Tic22-IV		1	1	2	2	1	2	1	1	3	1	1	1	0	1	1	0	1	1	1	1	2	1	1	1	2	1	1	1	1	4	0	1	1	2	1	1	1	2	2	0	0	0	0	0	0	0	0
	Unclassied Tic22		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
Tic20	Tic20 group 1	AT1G04940	2	2	2	2	2	3	2	4	5	1	2	2	1	2	4	2	2	2	2	2	2	2	1	2	3	2	2	2	2	6	2	1	2	2	2	1	1	2	2	0	0	0	0	0	0	0	0
	Tic20 group 2	AT2G47840	2	2	3	1	2	2	2	2	4	2	2	2	3	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	2	2	2	2	1	1	2	3	3	1	1	0	0
Tic21	Tic21	AT2G15290	1	1	2	2	1	2	1	1	2	1	1	1	2	2	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	6	1	2	2	2	1	2	1	2	4	3	1	2	0	1	0	0	0
Tic110	Tic110	AT1G06950	1	3	2	1	1	2	1	2	4	1	1	0	2	1	1	1	1	2	2	0	2	2	2	2	3	2	2	3	2	7	2	2	2	1	1	1	1	2	2	1	1	1	1	1	1	0	0
Tic40	Tic40	AT5G16620	1	1	2	2	1	2	2	1	2	1	1	1	2	1	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	3	1	1	1	2	1	1	1	2	2	2	1	1	1	0	0	0	0
Tic55	Tic55 clade	AT2G24820	1	1	1	2	1	2	2	1	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5	2	1	1	2	2	3	1	0	2	0	0	2	1	0	0	0	0
Others	Tic55-related (PTC52 clade)		2	2	2	2	2	12	2	4	5	3	4	3	6	3	2	3	5	3	6	5	5	5	2	3	5	4	9	5	2	12	3	4	3	2	2	2	2	4	3	6	3	7	1	0	0	0	0
	Tic55-related (CAO clade)		1	1	1	2	1	2	1	1	4	1	2	2	5	1	2	1	1	2	1	2	2	1	1	2	1	1	2	2	1	3	1	1	1	2	2	1	1	2	2	2	2	2	2	0	0	0	0

Continued

Table 1 Survey of the Number of Translocon Subunits in Algal and Plant Genomes—cont'd

			Dicots													*Monocots*																						*Flowering plant*	*Club-moss*	*Moss*	*Green algae*				*Red algae*			*Haptophyte*
Group	**Subunit**	**Query**	***Arabidopsis thaliana***	***Arabidopsis lyrata***	***Brassica rapa***	***Brassica oleracea***	***Theobroma cacao***	***Populus trichocarpa***	***Prunus persica***	***Medicago truncatula***	***Glycine max***	***Vitis vinifera***	***Solanum lycopersicum***	***Solanum tuberosum***	***Nelumbo nucifera***	***Sorghum bicolor***	***Zea mays***	***Setaria italica***	***Leersia perrieri***	***Oryza barthii***	***Oryza brachyantha***	***Oryza glaberrima***	***Oryza glumaepatula***	***Oryza longistaminata***	***Oryza meridionalis***	***Oryza nivara***	***Oryza punctata***	***Oryza rufipogon***	***Oryza sativa Indica***	***Oryza sativa japonica***	***Brachypodium distachyon***	***Triticum aestivum***	***Triticum urartu***	***Aegilops tauschii***	***Hordeum vulgare***	***Musa acuminata***	***Spirodela polyrhiza***	***Zostera marina***	***Amborella trichopoda***	***Selaginella moellendorffii***	***Physcomitrella patens***	***Chlamydomonas reinhardtii***	***Ostreococcus lucimarinus***	***Volvox carteri***	***Bathycoccus prasinos***	***Cyanidioschyzon merolae***	***Galdieria sulphuraria***	***Chondrus crispus***	***Chrysochromulina tobin***
Tic62	Tic62 (G-I)	AT3G18890	1	1	1	1	1	2	1	1	3	1	1	1	2	1	1	2	1	1	1	1	1	1	0	1	1	1	2	1	1	2	0	1	1	1	1	1	1	2	1	1	0	1	0	0	1	0	0
Others	Tic62-related (G-II)		1	1	1	1	1	1	2	1	2	1	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	2	1	1	3	1	0	1	1	1	1	1	3	1	1	1	2	0	0	0	0	0
	Tic62-related (G-IV)		1	1	1	1	1	1	1	2	2	1	1	1	1	1	4	1	1	1	1	1	1	1	0	1	1	1	1	0	1	5	1	2	1	1	1	1	1	2	1	1	1	1	1	0	0	0	0
	Tic62-related (G-V)		2	3	3	3	1	1	1	2	2	1	1	1	1	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	11	1	2	2	1	1	0	1	4	3	1	0	1	0	0	0	0	0
	Tic62-related (others)		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5	0	4	3	2	2	3	1	1	1	0	0
Tic32	Tic32 clade A	AT4G23430	5	4	7	10	4	8	7	10	19	5	8	8	3	6	7	7	4	5	6	10	3	10	3	8	5	6	10	7	4	22	6	7	4	8	6	4	4	4	0	0	0	0	0	0	0	0	0
	Tic32 clade B		3	3	6	5	2	4	2	3	9	2	3	3	4	2	2	2	2	2	2	2	2	2	1	2	3	2	2	2	2	7	3	4	1	3	1	2	2	15	4	0	0	0	0	0	0	0	0

