Chapter 9
Mechanisms of Chloroplast Protein Import in Plants

Paul Jarvis and Felix Kessler

Abstract Most chloroplast proteins are encoded as preproteins by the nuclear genome. Their import into chloroplasts occurs post-translationally. An N-terminal pre-sequence, the transit peptide, contains the organellar targeting information. It is specifically recognized by receptor components at the chloroplast surface. These receptors are components of the TOC (*t*ranslocon at the *o*uter envelope membrane of *c*hloroplasts) complex. Together with the TIC (*t*ranslocon at the *i*nner envelope membrane of *c*hloroplasts) machinery, this mediates the import of proteins into chloroplasts. In addition to the receptors, these complexes incorporate channel, motor and regulatory functions. Many putative or actual components have been identified. Multiple isoforms of the TOC receptors (and possibly of some other components) constitute the molecular basis of separate import pathways with distinct client preferences. This perhaps reduces competition effects between highly abundant and less abundant preproteins. Client preferences of different import pathways might also facilitate the differentiation of various plastid types. In addition to the canonical TOC/TIC-mediated import routes, alternative, mechanistically distinct pathways of protein transport to chloroplasts have been identified; one of these passes through the endoplasmic reticulum and Golgi apparatus. Other work has revealed several protein targeting pathways leading to the envelope membranes.

Keywords Chloroplast envelope · Chloroplast protein import · Plastid biogenesis · Protein targeting · Protein transport · TOC/TIC machinery · Translocon

Abbreviations

CAH1	Carbonic anhydrase 1
ceQORH	Chloroplast envelope quinone oxidoreductase homolog
cpHsc70	Chloroplast stromal Hsp70
MGD1	Monogalactosyldiacylglycerol synthase 1

P. Jarvis (✉)
Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
e-mail: paul.jarvis@plants.ox.ac.uk

F. Kessler
Laboratoire de Physiologie Végétale, Université de Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
e-mail: felix.kessler@unine.ch

S.M. Theg, F.-A. Wollman (eds.), *Plastid Biology,* Advances in Plant Biology 5,
DOI 10.1007/978-1-4939-1136-3_9, © Springer Science+Business Media New York 2014

OEP7/14	Outer envelope protein, 7/14 kD
PPI1	Plastid protein import 1
SPP	Stromal processing peptidase
SSU	Small subunit of Rubisco
TIC	Translocon at the inner envelope membrane of chloroplasts
TOC	Translocon at the outer envelope membrane of chloroplasts
TROL	Thylakoid rhodanese-like protein
UPS	Ubiquitin-proteasome system

9.1 Introductory Topics

9.1.1 Background

Plastids are the prototypical plant organelles. The chloroplast is the most prominent representative of the family. Chloroplasts derived from cyanobacteria by an endosymbiotic process during which a primordial photosynthetic bacterium was engulfed by a eukaryotic host cell. Over time, most of the cyanobacterial genetic material was transferred to host nucleus, both transforming the host genome and progressively reducing the chloroplast genome. The cyanobacterial model *Synechocystis* sp. PCC6803 [99] has 3168 predicted protein coding genes, whereas the chloroplast genome of *Arabidopsis thaliana*, the eudicot model system, retains 87 protein coding genes [178]. However, the number of chloroplast proteins has been estimated at around 1500 in recent proteomics studies [59, 113, 198]. The large difference is explained by the nuclear-encoded origin of the vast majority of chloroplast proteins. Therefore, a mechanism for importing proteins synthesized in the cytosol into chloroplasts is required [94, 105, 136, 187]. In this chapter, we will address how these nuclear-encoded chloroplast constituents are targeted to the organelle and translocated across the outer and inner envelope membranes.

