Protein Import in Chloroplasts: An Emerging Regulatory Role for Redox

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Advances in Botanical Research, Vol. 52 0065-2296/10 $35.00

DOI: 10.1016/S0065-2296(09)52010-1

ABSTRACT

Chloroplasts as well as mitochondria are surrounded by two envelope membranes that contain protein machineries to ensure well-organized communication and substrate distribution between the organelle and the rest of the cell. Protein import into organelles must be tightly coordinated with the internal protein synthesis machinery to ensure assembly of functional complexes of dual genetic origin. Import must also be coupled to the cellular metabolic state to guarantee requirements of the organelle in response to developmental changes and environmental conditions.

Several recently published findings point to a role for redox in regulating chloroplast protein import: light and cysteine-modifying reagents affect import, certain subunits of the protein import machinery contain redox-sensing components, and others are potential thioredoxin targets. Herein we review the recent structural, computational, genetic, and biochemical studies that have begun to identify key characteristics and properties underlying protein import in chloroplasts. The predicted topology of some components is discussed, pointing out conserved cysteines in the protein families that may play a role in linking redox to oxidative stress and changes in the metabolic state of chloroplasts.

I. INTRODUCTION

Chloroplasts, photosynthetic organelles in green plant cells, have developed different machineries during evolution to ensure a specific and efficient transport network for proteins that, while synthesized in the cytosol, exert their function within the plastid (Balsera *et al.*, 2009b; Cline and Dabney-Smith, 2008; Inaba and Schnell, 2008; Jarvis, 2008). Biogenesis of most chloroplast proteins implies, therefore, translocation through two lipid membranes (the outer and the inner envelope membranes) until they reach eventually the stroma of chloroplasts. In addition, some proteins are further

transported from the stroma to thylakoids, with a final destination to either the thylakoidal membrane or lumen. Hence, these proteins have to face the insertion or complete translocation through a third lipid bilayer membrane.

The chloroplast proteome is, however, composed not only of nuclear-encoded proteins, but also a number of subunits that are encoded in the chloroplast genome (cpDNA), reminiscent of the ancient cyanobacterial ancestors according to the endosymbiotic theory (Gould *et al.*, 2008; Gray, 1992). Some essential multimeric protein complexes in chloroplasts are composed of proteins of dual genetic origin, and their assembly into functional units obviously depends on the availability of all subunits. For instance, photosystem II (PSII) in plant chloroplasts is made up of more than 25 extrinsic and intrinsic different subunits of dual genetic origin (Barber, 2006). Three of these subunits (OE33, OE23, and OE17) function coordinately at the oxygen-evolving complex. They are extrinsically associated with PSII in the interior lumen, yet synthesized in the cytosol of the plant cell. The three subunits use the general import pathway at the envelope level, involving the TOC and the TIC complexes (Balsera *et al.*, 2009b; Inaba and Schnell, 2008; Jarvis, 2008). At least two different protein translocation machineries participate in their transport into thylakoids: cpTat (twin-arginine translocation complex, with prefix "cp" indicating pathway in chloroplasts) for the translocation of folded OE23 and OE17 (Robinson and Bolhuis, 2004), and cpSec (secretory pathway in chloroplast) for the translocation of unfolded OE33 (Schuenemann *et al.*, 1999b). A third translocation machinery in thylakoids, Alb3/cpSRP (single recognition particle), assures correct insertion and folding of the light-harvesting complex within the thylakoid membrane posttranslationally (Schuenemann *et al.*, 1998). On the other hand, insertion of the chloroplast-encoded reaction center protein PsbA (or D1) in thylakoids and its assembly with other PSII subunits occurs cotranslationally in an Alb3/SRP-dependent pathway in cooperation with cpSec, following a mechanism similar to that mediating the transport of proteins to the bacterial plasma membrane (Luirink *et al.*, 2001; Zhang *et al.*, 2001). Membrane insertion of the thylakoidal proteins cytochrome *f* and *PsaF* is, however, independent of Alb3 and SRP and seems to require only cpSec (Karnauchov *et al.*, 1994; Nohara *et al.*, 1996). Finally, small intrinsic subunits like PsbW and PsbX are spontaneously inserted into the thylakoid (Tissier *et al.*, 2002).

Coordination and regulation of protein import is, therefore, central to chloroplast biogenesis as both developmental changes and environmental signals are believed to influence the capability for transport of proteins into chloroplasts as well as their internal routing within the plastid. In this respect, organelles have developed efficient means of communication with other parts of the plant cell (Woodson and Chory, 2008). Present knowledge

indicates that chloroplast redox signals influence not only translation and translocation processes outside the chloroplast via retrograde signaling, but also the import of nuclear-encoded proteins. Light, temperature, and Cys-modifying reagents affect import (Bartsch *et al.*, 2008; Dutta *et al.*, 2009; Friedman and Keegstra, 1989; Hirohashi *et al.*, 2001; Pilon *et al.*, 1992; Row and Gray, 2001; Seedorf and Soll, 1995; Stengel *et al.*, 2009). The translocation machinery at the inner envelope membrane contains putative redox sensor components (Caliebe *et al.*, 1997; Hormann *et al.*, 2004; Kuchler *et al.*, 2002), and several members of the different protein transport complexes are linked to thioredoxin (Trx) (Balsera *et al.*, 2009a; Bartsch *et al.*, 2008; Mata-Cabana *et al.*, 2007)—findings that strongly suggest a role for redox in regulating and coordinating protein import with the rest of the plant cell.

II. PATHWAYS OF PROTEIN IMPORT IN CHLOROPLASTS

Most proteins targeted to chloroplasts are synthesized as preproteins or precursor proteins with a presequence (or transit peptide) at the N-terminus (Dobberstein *et al.*, 1977). The transit peptide is necessary for proper localization at the chloroplast surface (Bruce, 2000). Soluble proteins that are targeted to the thylakoid lumen contain a removable bipartite transit peptide, with the most N-proximal part analogous to the transit peptide of stromal proteins and the C-proximal counterpart specific for thylakoid transfer (Smeekens *et al.*, 1986). Thylakoid membrane proteins have a transit peptide for stromal targeting and contain internal motifs for insertion into the thylakoids. Preproteins are typically translocated in an unfolded state and after (or during) translocation, the transit peptide is removed by a specific protease. The mature protein is then folded in a functional competent conformation and/or targeted to the proper chloroplast subcompartment (Smeekens *et al.*, 1990).

An interesting question deals with the location of chloroplast protein synthesis in the cytosol and how preproteins are targeted or delivered to the outer envelope membrane surface of chloroplasts. Different experiments, including electron microscopy studies, have demonstrated that the process of protein import into chloroplasts is posttranslational (Carde *et al.*, 1982; Chua and Schmidt, 1979) and that a cytosolic guidance complex, formed either by chaperones (for proteins with a transit peptide) or the ankyrin repeat containing protein AKR2 (for certain outer membrane proteins),

accompanies the precursor protein to the chloroplast surface (Bae *et al.*, 2008; May and Soll, 2000; Qbadou *et al.*, 2006).

A. PROTEINS DESTINED TO THE STROMA

A general import pathway has been described for precursor proteins that contain a cleavable transit peptide and either reside in the stroma or have a stromal import intermediate.

The coordinate activities of the multimeric TOC and TIC complexes of the outer and the inner envelope membranes, respectively, facilitate the translocation of these substrates through these barriers (Agne and Kessler, 2009; Benz *et al.*, 2009) (Fig. 1A). Receptors at the outer envelope specifically recognize the chloroplast transit peptide and mediate precursor transfer to the pore and translocation at expense of GTP and ATP (Keegstra *et al.*, 1989; Olsen and Keegstra, 1992). Protein components in the intermembrane space assure the formation of supercomplexes between both translocases and the correct transfer of the precursor from TOC to TIC. Further transport across

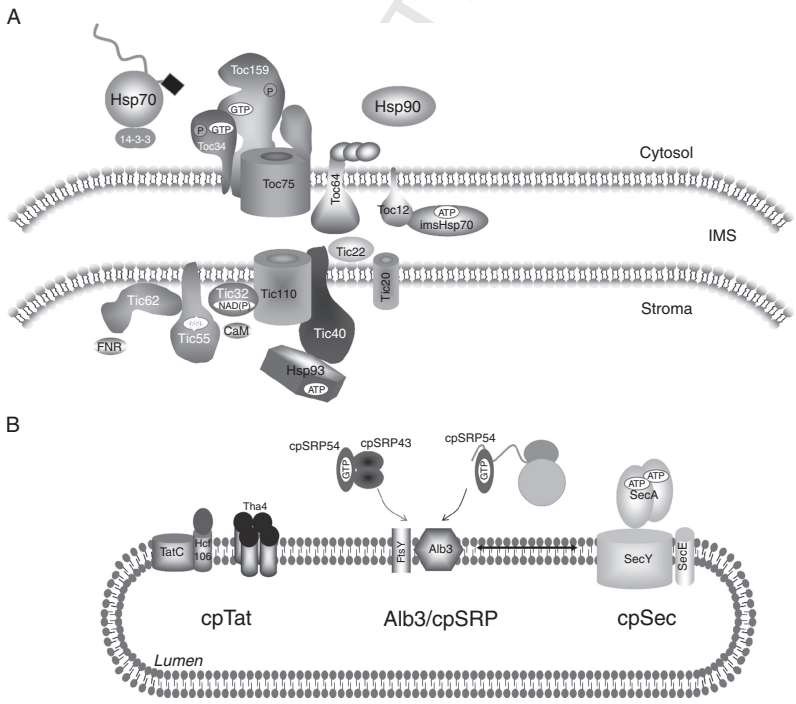

Fig. 1. (See color plate section at the back of the book.)

the inner envelope requires ATP for import motor at the *trans* side (Jackson-Constan *et al.*, 2001) until the preprotein finally reaches the stroma and the mature protein is folded and properly distributed.

B. PROTEINS DESTINED TO THE THYLAKOID MEMBRANE OR LUMEN

Following translocation to the stroma by the general import pathway, four different pathways mediate the insertion/translocation of proteins destined to the thylakoids and internal lumen compartment, three of which are dependent on proteinaceous machineries at the membrane, namely Alb3, cpSec, and cpTat. A fourth group, that includes certain small membrane proteins, are believed to be inserted within the thylakoids spontaneously (Aldridge *et al.*, 2009; Mori and Cline, 2001; Schunemann, 2004, 2007; Tissier *et al.*, 2002) **(**Fig. 1B**)**.

Pathways that mediate thylakoidal protein biogenesis involve either the Alb3 insertase, or the cpSec translocase, or both. Alb3, a homolog of the bacterial YidC and mitochondrial Oxa1 membrane insertases, functions together with the cpSRP system in the posttranslational targeting and insertion of the light-harvesting chlorophyll-binding proteins (LHCPs) into the thylakoids (Luirink *et al.*, 2001; Sundberg *et al.*, 1997). The cpSRP/Alb3 and cpSec systems also cooperate in the cotranslational integration of a number of chloroplast-encoded subunits (Nilsson and van Wijk, 2002; Nilsson *et al.*, 1999; Zhang *et al.*, 2001). In other situations, the cpSec system can function independent of Alb3/cpSRP posttranslationally for the insertion of nuclear- and chloroplast-encoded thylakoidal proteins (Karnauchov *et al.*, 1994; Mori and Cline, 2001; Nohara *et al.*, 1996; Schuenemann *et al.*, 1999a).

The transport of soluble proteins into the lumen is mediated either by cpTat or cpSec pathways, each specialized for a subset of substrates (Braun and Theg, 2008; Braun *et al.*, 2007; Cline *et al.*, 1992; Mori and Cline, 2001; Schuenemann *et al.*, 1999a). Whereas the former transports prefolded proteins, the latter translocates unfolded substrates in an ATP-dependent manner.

C. PROTEINS DESTINED TO THE INTERMEMBRANE SPACE

Information on the biogenesis of proteins in the intermembrane space between the two envelopes is beginning to emerge. Two nuclear-encoded protein residents of the space, Tic22 and monogalactosyldiacylglycerol synthase isoform 1 (MGD1), have been studied (Kouranov *et al.*, 1999; Vojta *et al.*, 2007a). Whereas both seem to enter via the TOC complex (an observation still controversial for Tic22), it has been proposed that MGD1 reaches the

stroma to the point at which its transit peptide is cleaved by the stromal processing peptidase and then retracts from TIC to the intermembrane space (Vojta *et al.*, 2007a). By contrast, Tic22 likely does not cross the inner envelope at any point of translocation.