Sum	Toc159	5[a]	7	6	7	4	9	3	5	8	4	5	5	9	4	7	5	4	5	5	4	4	5	5	4	5	5	5	5	4	20	4	4	4	6	5	4	4	4	4	0	1	3	5	1	1	0	0
	Toc34	2	7	2	3	1	2	1	2	2	2	2	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4	2	1	1	2	2	1	1	2	3	1	1	3	1	1	0	0	0
	Toc75	2	3	5	3	1	2	1	3	2	1	1	1	1	2	3	2	3	2	1	3	3	3	3	2	3	3	4	3	2	6	2	2	1	4	0	0	2	5	4	1	1	1	1	0	0	0	0
	Toc64	2	1	2	4	2	4	2	2	5	2	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	8	2	3	2	2	2	2	1	0	3	0	0	0	0	0	0	0	0
	Tic22	2	2	3	3	3	3	2	2	5	2	2	2	0	2	2	0	2	2	2	2	3	2	1	2	3	2	2	2	2	7	1	1	2	3	2	2	2	2	2	1	0	1	1	0	0	0	0
	Tic20	4	4	5	3	4	5	4	6	9	3	4	4	4	2	5	2	3	2	3	3	3	2	2	3	4	3	3	3	3	7	3	1	3	3	4	3	3	4	3	1	2	3	3	1	1	0	0
	Tic21	1	1	2	2	1	2	1	1	2	1	1	1	2	2	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	6	1	2	2	2	1	2	1	2	4	3	1	2	0	1	0	0	0
	Tic110	1	3	2	1	1	2	1	2	4	1	1	0	2	1	1	1	1	2	2	0	2	2	2	2	3	2	2	3	2	7	2	2	2	1	1	1	1	2	2	1	1	1	1	1	1	0	0
	Tic40	1	1	2	2	1	2	2	1	2	1	1	1	2	1	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	3	1	1	1	2	1	1	1	2	2	2	1	1	1	0	0	0	0
	Tic55	1	1	1	2	1	2	2	1	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5	2	1	1	2	2	3	1	0	2	0	0	2	1	0	0	0	0
	Tic62	1	1	1	1	1	2	1	1	3	1	1	1	2	1	1	2	1	1	1	1	1	1	0	1	1	1	2	1	1	2	0	1	1	1	1	1	1	2	1	1	0	1	0	0	1	0	0
	Tic32	8	7	13	15	6	12	9	13	28	7	11	11	7	8	9	9	6	7	8	12	5	12	4	10	8	8	12	9	6	29	9	11	5	11	7	6	6	19	4	0	0	0	0	0	0	0	0

[a]A pseudogene was removed (Shi and Theg, 2013a).
[b]These species are not included in Gramene database.
[c]This finding is significant different from other reports. Possibly due to different genome version.
The number of individual translocon subunits from 39 algal and plant species were identified from the Gene Trees in Gramene (http://www.gramene.org) based on the query using subunits from *Arabidopsis*. The subunit class was identified based on *Arabidopsis* subclasses. In addition, the numbers of subunits from eight additional genomes were retrieved with the query sequences using BlastP from either other databases: GenBank (*C. tobin*, *P. glauca*, *N. nucifera*), UniProt (*V. carteri*, *B. prasinos*, *C. crispus*), or Phytozome (*S. polyrhiza*, *Z. marina*). The retrieved sequences were aligned with the representative sequences from the Gene Trees and the phylogenetic tree information was used to classify the subunit subclass.