9.1.2 Transit Peptides

Some of the first studies on chloroplast protein import in the late 70s and early 80s of the past century focused on one of the most abundant and best known chloroplast proteins, the small subunit of Rubisco (SSU), establishing it as a model chloroplast import substrate [52, 67]. In cell-free translation experiments of isolated *Chlamydomonas* mRNA, Dobberstein and colleagues in 1977 observed that SSU was synthesized as a protein larger than that present in chloroplasts. They hypothesized that this was an extrachloroplastic form and that the additional amino acid sequence may be required for its transfer into the chloroplast. Since then many chloroplast proteins have been identified and studied, and as it turned out the majority of these are synthesized with N-terminal extensions as “preproteins”, abbreviated as pSSU

in the case of SSU. Furthermore, it has been demonstrated that the N-terminal extensions (known as transit peptides) are required for the targeting and translocation of preproteins into the chloroplast. Interestingly, transit peptides are not conserved in primary structure, and their length is also quite variable from approximately 30 to 70 amino acids [33, 34]. Certain physicochemical characteristics, however, such as a preponderance of hydroxylated and basic amino acids together with the underrepresentation of acidic amino acids are typical of transit peptides. It has also been found that transit peptides have a tendency to take on a natively unfolded structure [223]. Such an unfolded structure may facilitate the engagement of components of the chloroplast protein import machinery.

9.1.3 Energetics and Stages of Import

Before any of the components of the chloroplast protein import machinery were identified, the in vitro import system using isolated pea chloroplasts was extensively studied in terms of energetics and differentiable import stages. Early on it was recognized that preprotein import requires energy: light could be used as it leads to the production of ATP via the electron transport chain and the ATP synthase. Exogenous ATP is required when chloroplasts are depleted of energy in the dark and by the dissolution of the proton gradient using nigericin. Depending on the ATP concentration, at least three different stages of import were distinguished. In the absence of added ATP ("energy-independent stage"), the preprotein interacted with components at the chloroplast surface in a reversible, unstable way that could be trapped using covalent chemical crosslinking [115, 143, 163]. At low concentrations of ATP (100 micromolar), and GTP, the preprotein inserted across the outer envelope membrane in a stable way and was isolated as the so-called "early translocation intermediate" [106, 159, 228]. Later it was demonstrated that at this stage the preprotein is exposed to the cytoplasm but has also already engaged components at the inner envelope membrane. Complete translocation of the preprotein across both envelope membranes required "high" concentrations of ATP (1-5 millimolar) [160, 202]. At this stage the preprotein arrives in the chloroplast stroma where the transit peptide is cleaved by the stromal processing peptidase (SPP) (see Sect. 9.3.4). The preprotein can be arrested at this stage by chilling the chloroplasts on ice resulting in the "late translocation intermediate" [85, 133].

9.1.4 Identification of Translocon Components

The identification of translocon components long proved challenging: Eventually, three separate approaches turned out to be successful. A study using chemical crosslinking of pSSU in an in vitro import assay in the absence of added ATP at the "energy-independent stage" resulted in the cross-linking of an 86 kD protein [163]. At a later stage of import, in the presence of ATP, an additional 75 kD protein