D. PROTEINS DESTINED TO THE INNER ENVELOPE MEMBRANE

Two different insertion pathways have been described for proteins residing in the inner envelope membrane (Brink *et al.*, 1995; Firlej-Kwoka *et al.*, 2008) (Fig. 1A). The "stop-transfer" pathway is mediated by builtin hydrophobic signals that arrest them at the TIC level in the membrane and release them laterally into the lipid bilayer (Brink *et al.*, 1995; Firlej-Kwoka *et al.*, 2008). The "conservative sorting" pathway involves a soluble stromal intermediate that is retargeted to the envelope by an unknown mechanism (Li and Schnell, 2006; Lubeck *et al.*, 1996; Tripp *et al.*, 2007; Vojta *et al.*, 2007b).

E. DEVIATIONS FROM THE GENERAL TOC/TIC IMPORT PATHWAY

Proteomic studies have revealed many chloroplast proteins that are apparently transported without a canonical cleavage transit peptide (Kleffmann *et al.*, 2004). In these cases, the targeting information is contained in the mature protein sequence. Knowledge of the chloroplast import mechanism of these proteins is scanty, but novel pathways are being proposed. The import properties of two of these proteins, Tic32 and cQORH, that localize in the inner envelope membrane have been analyzed and, although energy in the form of ATP is required for import, no TOC/TIC components were found to be involved in their import (Miras *et al.*, 2002; Nada and Soll, 2004).

Distinct membrane insertion pathways are active for proteins residing at the outer envelope chloroplast membrane (Hofmann and Theg, 2005a; Inoue, 2007). The topological orientation for α-helical proteins anchored in the membrane by an N-terminal hydrophobic tail, represented by OEP7, is determined by the charged distribution flanking the transmembrane domain (Lee *et al.*, 2001; Salomon *et al.*, 1990; Schleiff *et al.*, 2001). These proteins might make use of the TOC channel for integration, without the need for the receptor components (Tsai *et al.*, 1999; Tu *et al.*, 2004). However, the insertion of proteins with an N_{out}–C_{in} topology, as Toc34, likely does not depend on protein machinery, but rather on the lipid composition of the membrane (Qbadou *et al.*, 2003). A mechanism for spontaneous insertion into the membrane is proposed for certain *β*-barrel outer membrane proteins, like OEP21 and OEP24, because neither proteinaceous machinery nor an obvious energy source seems to be required for their integration (Bolter *et al.*,

1999; Pohlmeyer *et al.*, 1998). The β-barrel protein Toc75 is unique among outer envelope proteins: it contains a cleavable bipartite transit peptide necessary for targeting to the outer envelope as well as for transport via an intricate transport pathway that likely involves stromal components (Tranel and Keegstra, 1996).

Another group of chloroplast proteins with secretory-pathway-targeting signal peptides has been identified and its relevance to their transport has been experimentally verified for a number of representatives (Chen *et al.*, 2004; Nanjo *et al.*, 2006; Villarejo *et al.*, 2005). How these proteins cross the envelope membranes and whether their transport involves vesicle fusion and the participation of TOC/TIC components is currently under investigation.

III. MOLECULAR MACHINERIES INVOLVED IN PROTEIN TRANSLOCATION THROUGH THE CHLOROPLAST ENVELOPE MEMBRANES: THE GENERAL IMPORT PATHWAY

Two multimeric protein complexes facilitate the translocation of most nuclear-encoded proteins into or across the two envelope membranes—the so-called TOC and TIC complexes (Fig. 1A). The subunits that comprise these complexes are designated by the molecular weight of the first subunit identified, usually in pea where much of the biochemical characterization has been done (Schnell *et al.*, 1997).

Protein import into chloroplasts has been divided into three distinct stages based on energy requirements (Perry and Keegstra, 1994): (a) Energy-independent stage during which proteins reversibly bind to the outer chloroplast surface (Kouranov and Schnell, 1997); (b) Early import intermediate stage, an irreversible step that corresponds to the stable binding/insertion and requires $\leq$20 μM ATP in the presence of GTP (Inoue and Akita, 2008; Kessler *et al.*, 1994); and (c) Translocation and processing, that requires $\geq$100 μM ATP (Pain and Blobel, 1987; Theg *et al.*, 1989). Upon emerging from the TIC complex, proteolytic cleavage of the transit peptide results in the formation of the mature, functional protein.

Toc75, the protein-conducting channel that is evolutionarily related to the family of Omp85 transporters in bacteria, is the central component of the TOC complex (Bolter *et al.*, 1998; Hinnah *et al.*, 1997, 2002; Schnell *et al.*, 1994). Toc75 associates with Toc159 and Toc34, two GTPases that act as import receptors at the outer membrane surface (Kessler *et al.*, 1994). These receptors receive the precursors from the cytosolic chaperones, preferentially the pair composed of Hsp70/14-3-3 that recognizes specific phosphorylated

residues in the transit peptide of certain substrates (May and Soll, 2000). Intrinsic GTPase activity gives the receptors the capability to function not only in preprotein recognition, but also as molecular switches and/or molecular motors in transport itself (Kessler and Schnell, 2002; Schleiff *et al.*, 2003). There is evidence that the GTPase cycle of these receptors is modulated by precursor binding (Reddick *et al.*, 2007). The mechanistic model of protein import holds that Toc GTPases (in their GTP-bound state) have high affinity for precursor proteins. GTP hydrolysis (and/or nucleotide exchange) is stimulated upon precursor binding (Becker *et al.*, 2004b; Jelic *et al.*, 2002), releasing the protein to the Toc75 channel due to a loss of affinity of the GDP-loaded form (Jelic *et al.*, 2002; Schleiff *et al.*, 2002). The GTPase receptors are also susceptible to modification by phosphorylation (Jelic *et al.*, 2003; Oreb *et al.*, 2008; Sveshnikova *et al.*, 2000b). Functional analyses have demonstrated, moreover, that phosphorylation and GTPase activity are mutually exclusive, prompting the idea that cycles of phosphorylation/dephosphorylation act as additional regulatory points in protein import. The functional significance of phosphorylation *in vivo* is, however, unknown (Aronsson *et al.*, 2006).

In addition to the GTPase receptors, the TOC complex contains a third receptor subunit, termed Toc64, composed of two distinct functional domains (Fig. 1A). Toc64 is a receptor for the cytosolic Hsp90 chaperone that delivers a subset of precursors to Toc34 (Qbadou *et al.*, 2006, 2007; Sohrt and Soll, 2000). According to *in vivo* studies, its presence may not be strictly required for import, but is essential for highly efficient translocation (Aronsson *et al.*, 2007; Hofmann and Theg, 2005b). On the other hand, Toc64 forms a ternary complex of unknown properties with Toc12 (Becker *et al.*, 2004a) and a chaperone of the Hsp70 family in the intermembrane space (imsHsp70) (Ratnayake *et al.*, 2008). Moreover, Toc64 also recruits Tic22, the only soluble subunit of the TIC complex in the intermembrane space (Qbadou *et al.*, 2007). It has been demonstrated that these four subunits (Hsp90, Toc64, imsHsp70, Tic22) form a complex in an ATP-dependent manner, leading to the proposal of a putative role of imsHsp70 in pulling the precursor from the TOC complex in cooperation with the Toc64/Toc12 pair. An association with Tic22 would assure the continuity of import to the Tic110 inner envelope translocon channel (Balsera *et al.*, 2009a; Gross and Bhattacharya, 2009; Heins *et al.*, 2002; Kessler and Blobel, 1996; Kouranov *et al.*, 1998). During TIC translocation, a putative import motor formed by stromal Hsp93 (or ClpC) chaperone develops in coordination with the cochaperone Tic40 (Chou *et al.*, 2006; Kovacheva *et al.*, 2005, 2007; Nielsen *et al.*, 1997; Stahl *et al.*, 1999). In the current mechanistic model, precursor interaction with Tic110 promotes the association of the

Tic40–Hsp93 complex with Tic110 that then supports transfer of the transit peptide to Hsp93 and stimulation of its ATPase activity, followed by delivery of the preprotein to the stroma.

Three redox sensor proteins, Tic62, Tic32, and Tic55, appear to regulate import according to the chloroplast metabolic redox state. Tic62 and Tic32 are classified as short-chain dehydrogenases (SDR)/reductases that associate with TIC in a regulatory manner dependent on NADPH, ferredoxin-NADP-oxidoreductase (FNR), and Ca^{2+} (Chigri *et al.*, 2006; Hormann *et al.*, 2004; Kuchler *et al.*, 2002; Stengel *et al.*, 2008). Tic55 contains a Rieske iron–sulfur center and a mononuclear iron-binding site (Caliebe *et al.*, 1997). It remains to be seen how these TIC-associated redox-active proteins function during import. The identification of their substrates and response to different cellular conditions also awaits further work.

IV. STRUCTURE–FUNCTION RELATIONS OF TOC AND TIC COMPONENTS: POTENTIAL FOR REDOX REGULATION

A. THE PORE-FORMING PROTEIN Toc75: POTRA MOTIFS AND CHANNEL PROPERTIES

Toc75, the protein-import channel in the TOC complex, is known as Toc75-III in *Arabidopsis thaliana*, referring to the chromosome where the protein is encoded (At3g46740). Two structurally and functionally different domains are recognized in the Toc75 protein sequence (Fig. 2A). The N-terminal soluble component, which functions as a receptor-binding site, harbors polypeptide-transport-associated (POTRA) domains involved in protein–protein interaction (Sanchez-Pulido *et al.*, 2003). The C-terminal membrane domain consists of antiparallel β-strands that form a β-barrel hydrophilic channel within the membrane through which precursor proteins are transported (Reddick *et al.*, 2008; Sveshnikova *et al.*, 2000a). Reconstitution experiments with recombinant Toc75 in planar lipid bilayer membranes allowed *in vitro* characterization of the channel (Hinnah *et al.*, 2002) and demonstrated that the N-terminal region has the capability to modulate channel properties (Bredemeier *et al.*, 2007; Ertel *et al.*, 2005).

Recently, high-resolution structures of the N-terminal POTRA domain and the C-terminal β-barrel pore for Toc75 bacterial homologs have been obtained by X-ray crystallography (Clantin *et al.*, 2007; Gatzeva-Topalova *et al.*, 2008; Kim *et al.*, 2007), providing a general insight into the structural rearrangement and mechanism of transport. These studies, together with

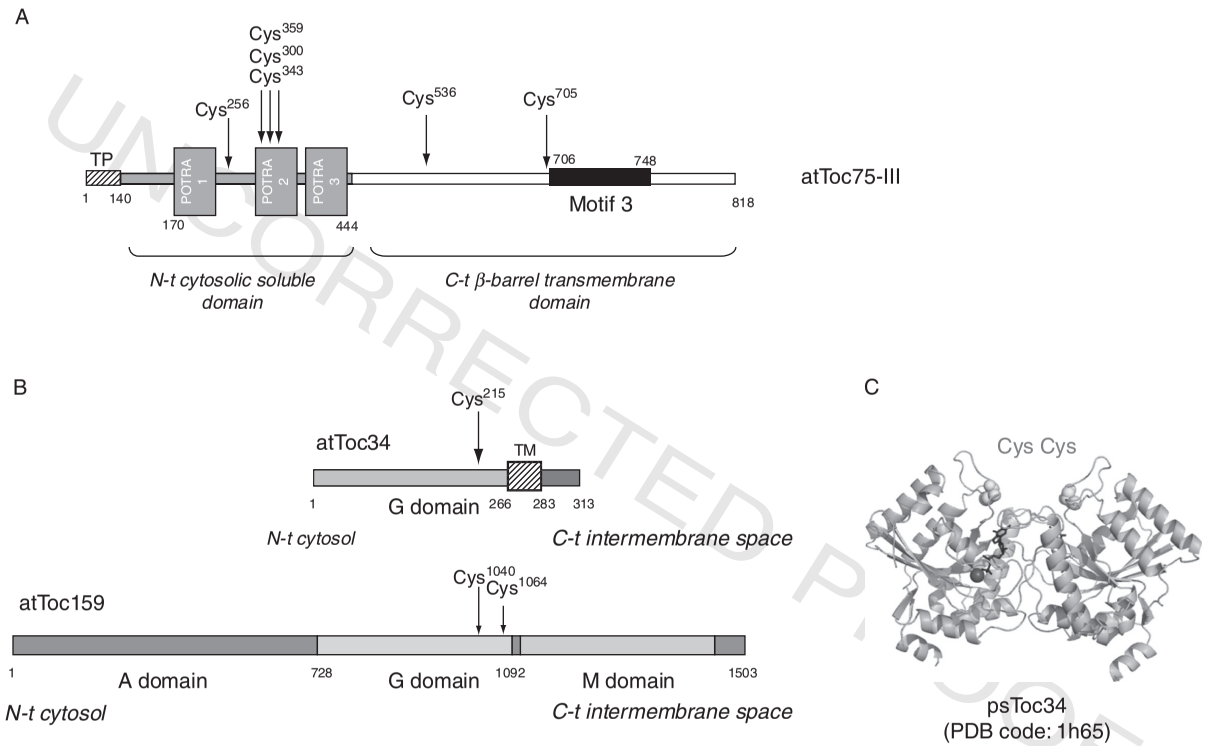

Fig. 2.