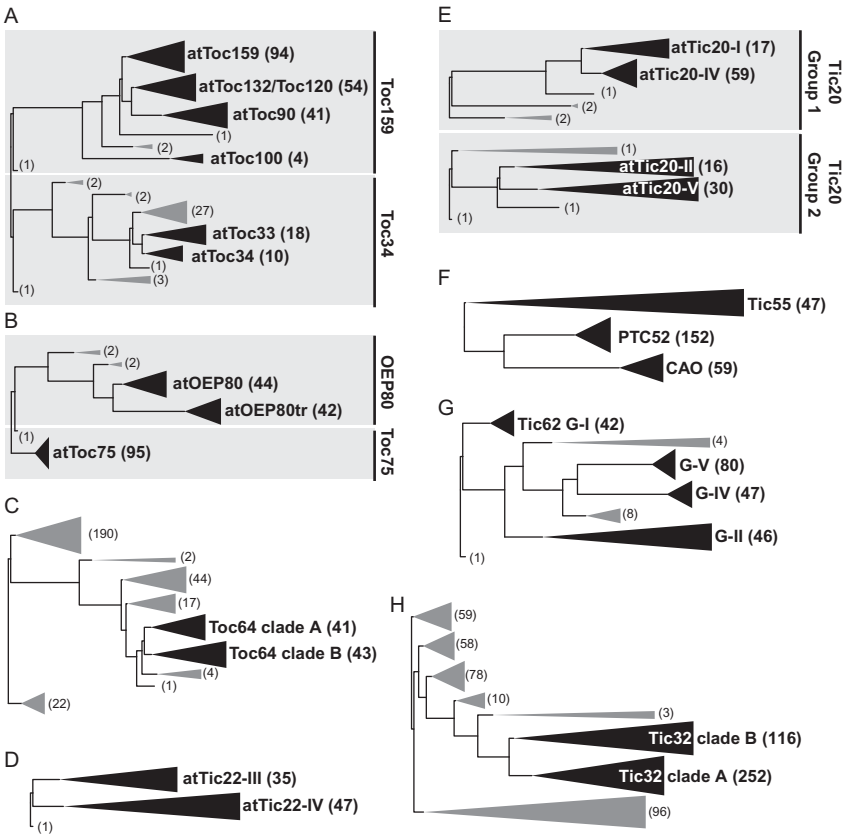

Fig. 10 Phylogenetic tree of the homolog of translocon subunits. The phylogenetic trees were derived from Gramene Gene Trees of Arabidopsis subunits. (A) Toc159 and Toc34 homologs. (B) Toc75 and OEP80 homologs. (C)–(H) Homologs of Toc64, Tic22, Tic20, Tic55, Tic62, and Tic32, respectively.

Most green lineage species show expansion of TOC159 subclass. Phylogentic analysis of TOC75 has been performed recently and classified TOC75 homologs into OEP80, OEP80tr, and TOC75 subclasses (Day et al., 2014). Following this classification, TOC75 was identified in all species of green lineage except *S. polyrhiza*. The red algae homologs were not grouped into any of TOC75 subclass (Table 1) similar to previous report (Day et al., 2014). The other TOC subunit TOC64 only present in land plants and were classified into two clades (Aronsson et al., 2007). Both clades of TOC64 seems to distribute evenly among species. The TIC component, TIC22, was classified into two subclasses (Kasmati et al., 2013). All lineage harbor at least a TIC22 homolog except *N. nucifera*, *S. italica*, and

O. lucimarinus. TIC20 homologs were classified into two groups (Kasmati et al., 2011). While most species harbor both groups of TIC20, the red and green algae only harbor TIC20 group 2. TIC110, TIC40, and TIC21 homologs each form a single clade among the homologs (data not shown) and present in all land plants but only in some algae. The homologs of TIC55, TIC62, and TIC32 form multiple clades (Fig. 10F–H) indicating that these TIC components diverged from protein with other functions (Balsera et al., 2007; Boij et al., 2009). The TIC55 and TIC62 clades have been defined previously (Boij et al., 2009). Both TIC55 and TIC66 were present in most algae and land plants. Previously, TIC32 clade has never been defined. In Fig. 10H, TIC32 clade A includes *Arabidopsis* TIC32 proteins reported by two groups (Reumann et al., 2005; Shi and Theg, 2013a) where as TIC32 clade B includes the proteins reported only by one group (Shi and Theg, 2013a). None of algae TIC32 homologs were grouped into either clade although others have identified TIC32 in both green and red algae (Reumann et al., 2005; Shi and Theg, 2013a). The limited number of components identified in our results from red algae and haplophyte may be affected by sequence divergence in red algae and haplophyte as mentioned previously (Hovde et al., 2014). Nevertheless, the haplophyte *C. tobin* has been reported to harbor TOC159, TIC110, TIC55, TIC22, and TIC20 (Hovde et al., 2014).

Similar to an earlier report (Kalanon and McFadden, 2008), most of the components of TOC and TIC were found in the green algal lineage but only some components were present in red algae. Green algae lack TOC64 while red algae lack both TOC64 and TIC40. In green lineage, TOC75 homologs were separated into TOC75 and OEP80 groups. Although some subunits are missing in some of the species, most TOC/TIC components are maintained in all land plant species suggesting the conservation of the general import pathway. It is interesting how the expansion of the subunits such as TOC159 and TIC20 in land plants plays a role in finetuning or altering the import mechanism and how the protein import occurs in algae without a complete set of the translocon subunits.

4.1.2 Evolution of TP of Small Subunit of RuBisCO

To understand the evolution of TPs in response to the evolution of translocon components, we aligned the TPs of the small subunit of RuBisCO (SStp) from 120 green plants (Holbrook et al., 2016). The representative sequences from green algae, moss, rice, *Arabidopsis*, pea, and tobacco are shown together with the sequence logo from different lineages (Fig. 11).