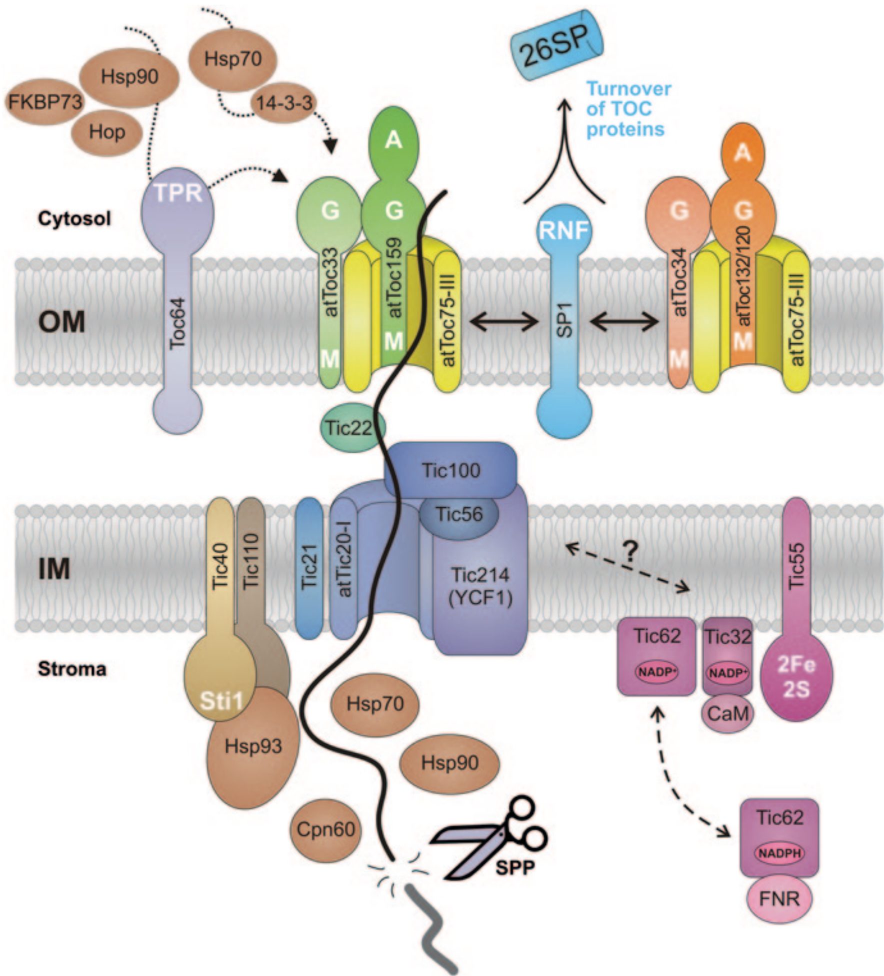

Fig. 9.1 The TOC and TIC complexes of the chloroplast protein import machinery. The TOC and TIC translocons in the outer and inner envelope membranes (*OM* and *IM*, respectively) are shown, as is a translocating preprotein (*black line*). Individual translocon components are identified by their molecular weights (*black text*), while some key functional domains are indicated (*white text*). Toc159, Toc34 and Toc75 together form the core TOC complex in the outer membrane (Toc159 is represented here by the *Arabidopsis* isoforms atToc159, atToc132 and atToc120; and Toc34 is represented by atToc33 and atToc34). While Toc159 and Toc34 are responsible for preprotein recognition, Toc75 (atToc75-III in *Arabidopsis*) forms the outer envelope channel. Different receptor isoforms enable the formation of different TOC complexes, and thus the operation of different import pathways with distinct client preferences. The RING finger (*RNF*) ubiquitin E3 ligase SP1 mediates the ubiquitination of TOC components, leading to their turnover by the 26S proteasome (*26SP*); this enables the dynamic reorganization of the protein import machinery. Various cytosolic chaperones and their cofactors (Hsp70 and 14-3-3; Hsp90, Hop and FKBP73) are proposed to interact with unfolded preproteins (forming so-called "guidance complexes"), to maintain their import competence and direct them to the Toc34 or Toc64 (OEP64) receptors. Tic22 is thought to provide a link between the TOC and TIC complexes, facilitating preprotein passage through the intermembrane space. Tic20 (atTic20-I) may participate in inner membrane channel formation, as