solution structures of the POTRA domain determined by small X-ray scattering and NMR (Knowles *et al.*, 2008), demonstrated the conformational flexibility of the POTRA domains that is essential for substrate binding. Functional analyses demonstrated that the POTRA domains differ in functional importance, consistent with the variation in the number of POTRA domains among species (Kim *et al.*, 2007). Toc75-III contains three POTRA domains **(**Fig. 2A**)**, each with a $\beta1$–$\alpha1$–$\alpha2$–$\beta2$–$\beta3$ topology (Kim *et al.*, 2007). The structure of the membrane-embedded C-terminal β-barrel domain revealed the presence of 16 β-strand modules in FhaC from *Bordetella pertussi*, a bacterial Toc75 homolog (Clantin *et al.*, 2007). Based on this structure, homology models have been built for POTRA2 and 3 as well as the β-barrel for pea Toc75 (Reddick *et al.*, 2008). The crystallographic structure also contributed to understanding the so-called motif 3, a highly conserved region within the β-barrel domain identified previously in a protein sequence analysis of the Toc75 protein family (Moslavac *et al.*, 2005). This motif is made up of a long loop, termed L6, and the first half of strand B12 (Clantin *et al.*, 2007). In the conformational state with which the structure was solved, motif 3 is inside the transmembrane β-barrel (Clantin *et al.*, 2007). The authors proposed that motif 3 is an active element in transport and that conformational changes would expel L6 from the inside, opening the channel for translocation. Interestingly, a number of conserved Cys residues are uniquely found in the second POTRA domain and at the beginning of motif 3 in the plant sequences **(**Fig. 2A**)**. The functional or structural importance of these residues is unknown. However, according to the homology model, they are not sufficiently close to participate in an intramolecular interaction, but could interact with other TOC subunits in regulating protein import.

B. THE Toc159 AND Toc34 GTPase RECEPTORS: A MULTIGENE FAMILY

Toc159 and Toc34 operate as receptors in the outer membrane and provide the import process with specificity and efficiency (Agne and Kessler, 2009; Kessler and Schnell, 2002). Sequence analysis revealed that the TOC GTPases, Toc159 and Toc34, are evolutionarily related and similarly organized (Hiltbrunner *et al.*, 2001a; Hofmann and Theg, 2003; Reumann *et al.*, 2005). Toc34 is anchored to the membrane by a short transmembrane helix at the C-terminal tail and exposes the GTP-binding domain (G domain) to the cytosol (Fig. 2B). Toc159 is characterized by a large N-terminal acidic domain (A domain), followed by the Toc34-related GTP-binding domain (G domain) and a membrane-protected C-terminal domain (M domain) (Fig. 2B), exposing the A and G domains to the cytosol (Becker *et al.*,

2004b; Chen *et al.*, 2000; Hiltbrunner *et al.*, 2001a; Hirsch *et al.*, 1994; Kessler *et al.*, 1994; Schnell *et al.*, 1994; Seedorf *et al.*, 1995). Studies on truncated versions of Toc159 have demonstrated that the A domain is dispensable for activity, and its role in protein import remains evasive (Agne *et al.*, 2009; Chen *et al.*, 2000). On the other hand, even though it has been shown that precursor proteins interact directly with the M domain of Toc159 (Kouranov and Schnell, 1997), its function, structure, and membrane topology remain unknown. Secondary structure predictions indicate that the M domain is composed mainly of β-sheets (data not shown), while topological prediction programs (TBBpred and TMBpro (Natt *et al.*, 2004; Randall *et al.*, 2008)) indicate the M-domain is likely forming a transmembrane β-barrel module.

High-resolution structures of the soluble G-domain of pea and *Arabidopsis* Toc34 in the GDP- or GTP-bound form have contributed useful information not only about the functional mechanism and recognition of peptide-binding pocket, but also about the quaternary structure and dimerization mode of the protein (Koenig *et al.*, 2008a; Sun *et al.*, 2002) (Fig. 2C). Independent experiments have demonstrated the ability of Toc34 and Toc159 to undergo homo- and heterodimerization via their GTPase domains (Bauer *et al.*, 2002; Becker *et al.*, 2004b; Hiltbrunner *et al.*, 2001a; Koenig *et al.*, 2008b; Smith *et al.*, 2002; Weibel *et al.*, 2003; Yeh *et al.*, 2007). Recently, an interaction between Toc34 and Toc159 demonstrated *in vivo* in *Arabidopsis* (Rahim *et al.*, 2009) prompted the proposal that interchangeable homodimeric and heterodimeric states may serve as a regulatory mechanism. The two subunits of the dimer may function as GTPase-activating proteins that reciprocally stimulate or coordinate the hydrolysis of GTP. Apropos this point, the transit peptide likely plays a prominent role in stimulating the GTPase activity of the receptors and influencing their interaction (Becker *et al.*, 2004b).

Even though extensive work has been done on TOC GTPase receptors, the actual sequence of events in preprotein recognition and transfer of the precursor to the channel is still under debate. Two models are being considered. Both agree with GTP-mediated regulation of the Toc34/Toc159 receptors and the function of the central channel Toc75, but they differ in the mode of preprotein recognition. The first model (targeting model) favors Toc159 as the initial preprotein receptor (Bauer *et al.*, 2002; Hiltbrunner *et al.*, 2001b; Smith *et al.*, 2004). Binding of the preprotein to Toc159 promotes oligomerization of both GTPases, Toc34 and Toc159, in their GTP-bound state. The interaction with Toc75 would stimulate their GTPase activity, resulting in transfer of the cargo protein to the channel. A second model (motor model) proposes Toc34 to be the initial preprotein receptor

and Toc159 to function as a GTP-driven motor that moves preproteins through the channel (Becker *et al.*, 2004b; Schleiff *et al.*, 2003; Sveshnikova *et al.*, 2000b). The mechanistic models proposed up to date undoubtedly need further improvement. Structural analysis of the functional complex at high resolution is essential to understanding the mechanism of preprotein recognition and translocation involving the GTPases receptors and central channel.

Plant Toc159 and Toc34 are encoded by a multigene family (Bauer *et al.*, 2000; Gutensohn *et al.*, 2000; Hiltbrunner *et al.*, 2001a; Jackson-Constan and Keegstra, 2001; Jarvis *et al.*, 1998; Kubis *et al.*, 2004). Only one protein is known for each Toc159 and Toc34 component in pea (psToc159 and psToc34, respectively), where most of the biochemical studies have been performed. However, other isoforms should not be ignored until the genome sequence is completed.

Several studies have demonstrated functional specialization among the Toc159 and Toc34 isoforms. Further, genetic analysis of knockout mutants of Toc159 homologs in *Arabidopsis* (atToc159, atToc132, atToc120, and atToc90) indicated functional redundancy between Toc132 and Toc120 and minimal functional overlap among Toc132/Toc120, Toc159, and Toc90 (Bauer *et al.*, 2000; Ivanova *et al.*, 2004; Kubis *et al.*, 2004). Characterization of the expression and accumulation of photosynthetic proteins in *Arabidopsis* mutants led to the conclusion that Toc159 is a receptor with specificity for highly abundant, photosynthetic proteins and that Toc132 and Toc120 are preferentially involved in the import of nonphotosynthetic proteins, especially in nongreen tissues (Bauer *et al.*, 2000; Kubis *et al.*, 2004). Toc90, the shortest member of the Toc159 family that lacks a yet uncharacterized A domain, may support the accumulation of photosynthetic proteins in plastids jointly with Toc159 (Hiltbrunner *et al.*, 2004). Further evidence for the existence of an import pathway with preference for photosynthetic precursors was gained by detailed studies on Toc34 mutant plants. In *Arabidopsis*, the Toc34 family is composed of atToc34 and atToc33, isoforms with different biochemical properties and tissue distribution (Jarvis *et al.*, 1998; Jelic *et al.*, 2003; Kubis *et al.*, 2003). atToc33 is mainly expressed in photosynthetic tissues and can be specifically phosphorylated, whereas atToc34 is the dominant isoform in roots and is not modified by phosphorylation. These differences are consistent with different modes of regulation—a proposal supported by genetic studies with *Arabidopsis* revealing that these two isoforms may function as receptors for different subsets of substrates. Thus, the *toc33* knockout mutant is deficient in expression, import, and accumulation of photosynthetic proteins. Specific and preferential association between the different Toc34 and Toc159 isoforms meets functional needs: atToc34 with

atToc132 and atToc120, and atToc159 with atToc33 (Bauer *et al.*, 2000; Gutensohn *et al.*, 2000; Jarvis *et al.*, 1998; Jelic *et al.*, 2003).

C. ACCESSORY TOC COMPONENTS: Toc64, Toc12, AND ImHsp70

A third preprotein receptor, termed Toc64, has been implicated in the protein import process in chloroplasts (Qbadou *et al.*, 2007; Sohrt and Soll, 2000). Toc64 has been described as having bimodular architecture and dual functionality, acting at both sides of the outer envelope membrane (Fig. 3A). Topological studies indicate that Toc64 is anchored to the membrane by three transmembrane helices in an N_{in}–C_{out} topology (Lee *et al.*, 2004b; Qbadou *et al.*, 2007). The C-terminal domain, composed of three tetratricopeptide repeats (TPR), is exposed to the cytosol and is involved in the recognition and interaction with cytosolic Hsp90 chaperones preloaded with precursor proteins (Mirus *et al.*, 2009; Qbadou *et al.*, 2006, 2007). The N-terminal amidase-like domain, oriented to the intermembrane space, seems to be involved in recruiting chaperones via interaction with a cochaperone, Toc12, and likely participates in coordination events at the intermembrane space side after preprotein translocation through Toc75 (Qbadou *et al.*, 2007). Functional analyses demonstrated that Toc64 lacks the capability to act as an amidase. Moreover, it seems like its amidase conformation has been dramatically disrupted by hydrophobic helices traversing the outer envelope membrane (Qbadou *et al.*, 2007; Sohrt and Soll, 2000) (Fig. 3A). Although perhaps essential for high-efficiency protein import, genetic studies have assigned a secondary role to Toc64 because it may not be required protein import per se (Aronsson *et al.*, 2007; Hofmann and Theg, 2005b). Furthermore, a detailed analysis of the *Arabidopsis* genome indicates the presence of a second Toc64 homolog in chloroplasts (J. Soll, unpublished data). Alternatively, it might have a very specific function for the import of just a subset of substrates, as demonstrated for its homolog in mitochondria (Lister *et al.*, 2007).

As the most recently described member of the TOC complex, little is known about Toc12 (Becker *et al.*, 2004a). It is anchored to the outer envelope membrane by a short motif at its N-terminus. The rest of the protein is exposed to the intermembrane space and shows sequence similarity to J-domains (Fig. 3B). In fact, biochemical studies have identified an interaction with Hsp70-like proteins in the intermembrane space (Becker *et al.*, 2004a). The exact nature and properties of the imsHsp70 that interacts with Toc12 in chloroplasts remain to be defined (Becker *et al.*, 2004a; Marshall *et al.*, 1990; Qbadou *et al.*, 2007; Ratnayake *et al.*, 2008; Schnell *et al.*, 1994; Waegemann and Soll, 1991).