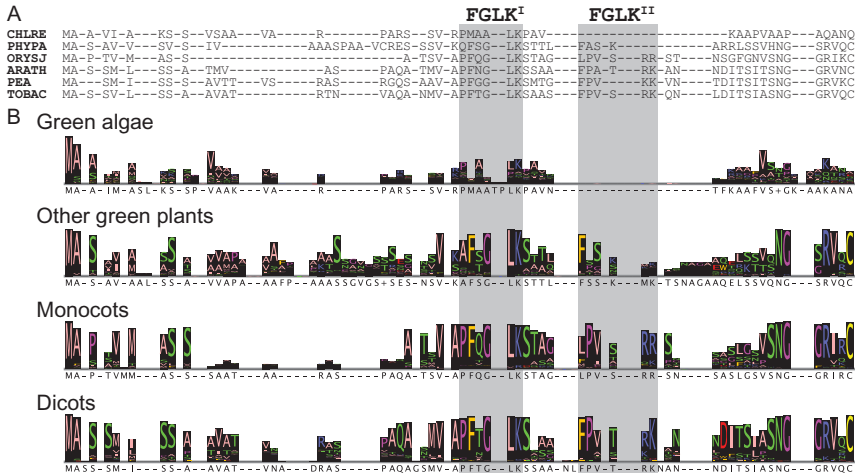

Fig. 11 Multiple alignment of SStp. Two semiconserved FGLK domains were identified and highlighted. (A) Representative sequences from different lineage groups. CHLRE, green alga *Chlamydomonas reinhardtii*; PHYPA, moss *Physcomitrella patens*; ORYSJ, rice *Oryza sativa* subsp. *japonica*; ARATH, *Arabidopsis thaliana*; PEA, pea *Pisum sativum*; TOBAC, tobacco *Nicotiana tabacum*. (B) Consensus sequences and sequence logos of the alignment separated by lineages.

Despite a diverse set of species, the SStp sequences are highly conserved. The only notable difference is the presecence of a single FGLK motif in green algae whereas two FGLK motifs are present in land plants. It is still unclear why higher plants evolved toward addition of an extra FGLK motif.

Although it is possible to expect that the highly conserved SStp sequences are due to the limited function of SStp in driving a RuBisCo subunit into the chloroplasts of photosynthetic tissue, the conservation of SStp also indicate that the TOC/TIC translocon presented in the photosynthetic tissue is conserved. This view is supported by the identification of *Arabidopsis* homologs of pea TOC159 and TOC34 that were found in leaves. The orthologs atTOC159 and atTOC33 remain expressed in photosynthetic tissues (Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004). In *Arabidopsis*, different subsets of TOC receptors were expressed in different tissues and each subset preferred specific TPs (Demarsy et al., 2014; Teng et al., 2012) indicating that TPs evolved in response to various combinations of TOC receptors. In the future, it will be interesting to analyze TPs of proteins that diverge and determine changes in the TP with the corresponding TOC/TIC components.

4.1.3 Comparative Nuclear-Encoded Plastid Proteomes

To understand the TP–translocon relationship through evolution, identification of TPs from multiple species is needed. While detailed prediction of plastid-targeted proteins has been performed in *Arabidopsis* and rice (Martin et al., 2002), it is lacking in other species. Here, we utilized three prediction programs, TargetP (Emanuelsson et al., 2000), Predotar (Small et al., 2004), and MultiLOC2 (Blum et al., 2009) to predict nuclear-encoded proteins that target to the plastid from 11 species including green alga, moss, 3 monocots, and 6 dicots. The protein sequences were downloaded from Phytozome database. The prediction results were cataloged into reliability class as defined by TargetP. The proteins determined by at least two programs with reliability class 1 or 2 convey a high degree of confidence in the class designation of the protein. The proteins predicted by at least two programs are considered as predicted plastid-targeted proteins. The results are shown in Fig. 12. Using these two prediction values, the nuclear-encoded plastid proteome was estimated to be in a range of 1400–3000 proteins, which accounted for 3–7% of the nuclear genomes. Note that we use the most inclusive criteria (i.e., the total number of proteins predicted by each program combined) the output yields a much higher number and averages ~7500 proteins or over 18% of the nuclear genomes (data not shown). However, considering the variability of these predictive tools it will require a more rigorous validation of the size and diversity of the plastid proteome. Yet, the TPs from these predictions could be valuable in understanding TP function and evolution.

4.2 Expression Control

Spatial and temporal expressions of the nuclear-encoded plastid precursor proteins under different internal and external conditions are well documented (Drea et al., 2001; Gesch et al., 2003; Harmer et al., 2000; Knight et al., 2002; Plumley and Schmidt, 1989; Vorst et al., 1990; Zhou et al., 2006). These regulations alter the cytosolic levels of precursors, which affect the rates of the precursor protein import. Because most nuclear-encoded plastid proteins utilize the general import pathway, changing the expression level of any of these proteins can also potentially affect the import rates of other proteins (Row and Gray, 2001a). Recent evidence indicates that some of the gene expression regulation of nuclear-encoded plastid proteins also involves retrograde signaling from the plastids (Estavillo et al., 2011; Gray et al., 2003; Kakizaki et al., 2009; Pesaresi et al., 2006).