was crosslinked. These results indicated that preprotein import proceeds through a sequence of interactions from the cytosol to the chloroplast stroma. However, this study did not molecularly identify either the 86 or the 75 kD components [163]. In a separate study, IgG directed against the 86 kD protein at the outer membrane was shown to inhibit preprotein import into the chloroplast [73]. This finding suggested that the 86 kD protein functioned as a preprotein import receptor. In a third study, recombinant purified pSSU fused to IgG-binding of ProteinA (pSSU-ProtA) was incubated on a large scale with isolated chloroplasts either in the presence of "low" concentrations of ATP to produce the early intermediate or with "high" concentrations of ATP to produce the late intermediate [106, 182]. Subsequently, the reactions were stopped on ice. The envelope membranes were isolated, solubilized and subjected to IgG-affinity chromatography. In the case of the early intermediate, this resulted in the co-isolation of three chloroplast envelope proteins (86, 75 and 34 kD) together with un-processed pSSU-ProtA. For the late intermediate, the corresponding experiment resulted in the co-isolation of five envelope membrane proteins (the same 86, 75, 34 kD bands and additional bands at 110 and 36 kD) together with the mature SSU-ProtA.

In hindsight, these three seminal studies together yielded the first evidence for components of the chloroplast protein import machinery. The components at the outer membrane were termed Toc (*t*ranslocon at the *o*uter membrane of the *c*hloroplast) [183]. The core of the TOC translocon consists of an apparently stable complex of Toc159 (of which the 86 kD protein is a fragment, see below), Toc75 and Toc34 that correspond to the proteins that were identified in the initial studies (Fig. 9.1) [107, 181]. One of the additional components that co-isolated with the late intermediate was later identified as Tic110 (*t*ranslocon at the *i*nner membrane of the *c*hloroplast) and was the first known component at the inner membrane [103, 140]. The 36 kD component remained unidentified.

At the early intermediate stage of translocation the preprotein is inserted across the outer membrane and already makes contact with components of the inner membrane. The components at the inner membrane are Tic20 and Tic22, which were both identified by covalent crosslinking to the trapped intermediate [115, 116]. Tic20 is an integral protein of the inner membrane, while Tic22 is one of a few known intermembrane space proteins and is only peripherally associated with the outer face of the inner envelope membrane (see Sect. 9.3).

part of a large TIC complex which also incorporates Tic214, Tic100, Tic56 and Tic21, the latter being only loosely associated with the complex. Thereafter, Tic110 functions together with Tic40 in the recruitment and regulation of stromal chaperones, such as Hsp93, Hsp90, Hsp70 and Cpn60, which may form motor complexes for protein import propulsion, facilitate protein folding, or aid intraorganellar routing. SPP removes the transit peptide (*grey line*) upon its arrival in the stroma. A redox-regulator, comprising Tic62, Tic55 and Tic32, might be involved in fine-tuning the import process, working in conjunction with FNR and calmodulin (*CaM*)

9.2 Translocation Across the Outer Membrane

9.2.1 Components of the TOC Complex

The three main components of TOC core complex have highly intriguing primary structures: Toc159 and Toc34 turned out to be homologous GTP-binding proteins of the septin class [106, 134]. While Toc34 consists of the conserved GTP-binding domain followed by a C-terminal transmembrane segment, Toc159 has an extensive N-terminal acidic (A-) domain and a large C-terminal membrane-anchoring (M-) domain [31, 40]. Toc75 is a beta-barrel type membrane protein of the Omp85 family, members of which are involved in the insertion of outer membrane proteins in bacteria [57, 63, 209]. Both Toc159 and Toc34 are located at the chloroplast surface and are accessible to exogenous protease. Crosslinking at the earliest stages of protein import as well as antibody inhibition suggested that Toc159 constitutes the primary import receptor at the chloroplast surface [73, 163]. But there is now ample evidence that Toc159 and Toc34 co-operate in the recognition of preproteins at the chloroplast surface although the exact sequence of events occurring at the chloroplast surface is not known [22, 104, 138]. Toc75 is resistant to exogenously added protease indicating that it is deeply buried in the outer membrane. Based on its topology and primary structure Toc75 was proposed to function as the preprotein-conducting channel in the outer membrane [182, 209]. Indeed, Toc75 has channel properties in electrophysiological setups [70]. In most graphic models, Toc159 and -34 are represented as GTP-regulated, preprotein-specific receptors providing access to Toc75 channel. This view is supported biochemically by the finding that non- or slowly-hydrolyzable GTP analogs block preprotein import into isolated chloroplasts [106, 228]. However, more recent reverse genetic studies in the *Arabidopsis thaliana* model system point to a very complex mechanism of GTP-regulation that we do not completely understand (see below) [1, 9, 129, 226]. A reconstitution study also suggested that the three components are sufficient to accomplish translocation in vitro [180]. Because of their central role in import and stable association, Toc159, -34 and -75 together were termed the TOC core complex (Fig. 9.1) [182].