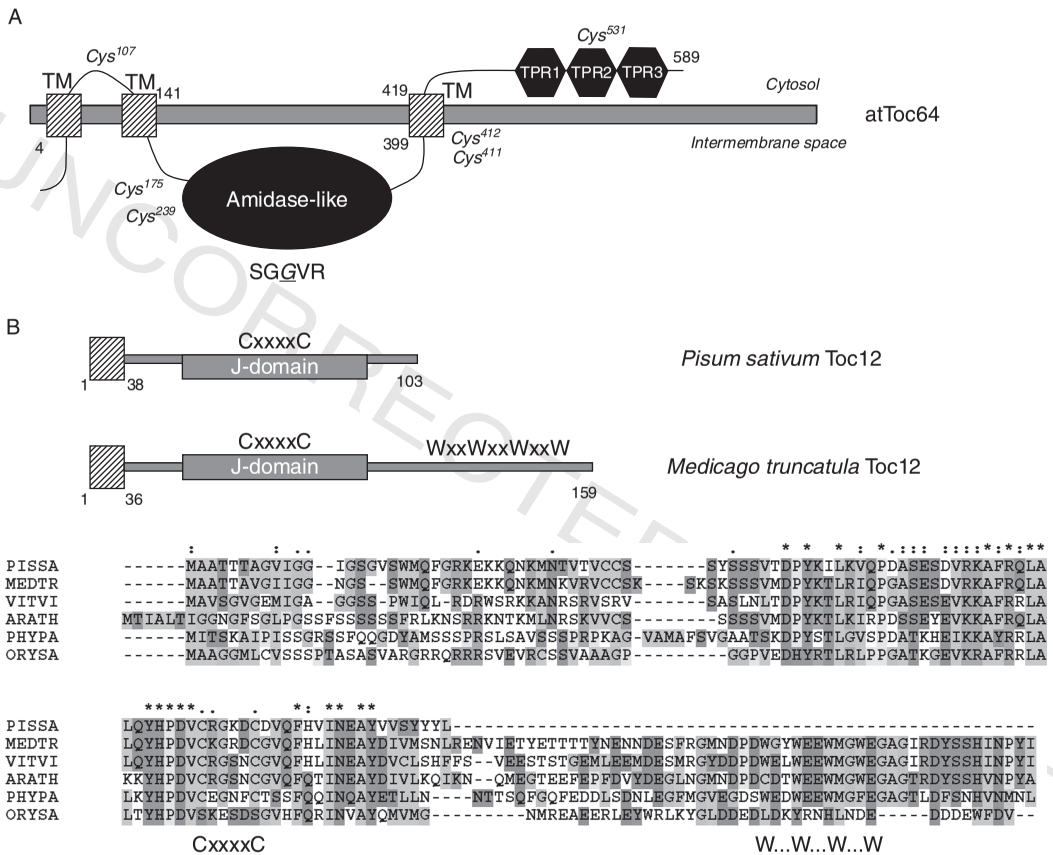

Fig. 3.

A three-dimensional model of pea Toc12 has been built from an NMR structure and a disulfide bridge involving the Cys of its characteristic CXGXXC motif has been proposed (Becker *et al.*, 2004a) (Fig. 3B). The disulfide bridge may be essential for its cochaperone activity because the mutation of the N-terminal Cys into serine dramatically affects Toc12 functionality in pea (Becker *et al.*, 2004a). Taking into account that reversible disulfide bridge formation is a common mechanism to alter chloroplast enzyme activity, the authors proposed that the bridge maybe important for the regulation of the translocation event. Although there is no direct evidence for this to date, a potential disulfide bridge constitutes an interesting statement on the basis of redox properties of the chloroplast intermembrane space. In pea, Toc12 is a protein of 103 amino acids, of which the first 38 residues may function as noncleavage targeting signal to chloroplasts (as reported in ARAMEMNON database; http://aramemnon.botanik.uni-koeln.de/) as well as membrane anchor (Becker *et al.*, 2004a). Homologs of Toc12 are found in *Arabidopsis*, *Zea*, *Medicago*, and *Physcomitrella*, albeit longer in sequence (160 residues). A multiple sequence alignment shows that pea lacks the last 52–54 amino acid residues found in other members of the family (Fig. 3B). Actually the *toc12* gene is composed of three exons, the third of which corresponds to the missing component in the Toc12 pea sequence (Fig. 3B). This structure suggests that either pea Toc12 sequence is incomplete or that the pea protein, indeed, lacks this domain possibly due to alternative splicing. Interestingly, a highly conserved tryptophan-rich motif (WxxWxxWxxW) is found in this domain. The functional significance of the absence of the C-terminal extension in pea Toc12 remains to be explored. The protein sequence from rice constitutes an exception as the Cys residues in the CXGXXC motif are replaced by serine and lacks the conserved tryptophans (Fig. 3B). Additional studies are necessary to analyze the role of the C-terminal region absent in pea and not conserved in rice as well as the significance of the Cys mutation in rice and possibly other cereals.

D. Tic22, A PUTATIVE LINKER IN THE INTERMEMBRANE SPACE

Little is known about the structure and function of Tic22—a soluble protein in the intermembrane space, peripherally associated with the outer face of the inner membrane (Fig. 1A) (Kouranov *et al.*, 1998). It has been found closely associated to Tic20, Tic110, and, as mentioned earlier, in a complex with a number of TOC components (Becker *et al.*, 2004a; Qbadou *et al.*, 2007). These findings have led to the proposal that Tic22 may participate as a molecular linker in the interaction between TOC and TIC during protein import. Phylogenetic analyses revealed that Tic22 is a protein of

cyanobacterial origin. Its homolog in *Synechocystis* (slr0924) has been experimentally located in the thylakoid lumen and, to a minor extent, in the periplasmic space (Fulda *et al.*, 2002). Tic22 is an essential protein in cyanobacteria and, interestingly, its content seems to be regulated by the redox state of the cell. Two putative roles were ascribed to slr0924 protein in *Synechocystis*, one in the transport of protein subunits and the other linked (directly or indirectly) to electron transfer (Fulda *et al.*, 2002). The relevance of these ideas awaits further experiments.

Two Tic22 isoforms are recognized in the genome of *Arabidopsis* (Fig. 4A): Tic22-IV (At4g33350) has two conserved Cys in two different motifs (Gly-X-X-Cys-Phe and Cys-Pro) and represents the ortholog of Tic22 in pea. The other isoform, Tic22-III (At3g23710), has one conserved Cys. Secondary structure predictions indicate that Tic22 is a mixed α/β protein (Fig. 4A). According to PHYRE, a protein structure prediction program (Kelley and Sternberg, 2009), templates for structural modeling with highest likelihood to be correct (45% estimated precision) are related to a number of proteins that contain a Trx-like fold and belong to the Trx superfamily. Two groups of proteins are recognized: one group is constituted of proteins that have evolved to bind metals to act as chaperones (metal-binding motif: Cys-Pro-X-X-Cys-Pro; PDB codes: 2cx4, 2ggt, 2b7k); the others are members of the DsbD-DsbC family (catalytic motif: Cys-X-X-Cys; PDB code: 1eej). The results from PHYRE are in accord with those obtained by the BIOINFO server (Ginalski *et al.*, 2003). Although the presence of redox-active Cys cannot be disregarded as both Tic22 isoforms have conserved Cys, their function as a Trx is not expected due to the absence of the canonical Trx catalytic motif, Cys-X-X-Cys. Apropos this point, it is noted that recent phylogenetic analyses have identified a number of Trx- and glutaredoxin-related sequences containing a single Cys at the putative active site (Fig. 4A) and the conserved metal-binding motif is not present. Moreover, due to the absence of Cys in the cyanobacterial homolog of Tic22, a relation with bacterial DsbD-DsbC is not appropriate (Fig. 4A). A putative function could be envisaged as a chaperone that involves a noncovalent interaction with substrates—like either protein disulfide isomerase-related ERp29 in the endoplasmic reticulum that contains an inactive Trx fold and assists in the maturation and transport of many secretory proteins (Barak *et al.*, 2009), or the Q8ZP25_SALTY and HYAE_ECOLI proteins that interact with the Tat signal peptide and function as molecular chaperones (Parish *et al.*, 2008). Structural and functional characterizations of Tic22 would give useful insight into the function of this putative chaperone in the chloroplast intermembrane space.

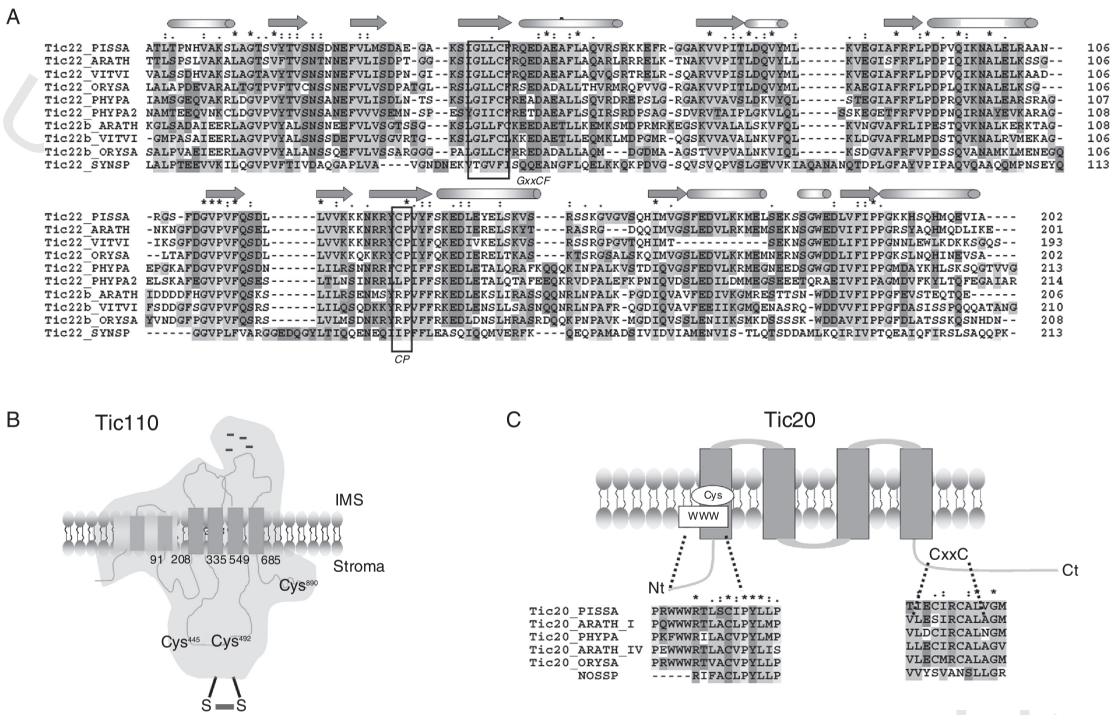

Fig. 4.

E. THE PROTEIN-CONDUCTING CHANNEL OF THE INNER ENVELOPE: Tic110 AND Tic20

Tic110 is an essential and specific protein in chloroplast-containing organisms (Inaba *et al.*, 2005; Schnell *et al.*, 1994; Wu *et al.*, 1994). It is found as a single copy in all plants, except for *Physcomitrella patens* in which a second isoform has been detected by genome sequence analysis (Kalanon and McFadden, 2008). There is extensive evidence that Tic110 forms the protein translocation channel at the inner envelope membrane of chloroplasts (Fig. 1A), although other specialized import pathways appear to be present, for example, Tic20 (Kouranov *et al.*, 1998).

Tic110 comprises two hydrophobic membrane-spanning α-helices at the extreme N-terminus, both necessary for anchoring into the inner envelope (Lubeck *et al.*, 1996). A new topological model of Tic110 incorporates four additional transmembrane helices, with amphipathic features, within the region of residues 92–959 in the pea sequence (Balsera *et al.*, 2009a) (Fig. 4B). This new model for Tic110 reconciles the results of numerous studies during the past several years. The model contains regions in the intermembrane space suitable to form supercomplexes with the TOC machinery and to receive the precursor as well as a large region in the stroma that could interact with molecular chaperones (Inaba *et al.*, 2003, 2005). However, other studies suggest that Tic110 forms a large globular domain on the stromal side, without channel activity, to recruit chaperones (Inaba *et al.*, 2003; Jackson *et al.*, 1998). Electrophysiological characterization of reconstituted planar lipid bilayer membranes of native Tic110 as well as recombinant mutants of the protein lacking the two hydrophobic transmembrane helices at the N-terminus favor the former topological model (Balsera *et al.*, 2009a; Heins *et al.*, 2002) (Fig. 4B). Experiments have shown that Tic110 has properties of a cation-selective channel that is sensitive to chloroplast transit peptides. These studies have assigned a prominent role to the amphipathic transmembrane helices in the channel structure of Tic110. Interestingly, Ca^{2+} seems to act as an effector of gating and selectivity in the Tic110 channel.