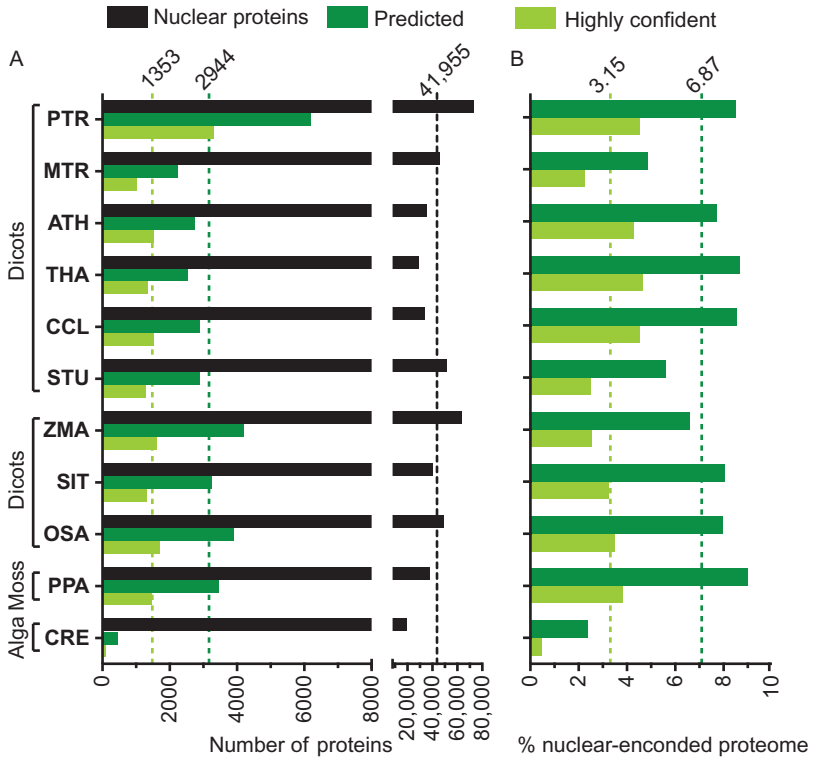

Fig. 12 Prediction of plastid proteome size. Three programs, TargetP, Predotar, and MultiLOC2, were used to predict nuclear-encoded plastid-localized proteins from 11 species: green algae model *Chlamydomonas reinhardtii* (CRE), a moss model *Physcomitrella patens* (PPA), rice *Oryza sativa* (OSA), maize *Zea mays* (ZMA), foxtail millet Setaria italic (SIT), *Arabidopsis thaliana* (ATH), *Thellungiella halophile* (THA), ligume *Medicago truncatula* (MTR), clementine *Citrus clementine* (CCL), potato *Solanum tuberosum* (STU), and poplar *Populus trichocarpa* (PTR). Proteins with at least two programs predicted with reliability class 1 or 2 are considered as highly confident (highly confident set) and proteins with at least two programs predicted are considered as predicted plastid-localized proteins (predicted set). (A) and (B) shown number and percentage of each set in each species, respectively.

In addition to the precursor proteins, the expression of the translocon components is also highly regulated. While green tissues express higher levels of atTOC33, atTOC159, atTOC64-III, atTIC55, atTIC62, and atTIC40, nongreen tissues express higher levels of atTOC34, atTOC132, atTOC120, atTIC20-I, and atTIC20-IV (Gutensohn et al., 2000; Vojta et al., 2004). A transcription factor CIA2 was also shown to upregulate atTOC33 and atTOC75-III expressions in leaves (Sun et al., 2001, 2009). Thus, cells

control spatial and temporal protein import rates by altering precursor proteins levels and generate different combinations of translocon components.

4.3 Precursor-Specific Import Pathways

4.3.1 Photosynthetic and Nonphotosynthetic Precursors

It was proposed that the multiple paralogs of the translocon subunits perform different functions (Jarvis et al., 1998). Currently, much evidence is shown to support this hypothesis. Knockout mutant phenotype analysis and biochemical characterization found that atTOC33 associates with atTOC159 in the TOC complex functioning in the import of photosynthetic proteins while atTOC34, atTOC132, atTOC120 are found in the TOC complex that functions in the import of nonphotosynthetic proteins (Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004). In spinach, two TOC34 isoforms were also identified (Voigt et al., 2005) suggesting that other plants may utilize specialized TOC receptors. In addition, the A domains of TOC159 have been shown to function in precursor selectivity of atTOC159 and atTOC132 (Inoue et al., 2010). This selectivity is further shown to rely on the TP sequence of the precursors (Wan et al., 1996; Yan et al., 2006). However, the element(s) of TP corresponding to precursor-class selection is still largely unknown (Jarvis, 2008). One of the elements discovered was a segment on the TP of *Arabidopsis* small subunit of RuBisCO (atSStp) from residues 41 to 49, which governs the TOC159-dependent pathway (Lee et al., 2009a). Another element was identified from microarray analysis of nuclear-encoded plastid protein genes in *ppi1* mutant, the *atTOC33* knockout plant (Vojta et al., 2004). Only the downregulated genes were shown to contain positively charged amino acids at the C-terminal of TPs (−8 and −1 positions) suggesting this element is involved in atTOC34 recognition (Vojta et al., 2004). The precursor-specific pathway seems to merge at the TIC complex where TIC components were found to associate with both photosynthetic and nonphotosynthetic proteins (Chen et al., 2002; Jarvis, 2008; Kovacheva et al., 2005). Nevertheless, atTIC20-IV was suggested to function in the alternative import pathway for housekeeping proteins (Kikuchi et al., 2013). Thus, the TOC/TIC complexes can dynamically modulate import of different classes of precursor proteins.

4.3.2 Age-Specific Precursors

Recently, the age-dependent regulation of protein import has been discovered (Teng et al., 2012). Based on the optimal import rates, precursors can be

classified into three different groups based on the age of chloroplasts: young chloroplast specific, old chloroplast specific, and age independent. The import efficiency into different ages of chloroplasts was shown to depend on the sequence of the TP (Teng et al., 2012). Import competition assays also found that TPs competed better within their own groups, suggesting each group utilizes a specific pathway (Teng et al., 2012). The attempt to determine the age-specific signal of TPs identified two consecutive positively charged residues as a signal for the old chloroplast-specific pathway (Teng et al., 2012). It is still unknown whether specific TOC receptor combinations participate in this recognition similar to that of Fig. 3 or posttranslational modification is involved in creating the age-dependent signal of TP (Teng et al., 2012). Although the physiological relevance of age-dependent import was shown by analyzing the precursor gene families, where each precursor contained a TP from a different age-selective group (Teng et al., 2012), it is unknown as to whether the aging of chloroplasts only depends on the age-selective import and/or differential expression of the precursors.

Nevertheless, the only reported components of translocons that differentially function at different ages are atTIC20 and atTIC21. Whereas atTIC20 function is important in the early development stage, TIC21 function becomes dominant in the mature stage (Li and Chiu, 2010; Teng et al., 2006).

4.4 Redox Regulation

The redox regulation of plastid protein import was shown to occur at both TOC and TIC translocons (Balsera et al., 2010). Earlier studies found that Cys-modifying agents (Friedman and Keegstra, 1989; Row and Gray, 2001b) and disulfide reducing agents (Pilon et al., 1992a; Stengel et al., 2009), inhibit and stimulate protein import, respectively. Protein import in *Physcomitrella* and *Chlamydomonas* were also enhanced in the presence of reducing agents (Stengel et al., 2009). In addition, the oxidant $CuCl_2$ was found to inhibit protein import by inducing disulfide bridge formation between TOC34, TOC75, and TOC159 (Seedorf and Soll, 1995). Disulfide bridge dimerization of TOC34 with a single conserved Cys has also been shown both in vitro and *in organello* (Lee et al., 2009b). These findings indicate the possibility of redox-dependent disulfide bridge regulation of protein import.

Another level of redox regulation involves TIC subunits. It was proposed that TIC components containing redox-related domains might be

involved in regulation (Bedard and Jarvis, 2005). While both dehydrogenases TIC62 and TIC32 harbor NADPH-binding sites (Chigri et al., 2006; Stengel et al., 2008), TIC55 has a Rieske 2Fe-2S center (Caliebe et al., 1997). Additionally, TIC62 contains a binding site for ferredoxin-$NADP^+$ reductase (FNR) (Stengel et al., 2008). The ratios of stromal $NADP^+$/NADPH have been shown to regulate the movement of TIC62 between stroma and inner envelope, and the interaction of TIC62 with FNR (Stengel et al., 2008). In reducing condition, TIC62 accumulates in the stroma and has a higher affinity to FNR (Stengel et al., 2008). Another study showed that NADPH abolished TIC62 and TIC32 interaction with TIC110 (Chigri et al., 2006). Lastly, the stromal $NADP^+$/NADPH ratio has been linked to regulate chloroplast protein import of a subgroup of precursors where higher ratios stimulate import (Stengel et al., 2009). This result confirms the role of redox regulation in protein import. Further studies would be required to determine the exact mechanism controlling the redox regulation.

4.5 Phosphorylation Regulation

Phosphorylation of TPs has also been shown to regulate protein import. Phosphorylation of multiple precursors has been observed (Chen et al., 2014; Lamberti et al., 2011; Waegemann and Soll, 1996) together with the identification of the kinases (Martin et al., 2006). The cytosolic guidance complex is proposed to recognize phosphorylated precursors before delivering them to the translocons (May and Soll, 2000). Although the phosphorylation consensus sequence (P/G (X) (K/R) X(S/T) X(S*T*)) (May and Soll, 2000) is not present in the sequence of all chloroplast-targeted proteins, Ser/Thr residues are highly abundant in TPs. Furthermore, knockout of cytosolic kinases shows a clear effect in chloroplast differentiation (Lamberti et al., 2011). A phosphorylation-dephosphorylation cycle has been suggested, where the incoming precursors are in phosphorylated forms and the translocon-associated phosphatase dephosphorylates the precursors to initiate translocation (Waegemann and Soll, 1996). However, the phosphatase has not yet been identified. Nevertheless, the mutants of three TPs lacking the phosphorylation sites were able to direct the import of GFP into the plastids, which indicates that phosphorylation of TP is not required for plastid targeting (Nakrieko et al., 2004). Phosphorylation–dephosphorylation cycles may ensure that a subset of highly expressed proteins reach the plastid efficiently (Holbrook et al., 2016; Nakrieko et al., 2004).