Tic110 is composed mainly of α-helices (Balsera *et al.*, 2009a; Inaba *et al.*, 2003). Computational analyses have detected the presence of two helical unit repetitions in the stroma that resemble those of the so-called HEAT motifs (Andrade *et al.*, 2001; Balsera *et al.*, 2009a). Interestingly, a large number of the HEAT repeats are involved in cytoplasmic transport, where the two helical units appear to function as flexible joints that can wrap around target substrates and act as scaffolding on which other molecular components assemble (Andrade and Bork, 1995; Andrade *et al.*, 2001). A highly

disordered, negatively charged region may be important for protein interaction, likely in the intermembrane space (Balsera *et al.*, 2009a). In addition, a number of fully conserved Cys are spread over the sequence and, according to the proposed topological model (Balsera *et al.*, 2009a), they are likely localized in the stromal region of the protein (Fig. 4B). Biochemical experiments uncovered a redox-active disulfide bridge potentially functional in regulating Tic110 via stromal Trx. Owing to its presence in the reduced state in chloroplasts isolated from darkened plants, Tic110 was assigned a role in allowing the channel to adapt to environmental change (Balsera *et al.*, 2009a). Thus, when channel activity is disturbed by oxidation, Trx could restore the system to normal activity. The validity of this interpretation awaits further experiments.

Tic20 (Kouranov *et al.*, 1998) and Tic21 (Teng *et al.*, 2006) are two other candidates proposed to constitute translocation channels at the inner envelope membrane of chloroplasts. Participation of Tic20 in the import of preproteins has been assessed by crosslinking experiments in which the protein was found to form part of an active import supercomplex in the chloroplast envelope together with other TOC and TIC components (Kouranov and Schnell, 1997; Kouranov *et al.*, 1998). Although Tic20 knockout plants are not viable, *in vivo* analyses have been carried out in transgenic *Arabidopsis* plants in which levels of expression of the protein were altered (Chen *et al.*, 2002). It was concluded that, while the import of certain nuclear-encoded plastid proteins was impaired, others were not affected. More recently, analyses of a conditional mutant generated in *Toxoplasma gondii* showed that Tic20 is essential for protein import albeit likely not as the general import pore, but as an accessory or regulatory component of the import complex (van Dooren *et al.*, 2008). Topological studies have not yet been addressed, but four α-helical transmembrane regions are predicted by hydrophobicity analyses (Fig. 4C). GFP fusion at the Tic20 C-terminus in *Toxoplasma gondii* demonstrated that this region is oriented toward the stroma, suggesting an N_{in}–C_{in} topology (van Dooren *et al.*, 2008).

Databases reveal two isoforms of Tic20 in *A. thaliana*: Tic20-I (At1g04940) is the ortholog of psTic20 and Tic20-IV (At4g03320). Two other *Arabidopsis* proteins distantly related to Tic20 (At2g47840 and At5g55710) represent the closest orthologs to cyanobacterial counterparts (Kalanon and McFadden, 2008). A chloroplast location is predicted for all four proteins by the ARAMMENON database (http://aramemnon.botanik.uni-koeln.de/). Given that the function of these isoforms is not known, further characterizations of the cyanobacterial counterparts may help understanding the role of Tic20 in plants (Gross and Bhattacharya, 2009). Tic20 may form a channel independent of Tic110.

Analysis of protein sequences of Tic20-I family members reveals a highly conserved C-terminal CXXC motif (except in *Arabidopsis* where it is replaced by an SXXC motif) and a highly conserved CXP motif close to a WWW motif within the first putative transmembrane helix. Assuming four transmembrane helices in Tic20 and topology similar to *Toxoplasma gondii* (van Dooren *et al.*, 2008), the CXXC motif would face the chloroplast stroma (Fig. 4C). However, whether these Cys act in a regulatory capacity remains to be experimentally verified.

The other inner membrane component proposed to function as channel for protein import, Tic21 (At5g15290), has four predicted transmembrane helical segments (Teng *et al.*, 2006). However, its affiliation to the TIC complex is a matter of debate as the protein has been identified as a metal permease (Duy *et al.*, 2007).

F. ENERGY DRIVING FORCE IN THE TIC COMPLEX: Tic40 AND Hsp93/ClpC

One of the functional aspects of Tic110 is its capability to recruit the stromal motor complex composed of the membrane-attached Tic40 cochaperone and the stromal Hsp93 (or ClpC) chaperone at the inner envelope membrane (Fig. 1A) (Nielsen *et al.*, 1997; Stahl *et al.*, 1999). Tic40 is anchored to the inner membrane by a single transmembrane helix at its N-terminus, exposing a large C-terminal domain to the stroma that contains a putative TPR domain followed by a Sti1 domain that is characteristic of cochaperones Hsp70-interacting protein (Hip) and Hsp70/Hsp90-organizing protein (Hop) (Bedard *et al.*, 2007; Chou *et al.*, 2003; Stahl *et al.*, 1999) (Fig. 5A). The TPR motif is a degenerate 34-amino acid sequence that structurally consists of two antiparallel α-helices often found in tandem repetitions in a protein sequence. This motif is found in certain cochaperones, especially for interaction with Hsp90 (Odunuga *et al.*, 2003). It has been demonstrated that the Tic40 Sti1 domain is functionally equivalent to the Hip/Hop family of cochaperones (Bedard *et al.*, 2007; Chou *et al.*, 2006). The mode of action currently envisaged involves the ternary interaction of Tic40 with Hsp93 and Tic110 during a late stage of translocation. A region that includes the putative TPR domain of Tic40 has been implicated in interaction with Tic110, while the Sti1 domain seems to stimulate the ATPase activity of the Hsp93 chaperone. Analyses of mutant plants suggest that Tic40 might not essential for protein import, but is important for its efficiency (Chou *et al.*, 2003). Refinements of the current model are awaited with interest (Jarvis, 2008).

An analysis of its amino acid sequence with the SMART search engine (Letunic *et al.*, 2006) revealed that Tic40 likely consists of two Sti1 modules (Karpenahalli *et al.*, 2007). Surprisingly, the TPR motifs were not

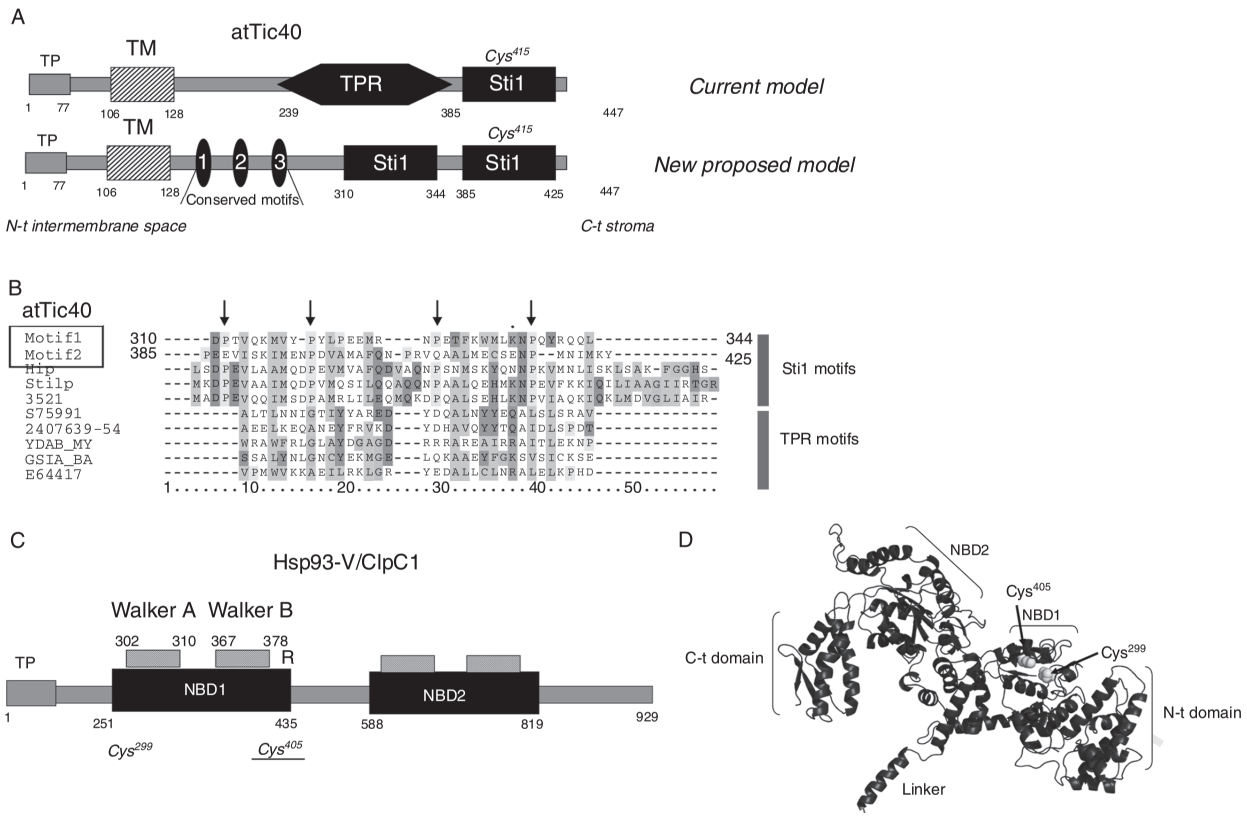

Fig. 5.

recognized. A further search with the TPRpred and REP programs (Andrade *et al.*, 2000) yielded negative results, suggesting that, if TPR motifs are present in Tic40, they are largely degenerated. In fact, the protein sequence region where putative TPR motifs were identified in Tic40 is composed of strategically situated proline residues that fit better with Sti1 motifs than with TPR motifs (Fig. 5B). Based on this finding, an alternative module organization is proposed here in which two Sti1 motifs are arranged in tandem at the C-terminus of Tic40 (Fig. 5A). Considering that Sti1 modules are also composed of α-helices, the secondary structure of Tic40 would fit with the second alternative model presented here. Structural analyses are, however, necessary to confirm a given model. Bedard and collaborators pointed out that the Tic40 family of proteins contains only one conserved Cys within their Sti1 domain (Bedard *et al.*, 2007). This observation may explain how Tic40 covalently interacts with Tic110 in the presence of copper chloride (II)—that is, by forming a crosslink between this Cys and a counterpart in Tic110 (Stahl *et al.*, 1999). The Tic40 Cys residue is, however, in the region proposed to interact with Hsp93, not Tic110. This point should be further studied with other interaction partners under controlled redox conditions.

Hsp93 (or ClpC) belongs to class I of the Hsp100/Clp superfamily—molecular chaperones that use ATP to promote changes in the folding of proteins and belong to the functionally heterogeneous AAA+ family of proteins (ATPases associated with various cellular activities). They can function as independent molecular chaperones or as regulatory components of the Clp protease. The Hsp93 population associated with the inner envelope in chloroplasts is recognized as a component of the TIC machinery that acts as the protein motor in the final stages of import (Akita *et al.*, 1997; Kovacheva *et al.*, 2005, 2007; Nielsen *et al.*, 1997). At the protein sequence level, Hsp93 in chloroplasts is very similar to the counterparts in cyanobacteria (data not shown), and no obvious motif can be identified at first sight as a determinant for a specific function in protein import transport. *Arabidopsis* has two isoforms of the protein: Hsp93-V or ClpC1 (At5g50920) and Hsp93-III or ClpC2 (At3g48870) (Constan *et al.*, 2004; Kovacheva *et al.*, 2007). Dimers of ClpC1, but not ClpC2, were detected in the *Arabidopsis* stroma by proteomics (Peltier *et al.*, 2004). On the other hand, in maize mesophyll chloroplasts, a hexameric ClpC was observed (Majeran *et al.*, 2008) in accord with the expected functional assembly (Andersson *et al.*, 2006).

Recently, Hsp93 was identified as a potential Trx target in cyanobacteria, although the physiological significance of this observation is not known (Mata-Cabana *et al.*, 2007). Moreover, the expression of *clpC* seems to respond to redox state in *Synechocystis* (Hihara *et al.*, 2003). Interestingly, knockout mutants plants of chloroplast NADPH-Trx reductase (NTRC), a

protein involved in the response to environmental stress, showed increased levels of transcripts for Hsp93-V in short-day *ntrc* rosette leaves (Lepistö *et al.*, 2009).

The Hsp93 sequence has two ATP-binding domains (NBD1 and NBD2), each containing two conserved motifs (known as Walker A and Walker B) that participate in the binding of nucleotide and the Mg^{2+} cation (Schirmer *et al.*, 1996). NBD1 and NBD2 are linked by a central spacer that provides flexibility to the molecule. Further, the so-called arginine finger (R-finger) senses ATP binding and hydrolysis and transmits conformational changes essential for Hsp93 activity (Fig. 5C). Cyanobacterial Hsp93 contains a single conserved Cys, suggesting that a regulatory intermolecular disulfide bridge links activity to Trx. The conserved Cys residue is located at the beginning of the Walker A motif in the first nucleotide-binding domain (Fig. 5C) and thus may have a direct effect on ATPase activity. In plants, the situation is different: a second conserved Cys is present in the NBD1 domain (Fig. 5C). According to a theoretical structure built by homology modeling using a high-resolution structure of ClpB as template (PDB code: 1QVR; 49% identity), these two Cys residues (Cys299 and Cys405 in Hsp93-V from *Arabidopsis*) are sufficiently close to form an intramolecular disulfide bridge (Fig. 5D). If so, the disulfide bridge could either be structural or regulate direct ATPase activity. The applicability of either of these views awaits future experiments.