4.6 Potential Role of Proline Isomerization

The TP FGLK physicochemical domains have an established role in import efficiency (Chotewutmontri et al., 2012; Lee et al., 2006; Pilon et al., 1992a); these domains often contain Pro residues. Pro residues can exist in two isomeric forms, *trans* and more uncommonly *cis* (Song et al., 2006). The *cis* Xaa-Pro peptide bond (where Xaa can be any amino acid) can introduce a "kink" into a protein sequence, a structural element that has been suggested to contribute to several important cellular processes, including cell signaling, replication, and regulating protein activity (Andreotti, 2003; Dugave and Demange, 2003). Interestingly, TP Pro residues have been previously suggested to play a role in both recognition and efficient import of precursor proteins (Chotewutmontri et al., 2012). Furthermore, FKBP, a peptidyl proline isomerase was previously suggested as a putative guidance complex component (Fellerer et al., 2011; Lee et al., 2013). We suggest that the Pro-induced "kinks" may enable recognition or attachment of one or more proteins functioning as molecular ratchets which could facilitate efficient precursor import into the plastid stroma.

We hypothesize that specific *cis* Xaa-Pro residue(s) could alter the structure of the TP to increase or decrease recognition with one or more of the TOC components and affect precursor import efficiency. To further investigate the predicted isomeric form of Pro residues in the vicinity of the FGLK domains, we used CISPEPpred prediction software to predict Pro *cis*/*trans* peptide bond conformation in the RuBisCo small subunit TP (Fig. 13A and B) (Song et al., 2006). We aligned 24 SStp sequences from green plants using ClustalX and overlayed the CISPEPpred prediction for *cis* Xaa-Pro residues. Although only three Pro positions were largely conserved (near positions 22, 30, and 41) only the Pro at position 30 was predicted to have a potential *cis* confirmation (Fig. 13A and B). This residue was predicted to be a *cis* Xaa-Pro in approximately 70% of the TPs. Interestingly, all of the *cis* Xaa-Pro residues are predicted to occur in the middle of the SStp sequence.

Next, we wanted to investigate the occurrence of *cis* Xaa-Pro residues in a database of 912 confidently predicted TPs using CISPEPpred (Chotewutmontri et al., 2012). Interestingly, this program predicted that ~35% of the TPs contained at least one *cis* Xaa-Pro. This is significant since only ~5% of all Pro are found in the *cis* confirmation of proteins with known structures (Jabs et al., 1999; Pall and Chakraabarti, 1999; Weiss et al., 1998). Recent studies have suggested that *cis* Pro conformation correlates with local