G. REGULATORY COMPONENTS OF THE TIC COMPLEX: Tic55, Tic62, AND Tic32

Three modular proteins, Tic55, Tic62, and Tic32, have been classified as members of a redox regulatory network at the TIC machinery (Caliebe *et al.*, 1997; Hormann *et al.*, 2004; Kuchler *et al.*, 2002). Tic55 is a multi-domain integral membrane protein that consists of: (i) a Rieske domain that binds a [2Fe-2S] cluster via the motif Cys-X-His-X16-Cys-X2-His; (ii) a catalytic domain that contains a mononuclear nonheme mononuclear Fe(II) ion active site, likely coordinated by two histidine residues and one carboxylate ligand from an aspartate residue, a type of structural motif known as ''2-His-1-carboxylate facial triad''; (iii) a hydrophilic CXXC domain of unknown function; (iv) a C-terminal transmembrane domain predicted to contain two transmembrane hydrophobic helices, TM (Caliebe *et al.*, 1997) (Fig. 6A). *In vitro* protein import experiments in chloroplasts showed that the translocation of precursor proteins into chloroplasts was diminished by diethylpyrocarbonate, possibly via modification of the Rieske iron–sulfur cluster. However, the role for Tic55 in protein import is not

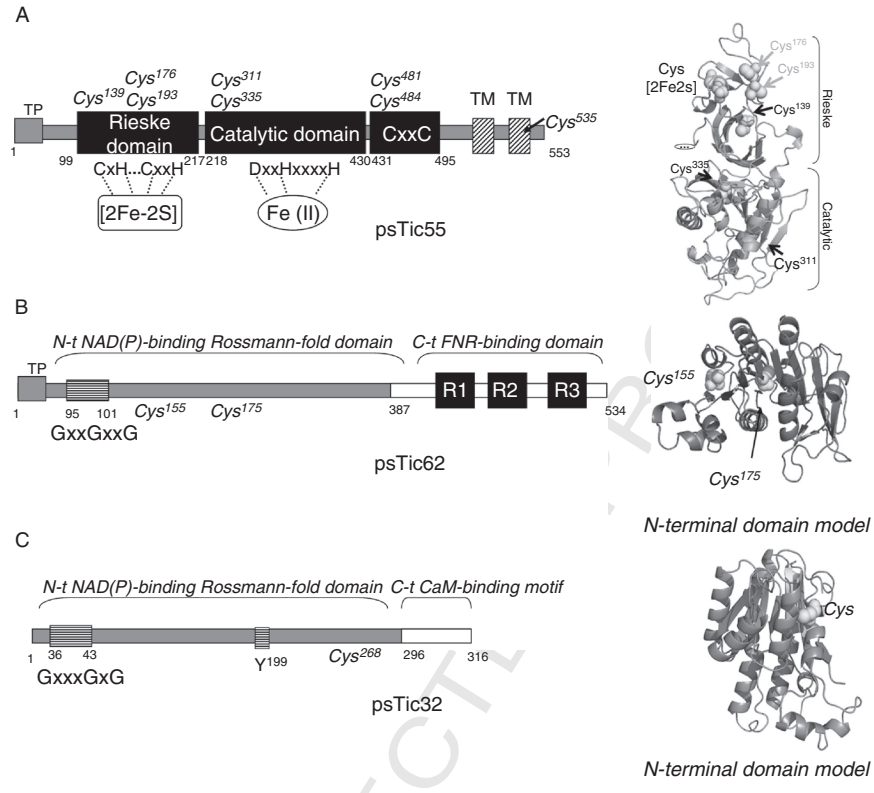

Fig. 6. (See color plate section at the back of the book.)

known. It is of cyanobacterial origin evolutionarily and is related to the LLS1 (*Lethal-leaf spot 1*)-family of oxygenases in plants (Gray *et al.*, 2004).

This type of Rieske nonheme protein, which is widespread among electron transfer proteins, participates in widely divergent enzyme reactions. Whether Tic55 participates in an electron transfer reaction or as a redox sensor element that controls TIC activity, or both, remains to be investigated (Caliebe *et al.*, 1997; Hidalgo *et al.*, 1997; JagerVottero *et al.*, 1997). There is evidence that the oxidation state of the Rieske center acts as a regulatory element based on findings with similar proteins (Martins *et al.*, 2005). Interestingly, Tic55 has recently been identified as a potential Trx target (Bartsch *et al.*, 2008). While this identification needs to be supported by biochemical or genetic studies, it is in accord with the presence of a number of conserved Cys not involved in iron binding in the chloroplast protein (Fig. 6A). Of particular interest is a fully conserved CxxC motif located in a domain of Tic55, LLS1, and PTC52 and not found in other Rieske proteins (Gray *et al.*, 2004).

Relevant possibilities also come from initial homology modeling of the Rieske domain which pointed to a thiol pair (Cys 176 and 193 in the pea Tic55 sequence, Fig. 6A) as possible participants in disulfide bridge formation and, therefore, in interacting with Trx. These Cys residues are located in the so-called proline loop characteristic of Rieske proteins that connects the cluster-binding domain with the basal domain (Colbert *et al.*, 2000). How the Rieske cluster regulates the active site, whose regulatory redox motif is recognized by Trx and elucidation of the role of Tic55 in regulating the complete TIC machinery are very interesting questions to pursue in the future.

Tic62 and Tic32 activities are linked to NADPH (Hormann *et al.*, 2004; Kuchler *et al.*, 2002). They belong to the extended and classical families of the SDR superfamily, respectively, and are predicted to contain a Rossmann-type fold. Their reductase activity has been demonstrated experimentally by *in vitro* assays using NBT as substrate, but endogenous substrates are still unknown. To date, no direct functional link or interaction has been detected between them, and they may possibly function independently. Both are extrinsically attached to the inner envelope membrane at the stromal side and show similar modes of dynamic binding to the TIC complex: more oxidizing conditions (or lower NADPH/NADP ratio) favor their association with TIC, whereas more reducing conditions (or higher NADPH/NADP ratio) dissociate the subunits from the complex (Chigri *et al.*, 2006; Stengel *et al.*, 2008). Despite their similarities and common cyanobacterial origin, they are not evolutionarily related and interact with different partners: Tic62 interacts with FNR, and Tic32 binds to calmodulin (CaM)—a primary transducer of intracellular Ca^{2+} signals in eukaryotes (Chigri *et al.*, 2006; Kuchler *et al.*, 2002).

Tic62 is characterized by a bimodular structure: the N-terminal domain contains the NADPH-binding motif, while the C-terminal domain is predicted to be highly disordered in solution and to contain several repetitive proline-rich modules (Kuchler *et al.*, 2002) (Fig. 6B). These repetitive modules have been shown to interact with FNR. Evolutionarily, only vascular plants possess the "full-length Tic62" protein, that is, they contain the C-terminal, FNR-binding domain that is missing in other plants like *Physcomitrella patens* (Balsera *et al.*, 2007). This leads to the question of what defines Tic62 as a TIC component: is it reductase capacity or FNR-binding capacity? As noted earlier, the redox environment, reflected by the stromal NADPH/NADP ratio and FNR availability, strongly influences Tic62 functionality and its binding to the TIC complex (Stengel *et al.*, 2008). However, not only these factors affect the reversible binding of Tic62 to the TIC complex: its distribution between the soluble stromal compartment and the inner

envelope membrane: more reducing conditions released Tic62 from the membrane to the stroma, thereby possibly increasing its affinity for FNR. The significance of this stromal effect is not known.

Tic32 consists of a catalytic (NADP-binding) N-terminal domain and a C-terminal CaM-binding extension (Fig. 6C) (Chigri *et al.*, 2006). It is not known whether CaM regulates Tic32 oxidoreductase activity or provokes a conformational rearrangement that perturbs interaction with its partners. The second option seems plausible because coimmunoprecipitation experiments showed that NADPH abolished the interaction of Tic32 with the TIC machinery. It was assumed, therefore, that CaM would favor binding to the translocon complex. Protein sequence analyses showed the presence of a number of Tic32 homologs in plants (Kalanon and McFadden, 2008). However, because no functional data are available for these homologs, a relation with the TIC complex cannot be ensured. Further, due to lack of a transit peptide (Nada and Soll, 2004), a chloroplast location cannot be predicted for the Tic32 homologs. Thus, much remains to be done to understand the role of the redox-sensitive Tic62 and Tic32 molecules that are switches closely associated with the TIC complex. Interestingly, both Tic62 and Tic32 contain conserved Cys in their Rossmann-fold domain (Fig. 6B and C). Based on homology modeling, they seem unfit for an intramolecular interaction (Fig. 6B and C). It is of interest to learn whether they participate in intermolecular binding.

V. REGULATION OF CHLOROPLAST PROTEIN IMPORT BY METABOLIC AND ENVIRONMENTAL REDOX STATE

A. THE OUTER ENVELOPE, THE FIRST MEMBRANE BARRIER IN PROTEIN IMPORT

Biochemical characterization of protein import in chloroplasts with *in vitro* systems has shown that chloroplast protein translocation is affected by Cys-modifying reagents such as *N*-ethyl-maleimide (NEM) and dithiothreitol (DTT). Experiments with the precursor of the small subunit of RuBisCO (pSSu) as a model protein for import showed that the affinity for the receptor-binding sites was dramatically reduced after NEM treatment, while the number of binding sites was not affected (Friedman and Keegstra, 1989). These results were later confirmed not just for pSSu, but also for the precursor of ferredoxin-NADP-oxidoreductase (pFNR) (Row and Gray, 2001). Significantly, mutating the Cys residues of the transit

peptides did not affect import. These findings thus let us to the view that at least one component of the chloroplast protein import machinery participating in the formation of early import intermediates contains one or more functionally important Cys residues.

Subsequent work showed that import yield was stimulated after chloroplasts were treated with DTT or reduced glutathione (Pilon *et al.*, 1992; Stengel *et al.*, 2009). In another set of experiments, the oxidizing agent copper chloride (II) was shown to inhibit the formation of an early import intermediate and subsequent import of pSSu (Seedorf and Soll, 1995). Inhibition was relieved by DTT. In this case, the effect was ascribed to the formation of a crosslinked complex consisting of Toc159, Toc34, and Toc75. The opposite effects of DTT and $CuCl_2$ treatments point to the presence of a reversible disulfide bridge at the level of outer envelope membrane in land plant chloroplasts. By means of diagonal 2D redox SDS-PAGE, different redox treatments on outer envelope vesicles isolated from pea chloroplasts confirmed the formation of intermolecular disulfide bridges among the TOC components. It was concluded that the redox state of Cys in the TOC complex affects the efficiency of protein import (Stengel *et al.*, 2009). The longstanding question of whether DTT can influence the chloroplast protein import machinery has thus at long last been answered.

The only subunit of the import machinery in chloroplasts whose structure is known to date at high resolution is Toc34 (Koenig *et al.*, 2008b; Sun *et al.*, 2002). X-ray crystallography structural studies revealed that the Toc34 monomer is divided into a globular GTP-binding domain (G domain) and a small C-terminal α-helical part (Figs. 2C and 7). The core is defined by six β-sheets flanked by six α- and two 3_{10}-helices. A feature of the Toc34 structure is a long loop connecting $\beta 6$ and $\alpha 6$ secondary elements. Many conserved residues in the Toc34 sequence are localized in this loop, and a role in the nucleotide exchange process is proposed for this region (Sun *et al.*, 2002). As noted earlier, this cytosolic loop contains the fully conserved Cys residue in the Toc34 family that was shown to interact with other TOC components in the presence of copper chloride using pea as a model plant (Seedorf and Soll, 1995; Sun *et al.*, 2002). Interestingly, the long loop and the conserved Cys residue are also predicted in the Toc159 family (Figs. 2C and 7).

In view of these findings, determination of the structure of the outer envelope channel protein, Toc75, becomes even more timely. A high-resolution structure of a bacterial homolog of Toc75 (Gatzeva-Topalova *et al.*, 2008) highlights the need to analyze the conformation of the complete N-terminal POTRA-containing domain and its organization with respect to the pore domain and the membrane. As seen earlier, a number of conserved

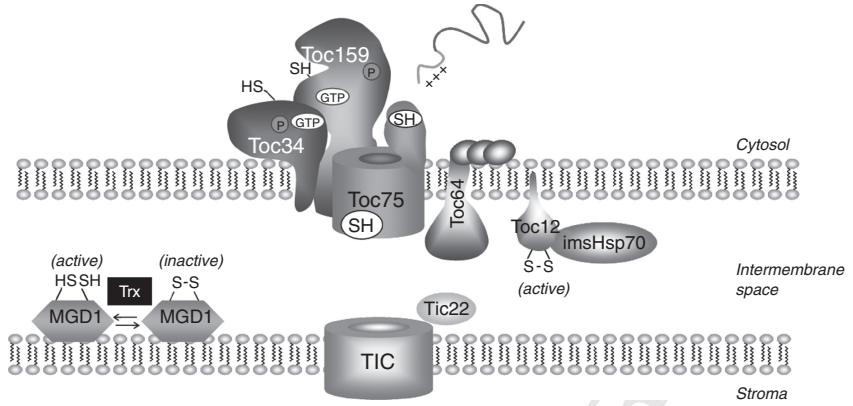

Fig. 7. (See color plate section at the back of the book.)

Cys residues are found in the second POTRA domain—in the cytosol and at the beginning of motif 3 within the channel (Fig. 2A). As noted earlier, these residues may participate in the interaction with Toc34.

Finally, the Toc64 receptor has also been implicated in covalent interactions with other TOC components using copper chloride (II) as a specific Cys crosslinker (Sohrt and Soll, 2000). Of the six Cys conserved in land plants (Fig. 3A), only two are predicted to be located to the cytosolic side (Qbadou *et al.*, 2007), one in the loop between the first two putative transmembrane helices and the second in the second TPR domain, the latter also conserved in the mitochondrial isoform (Kalanon and McFadden, 2008). These residues may be responsible for interacting with other TOC components. Mutation studies would be useful to assess the role that these Cys play at the outer envelope interface.

B. BETWEEN TWO BARRIERS, THE INTERMEMBRANE SPACE

It has been known for some time that at least two chloroplast precursor proteins, nonphotosynthetic ferredoxin (FdIII) and FNR isoform II (FNRII), are differentially imported into chloroplasts under light and dark conditions (Hirohashi *et al.*, 2001). High light resulted in mistargeting and accumulation of these preproteins in the intermembrane space, whereas the import of the precursor forms of photosynthetic counterparts (FdI and FNRI) was not affected. This effect of protein arrest in the intermembrane space under high light was ascribed to a folding event, likely due to the sensitivity of the folding machinery to redox state in the intermembrane space (Becker *et al.*, 2004a).

Furthermore, two residents of the intermembrane space, Toc12 and MGD1, potentially contain disulfide bridges (Fig. 7). The redox state of the Cys residues in MGD1 has been linked to function, showing that the enzyme is active only in its reduced form. Further, Trx has been reported to modulate the activity of MGD1, resulting in the proposal that redox regulation may be essential to coordinate galactolipid biosynthesis and chloroplast development (Benning and Ohta, 2005; Yamaryo *et al.*, 2006). By contrast, the oxidized form of Toc12 is believed to be the form active in coordinating the chaperone system of the intermembrane space with protein import (Becker *et al.*, 2004a). However, the presence of a disulfide bridge in Toc12 has yet to be confirmed. The intermembrane studies raise a number of interesting questions. First, how does MGD1 sense the redox state of the inner membrane space? One could think of the presence of Trxs, and a proteomic study has indeed detected a form of m-type Trx associated with the *Arabidopsis* envelope membrane. It is likely, however, that it is present at the stromal face of the inner envelope membrane (Ferro *et al.*, 2003). Even so, one could envisage a mechanism similar to the one reported for thylakoids for which a transmembrane reducing equivalent transfer system has been proposed to link the stroma to the lumen (Motohashi and Hisabori, 2006). There is, however, no evidence for such a system in the envelope. Finally, machineries are known to catalyze the oxidation of proteins in the intermembrane space of mitochondria and the periplasmic space of bacteria (Herrmann *et al.*, 2009). Here again, although the presence of semiquinone radicals, flavoproteins, and iron–sulfur proteins has been experimentally detected at the inner envelope membrane of chloroplasts and an electron transfer chain employing these carriers might be present (JagerVottero *et al.*, 1997), no equivalent systems have been detected so far in this region.

At this point it would be necessary to identify if a disulfide bridge is present in Toc12, to get additional insight into the regulatory disulfide bridge in MGD1 and look for associated elements in the intermembrane space. Further experiments are also needed to understand how light affects the transport of a subset of proteins resulting in their folding and arrest in the intermembrane space. It would be interesting to know whether Cys are implicated and the identity of neighboring proteins that participate in this process.

C. CHLOROPLAST REDOX STATE AND THE INNER ENVELOPE BARRIER

It is now accepted that the activity of the TIC complex is likely linked to both metabolic and oxidative regulation (Fig. 8). Recently, three TIC components have been functionally linked to stromal thioredoxins. The finding that

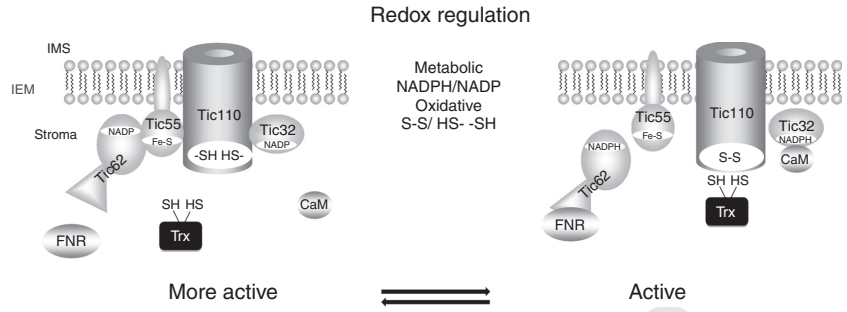

Fig. 8.

Tic110, the putative translocation channel, contains a regulatory disulfide bridge linked to specific stromal Trxs has opened a new line of investigation in the regulation of protein import. It has been proposed that Tic110 may respond to environmental factors via a change in redox and that Trx then reactivates Tic110 by reduction (Balsera *et al.*, 2009a). Future experiments will determine whether a particular Trx acts *in vivo* in this capacity as well as the functional consequences of the effect of change in redox state. Another TIC subunit, the redox sensor Tic55, has been also classified as a Trx target based on its interaction with a mutant Trx protein (Bartsch *et al.*, 2008). Although sequence analysis demonstrated the presence of well-conserved Cys in strategic positions (Fig. 6A), further experiments are needed to confirm the presence of regulatory disulfide bridge(s) in Tic55 and, eventually, its functional significance.

Evidence for a final component of the TIC machinery as a putative Trx target, Hsp93, was obtained with a cyanobacterial homolog (Mata-Cabana *et al.*, 2007). Although the TIC machinery is not present in cyanobacteria, work is justified to determine whether plant and cyanobacterial Hsp93 chaperone functions are comparable. As mentioned earlier, a proteomic study has identified a Trx isoform closely associated with the inner envelope membrane, raising the possibility of a functional link between these groups of proteins (Ferro *et al.*, 2003).

On the other hand, a dynamic interaction between Tic62 and Tic32 and the TIC machinery is dependent on the ratio of another redox pair, NADPH/NADP (Chigri *et al.*, 2006; Stengel *et al.*, 2008). This ratio appears to play a central role due to their functionality both as redox carriers and signaling components (Fig. 8). Although variations in the NADPH/NADP ratio in chloroplasts are not extensive due to the photosynthetic machinery operating during the day and the oxidative pentose phosphate pathway at night, the equilibrium may be significantly perturbed during metabolism, particularly

under stress conditions. It thus seems feasible that by a dynamic association, the Tic62 and Tic32 subunits could influence the import properties of, at least, a subset of proteins by altering the composition of the TIC machinery in response to changing conditions of the chloroplast. Indeed, recent results showed that the protein import efficiency of a subset of protein substrates is affected after altering the NADPH/NADP ratio in isolated chloroplasts by the addition of different metabolites—a decrease in the NADPH/NADP ratio enhanced protein import (Stengel *et al.*, 2009).

The metabolic factors, FNR and CaM, also affect Tic62 and Tic32 binding, respectively, as mentioned earlier (Chigri *et al.*, 2006; Kuchler *et al.*, 2002). Again, the molecular mechanisms of these interactions remain largely unknown. Thus, it remains to be seen whether FNR transmits redox information to Tic62 when thylakoid bound and part of the photosynthetic machinery or when dissociated and soluble in the stroma (Forti and Bracale, 1984). Alternatively, a signal could be generated when the enzyme undergoes binding and induces a conformational change in Tic62.

On the other hand, the functional interpretation of the Tic32–CaM interaction is also elusive. CaM responds to Ca^{2+} signals and transmits information to a receptor protein whose activity is then affected (Zielinski, 1998). It is known that light/dark conditions influence the level of Ca^{2+} in the chloroplast (Sai and Johnson, 2002) and that the import of a subset of proteins that contain a cleavable transit peptide is inhibited either by calmodulin inhibitors or calcium ionophores. Further, the addition of external calmodulin or calcium restores import activity (Chigri *et al.*, 2005) and calcium acts as an effector in control of gating and selectivity of the Tic110 protein channel (Balsera *et al.*, 2009a). It becomes interesting to know if Ca^{2+}, CaM, Tic32, and Tic110 are functionally connected.

A role for redox in controlling the composition of the TIC machinery has been strengthened by a recent proteomic study on envelope membranes of C3 (pea) chloroplasts and C4 (maize) mesophyll chloroplasts (Brautigam *et al.*, 2008). Whereas the channel-forming proteins Tic110 and Toc75 showed a relatively high spectral abundance in both samples, the study showed that the relative abundance of Tic55, Hsp93, and FNR is markedly decreased in C4 mesophyll envelopes. On the other hand, Tic32 and Tic62 were not identified in the envelope of C4 mesophyll chloroplasts. The authors proposed that, due to a spatial separation of reduction equivalents produced in mesophyll and bundle sheath chloroplasts, C3 and C4 mesophyll chloroplasts have different modes of redox-dependent import. Independent proteomic experiments with total chloroplast membranes from maize mesophyll and bundle sheath cells revealed high levels of Tic110, Tic40 (and Tic21) in mesophyll chloroplasts under conditions attributed to increased protein influx (Majeran

and van Wijk, 2009; Majeran *et al.*, 2008). In sharp contrast to the Brautigam study (Brautigam *et al.*, 2008), subunits of the translocation machinery other than Hsp93 were not detected specifically in mesophyll cells. At this point, no further conclusions can be drawn regarding the distribution of the redox regulatory subunits.

VI. FURTHER POSSIBLE REDOX TARGETS IN CHLOROPLAST PROTEIN IMPORT

A. THE DRIVEN MOTOR FORCE FOR THE SEC MACHINERY

The cpSec system in thylakoids consists of a membrane-embedded protein-conducting channel, formed by the SecE and SecY subunits, and the peripherally associated ATPase motor SecA (Laidler *et al.*, 1995; Nakai *et al.*, 1994; Schuenemann *et al.*, 1999a; Yuan *et al.*, 1994) (Fig. 1B). Upon complex formation with the precursor protein in the stroma, SecA associates with the thylakoidal SecE/Y channel. Cycles of ATP hydrolysis together with proton motive force energy provide the driving force for the complete translocation of the preprotein through the channel (Economou and Wickner, 1994; Karamanou *et al.*, 1999; Nakai *et al.*, 1994).Out of the two SecA-related sequences in *Arabidopsis* (At4g01800 and At1g21650), the former represents the true homolog to cyanobacterial SecA and its localization in chloroplasts has been confirmed by proteomics (Kleffmann *et al.*, 2004).

Little is known specifically about SecA in chloroplasts but its functional equivalence and high sequence similarity to bacterial homologs allows direct comparisons with many functional and structural studies performed on the bacterial system (Yuan *et al.*, 1994). Under physiological conditions, SecA forms homodimers in solution. However, the oligomerization state of the protein during translocation is a matter of debate since certain groups have proposed that the dimer dissociates during translocation. This view is not shared by others, and it has been proposed that a dynamic equilibrium between different oligomerization states exists in SecA that depends on environmental conditions, lipids, or even the presence of transit peptides (Benach *et al.*, 2003; Driessen, 1993; Duong, 2003; Or and Rapoport, 2007; Osborne *et al.*, 2004; Shin *et al.*, 2006; Woodbury *et al.*, 2002).