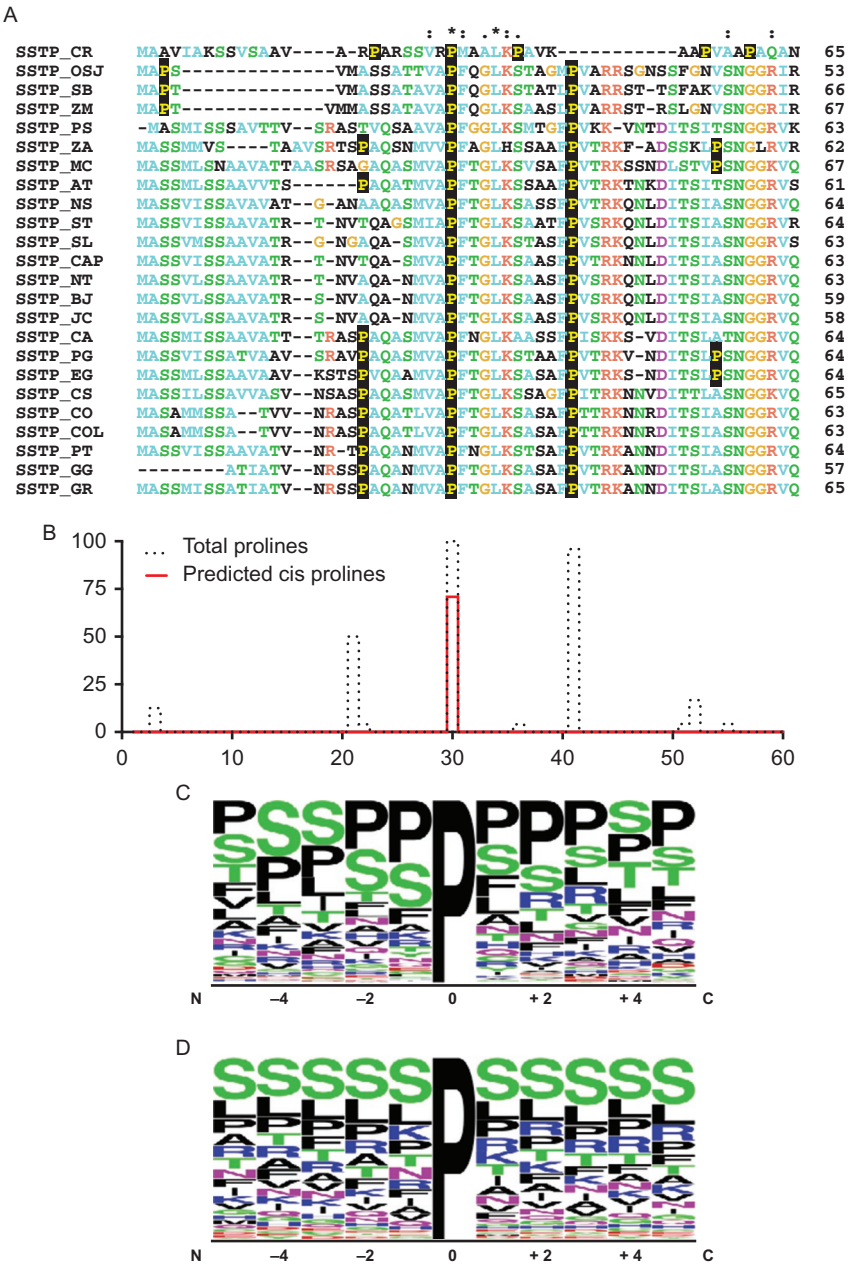

Fig. 13 *Cis*-proline function. (A) Alignment of 24 RuBisCo small subunit TPs using ClustalX; Pro residues are highlighted in *black*. (B) Graph summarizes the locations of aligned Pro residues from (A). Cis–Pro were predicted using CISPEPpred online tool at http://sunflower.kuicr.kyotou.ac.jp/~sjn/cispep/. (C–D) Logo plots of +5 and −5 amino acid positions around a *cis*-predicted Pro (C) and a *trans*-predicted Pro (D). Dataset is 912 Arabidopsis TP sequences as identified in Chotewutmontri et al. (2012). Cis–Pro were predicted in the dataset using CISPEPpred.

amino acid sequence content (Song et al., 2006). When we looked at the flanking sequences (± five residues, xxxxxPxxxxx) of the predicted *cis* (Fig. 13C) and predicted *trans* (Fig. 13D) Pro sequences from the Arabidopsis 912-TP database using a logo plot, we saw a clear difference in the frequency of flanking amino acids. The *cis* Pro residues have a very high occurrence of additional Pro residues that seem to be found on both sides of the *cis* Pro. We also see higher occurrence of Phe residues immediately flanking the *cis* Pro. However, the *trans* Pro residues seem to have a very uniformly distributed pattern with S > L > P > R/K in the logo plot, occurring nearly equally on both sides of the *trans* Pro. It is difficult to discern the causality of these differing amino acid distributions. Do the flanking sequences simply influence the Pro isomeric form; or does recognition of the *cis* Pro isomeric form require some additional residues for its maximum specificity or recognition by the translocon(s)? Although how processing is coupled to translocation is not known, it is not surprising that this may be an intimate interaction that requires subtle spatial control between the emerging preprotein and SPP in the stroma. Investigating the potential role of *cis* Pro racheting "kinks" in the TP sequence will be a topic of future study.

4.7 Regulation by Ubiquitin–Proteasome System

The level of precursor proteins in cytosol has been shown to be regulated by the ubiquitin–proteasome system, specifically through the cytosolic Hsc70 and the C-terminus of Hsc70-interacting protein (CHIP) E3 ubiquitin ligase pathway (Lee et al., 2009c; Shen et al., 2007). Interestingly, other evidence also indicated that a putative C3HC4-type really interesting new gene (RING) E3 ubiquitin ligase SP1 interacts with all of the TOC components and initiates their degradation via proteasome (Ling et al., 2012). It was proposed that E3 ubiquitin ligase SP1 regulates the turnover of TOC components through ubiquination and degradation pathways. In combination with the differential expression of TOC components, this results in modulation of the composition of TOC components (Ling et al., 2012). SP1-mediated TOC composition change was also suggested to control the transition between plastid types (Ling et al., 2012). Clearly, regulation of both the complex components and precursor proteins work together to modulate import to the plastid. This is a highly complex system and additional analysis of regulation pathways are still required to fully understand how these pathways interplay.

5. CONCLUDING REMARKS

Significant research has focused on the mechanisms that control import of nuclear-encoded precursor proteins. The general import pathway represents a central hub for recognition, regulation, and membrane translocation of precursors; this system plays a key role in organelle function and homeostasis. However, it is clear that noncanonical pathways work in concert with the TOC/TIC system to respond to developmental and physiological responses in the cell. Although we have developed a strong basis for understanding plastid import, more detailed analysis of the components mediating cytosolic recognition, TOC assembly, and regulation of the import process are required. Furthermore, understanding how diverse TPs mediate the selective targeting and translocation of precursors will reveal important regulation and quality control elements of this pathway. In depth understanding of TP domain architecture may allow for the design of novel synthetic TPs in the future. In summary, emerging studies that integrate the assembly and regulation of the plastid import pathways will contribute to uncovering the mechanisms that mediate the plasticity of organelle development.

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