High-resolution structures have been obtained by X-ray crystallography for several bacterial SecA proteins in different nucleotide-bound states (Hunt *et al.*, 2002; Osborne *et al.*, 2004; Papanikolau *et al.*, 2007; Sharma *et al.*, 2003; Vassylyev *et al.*, 2006; Zimmer *et al.*, 2008). SecA is composed of two domains: an N-terminal motor domain and a C-terminal translocation

domain. The motor domain shares similarities with the DEAD motor domains of ATPase helicases. With this type of protein, the binding of ATP and release of ADP generate a force that is translated into large protein conformational changes and movement of the protein.

Based on studies with SecA homologs, several structural elements can be identified in the chloroplast SecA protein from *Arabidopsis* (Fig. 9A). Two nucleotide-interacting regions, NBD1 and NBD2 (or IRA2), have been identified in the motor domain (Kull *et al.*, 1996) as forming a mononucleotide cleft (Sharma *et al.*, 2003). Whereas NBD1 contains the minimal ATPase catalytic machinery, the highly flexible NBD2 (or IRA2) subdomain acts as intramolecular receptor for ATPase hydrolysis (Sianidis *et al.*, 2001). The motor region also contains a family-specific variable region (VAR) that

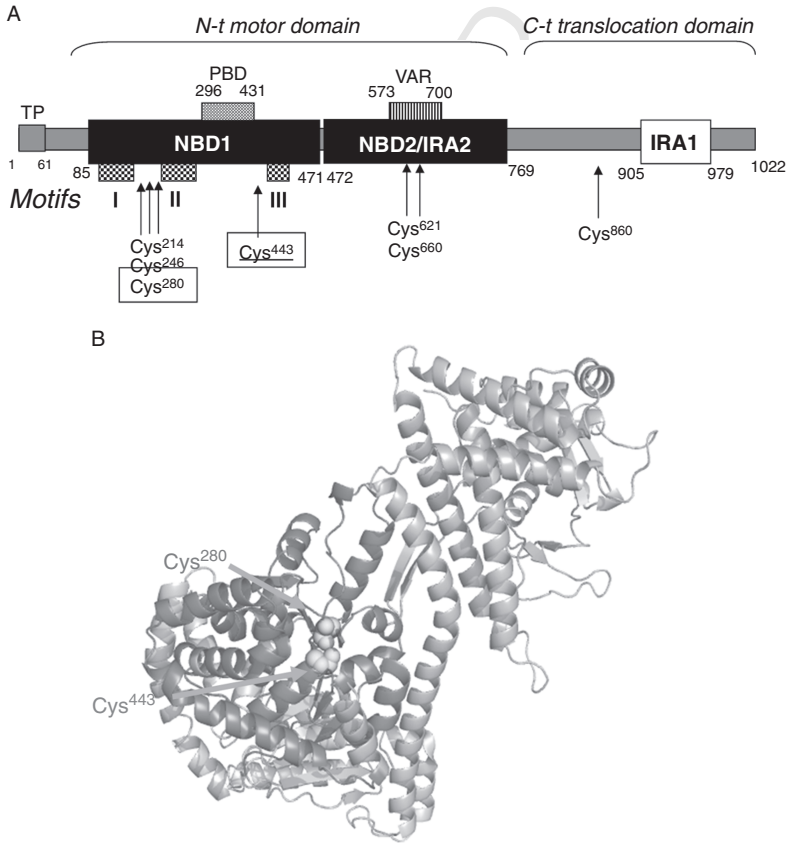

Fig. 9.

shows significant length and sequence variability in the different SecA proteins. Cyanobacterial and plant SecA present one of the longest VAR regions in the SecA superfamily (Papanikolau *et al.*, 2007) that, interestingly, is the most divergent region between cyanobacterial and plant SecA. Although a function has not been identified, this region may be involved in SecA oligomerization (Papanikolau *et al.*, 2007). Another region identified in the motor domain of chloroplast SecA is the preprotein-binding domain (PBD). On the other hand, the C-terminal translocation domain functions as a regulatory domain that physically associates with NBD2 (or IRA2) and restricts its activator function (Sianidis *et al.*, 2001).

Recently, a proteomic study carried out with membrane preparations from a cyanobacterium has identified SecA as a potential Trx target (Mata-Cabana *et al.*, 2007). The high sequence similarity between the bacterial SecA homologs and *Synechocystis* and *Arabidopsis* SecA proteins (42–49% identity) prompted us to apply homology modeling to build a three-dimensional structure for cpSecA to obtain insight into the conformational organization of the conserved Cys (Fig. 9B). Independent of the template used for the modeling (PDB codes: 2fsf, 1nkt, 2vda, 2ibm, 1tf5), two conserved Cys (Cys280 and Cys443) in the *Arabidopsis* model were found to be located in a favorable position for forming a disulfide bridge. Interestingly, the first of these Cys precedes the catalytic motif II or Walker B DEAD-motif, whereas the second is positioned closed to the so-called motif III (Hunt *et al.*, 2002; Sharma *et al.*, 2003) (Fig. 9A). It was found that motif III is intertwined with the catalytic motif II and IRA2. The formation of a putative disulfide bridge close to the catalytic site places it in strategic position for regulation. However, due to the lack of the first Cys, a similar disulfide bridge is not expected for the cyanobacterial SecA counterpart (see also the paper by Nishiyama and Hisabori in this volume concerning the distribution and specificity of Cys residues distribution in cyanobacterial enzymes) (Fig. 9A). Considering that SecA from *Synechocystis* was experimentally identified as a potential Trx target (Mata-Cabana *et al.*, 2007), further analyses are necessary to determine whether the cyanobacterial Cys targeted by Trx are linked in an intermolecular disulfide bond.

B. TRANSPORT AND FOLDING OF REDOX-ACTIVE PROTEINS IN THE THYLAKOIDAL LUMEN

Since the turn of the current century, a number of groups have contributed to the elucidation of the composition, mechanisms, and metabolic activities of proteins in the thylakoidal lumen—a long-neglected compartment of thylakoids. To date, more than 70 different proteins have been detected in the

lumen, all nuclear encoded, that function not only in the light reactions, but also in folding, proteolysis, and protection against oxidative stress (Peltier *et al.*, 2002; Schubert *et al.*, 2002). Recent observations provide evidence that a number of these proteins contain disulfide bridges and have the potential to participate in redox signaling (Gopalan *et al.*, 2004; Hall *et al.*, 2008; Marchand *et al.*, 2006). However, in contrast to their stromal counterparts that are usually active in the reduced state, lumenal proteins are active when oxidized (Buchanan and Luan, 2005).

Import behavior has been analyzed for two lumenal proteins that contain disulfide bridges essential for activity—the immunophilin FKBP13 and oxygen-evolving complex extrinsic subunit OE33 (or OEE1/PsbO) (Fig. 10A). Both proteins have been identified as Trx targets and are thus candidates for redox regulation (Gopalan *et al.*, 2004; Lee *et al.*, 2004a; Lemaire *et al.*, 2004; Marchand *et al.*, 2006). *In vitro* import experiments suggested that FKBP13 uses cpTat for import (Gupta *et al.*, 2002)—a pathway that employs folded substrates, thereby raising the question of whether FKBP13 is transported in the oxidized or reduced state. Both scenarios are plausible since biochemical and structural studies revealed that the disulfide bridge formation is essential not for folding but for enzymatic activity. By contrast, OE33 follows a cpSec-dependent pathway of translocation (Cline *et al.*, 1992). OE33 contains disulfide bridges that are essential both for activity and incorporation as part of PSII structure (Tanaka and Wada, 1988). OE33 has thus to be kept in an unfolded, competent state in the stroma as well as when

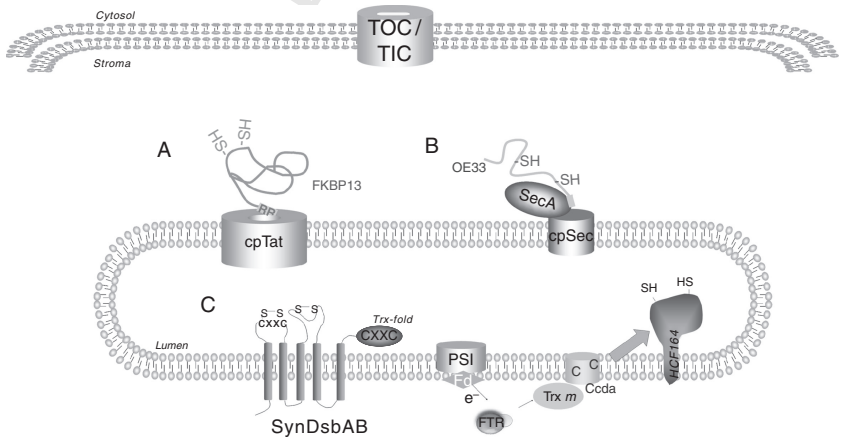

Fig. 10. (See color plate section at the back of the book.)

crossing the thylakoidal membrane. Once in the lumen, the protein is oxidized and folded (Fig. 10B).

The evolutionary and molecular basis for the acquisition of a transit peptide specific for the cpTat or cpSec import pathway is not clear. The situation is complicated by the requirement of particular proteins for transport in either the folded or unfolded state. The disulfide bond-containing aprotinin, a therapeutic protease inhibitor, is an interesting case in point. By fusing the protein to transit peptides specific for each pathway, aprotinin has been successfully translocated into the lumen of tobacco plastid transformants by using either the cpSec or cpTat pathways (Tissot *et al.*, 2008). In each case, the protein was fully functional after translocation—an indication that disulfide bridges were correctly formed, independent of import pathway. At least for this example, the efficiency of transport was comparable and a functional structure was similarly acquired in the stroma and the lumen. A requirement for specific lumenal chaperones may be an important factor in defining the path of translocation. On the other hand, keeping in mind that SecA is a putative Trx target, selection of a particular pathway may be linked to regulation of import for a subset of substrates (Fig. 10). This is an attractive idea that needs in-depth experiments. First, SecA should be confirmed as a Trx target biochemically or genetically and its regulatory disulfide bridge(s) should be identified. These studies should include demonstration that Trx alters SecA activity and protein import. Different situations can be envisaged for an effect on import: the redox state of SecA could influence its oligomerization state (a subject highly debated in the literature), its interaction with the transit peptide or its catalytic activity, the most common mechanism of action of Trx in the stroma (Schurmann and Buchanan, 2008).

Little is known about how newly imported proteins are correctly folded for disulfide bridge formation once in the lumen. This could be either enzyme mediated or achieved spontaneously in an environment that is highly oxidizing, particularly in the light (Fig. 10C). The mystery is heightened by the apparent absence of Trx in a compartment that, so far, appears to house a number of Trx targets. Recent work, however, offers relevant possibilities.

HCF164, a protein residing in the thylakoid, that contains a soluble Trx-like domain exposed to the lumen, has been proposed to act as transmembrane transducer of reducing equivalents from photoreduced stromal Trx (Motohashi and Hisabori, 2006) (Fig. 10C). In a separate report, structural analysis prompted the suggestion that cytochrome *c*6*A*, a redox-active lumenal component, could act as catalyst for the oxidation of protein dithiols by molecular oxygen (Marcaida *et al.*, 2006). Finally, more recently, a protein required for disulfide bond formation in the extracytosolic space has been identified in the inner membrane of the cyanobacterium

Synechocystis, SynDsbBA (Singh *et al.*, 2008) (Fig. 10C). The full-length protein is the result of the evolutionary fusion of two homologs from Gram-negative bacteria: (i) DsbA, a soluble periplasmic protein with a catalytic Cys-X-X-Cys motif that acts as a disulfide bond carrier and rapidly oxidizes Cys residues in protein substrates, and (ii) DsbB, a membrane-localized thiol oxidoreductase protein that transfers redox equivalents to DsbA. Genome analyses revealed that this fused system is conserved in all oxygenic photosynthetic organisms (Singh *et al.*, 2008). Further, its ortholog in *Arabidopsis* (At4g35760) seems to be localized in the thylakoidal membrane according to the plastid proteome database (http://ppdb.tc.cornell.edu). Future studies on the location, function, and substrate specificity of this fused protein will surely give new insight into redox regulation. Establishing the system in chloroplasts would give also support to the believed prokaryotic evolutionary origin of this compartment (Herrmann *et al.*, 2009). A tangential area awaiting exploration concerns the role of thiol/disulfide exchange in regulating the activity of lumenal proteins and the mechanism(s) by which these changes are achieved.

ACKNOWLEDGMENTS

B. B. B. gratefully acknowledges receipt of a Research Award from the Alexander von Humboldt Foundation. Work in the laboratory of J. S. was supported by Deutsche Forschungsgemeinschaft Grant SFB594.

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