9.2.2 TOC Components in Arabidopsis thaliana

The research described so far was carried out using the pea (*Pisum sativum*) chloroplast in vitro system. This system allows large-scale chloroplast isolation, which in turn is useful for all sorts of biochemical experimentation. But up to now pea has not been amenable to genetic techniques. Here, *Arabidopsis thaliana* has proven incredibly powerful. A first mutant was identified in a forward screen of T-DNA insertion lines to find pale-green chloroplast biogenesis mutants—this was the *plastid protein import 1* (*ppi1*) mutant [95]. The PPI1 protein (atToc33) turned out to be highly homologous to pea Toc34, thereby providing the first insight into its role in chloroplast biogenesis. Moreover, the *Arabidopsis* genome contained a close

homolog of atToc33, atToc34, that was able to functionally complement the *ppi1* mutant. Indeed, the atToc33 and -34 proteins share considerable redundancy, with the double knock-out being embryo lethal [50]. This finding indicates that the two proteins together fulfil an essential role in plastid development, presumably by their participation in protein import. Since then, many more details of a complex TOC system in *Arabidopsis* have emerged: For instance, a total of four Toc159 homologs exist (atToc159, -132, -120 and -90). This GTPase subfamily shares the A-, G- and M-domain features of pea Toc159, with the exception of atToc90 that lacks the A-domain [19, 68].

Inevitably, questions regarding the possibility of distinct functions of these homologs emerged. Evidence for such a scenario stems from the analysis of the *ppi2* mutant in which the *atTOC159* gene is disrupted by a T-DNA insertion [19]. The *ppi2* mutant has a tell-tale seedling-lethal, albino phenotype pointing to a defect in chloroplast biogenesis. Moreover, a strong reduction in the levels of many, but not all photosynthesis-associated proteins was observed. This suggested that atToc159 is a major receptor required for the massive accumulation of photosynthesis-associated proteins. While this may hold true, the *ppi2* mutation also affects the accumulation of proteins that are not associated with photosynthesis [29]. Therefore, the functional boundaries of atToc159 are not as clear cut as originally believed. The single T-DNA insertion mutants of the other Toc159 homologs have milder (Toc132) or no phenotypes (Toc120/-90) [69, 91, 124]. However, the *toc132 toc120* double mutant resulted in a very severe phenotype, indicating redundancy. Because these two genes are expressed predominantly in non-photosynthetic tissues such as roots, this suggested that the two homologs together may have a central role in the import of non-photosynthesis-associated, "house-keeping" proteins [89, 91, 124]. Preferential assignment of the Toc34 isoforms to different import pathways has also been proposed (Fig. 9.1) [50, 91, 123]. While along rather general lines, the respective functional assignments of Toc159 and Toc132/-120 to specific groups of preproteins are probably more or less correct; nonetheless, a much more differentiated view must be developed.

Just recently, it has been demonstrated that chloroplast preproteins fall into three age-dependent classes, with the optimal import efficiency of each correlating with different chloroplast ages [204]. Intriguingly, the molecular determinants of age-dependent import lie within the transit peptide. In light of these findings, the question arises as to what extent age-dependent import pathways correspond to the separate import pathways that have been identified in the context of TOC receptor-dependent client-specificity.

9.2.3 TOC Complexes

In the absence of genome sequence information, the situation in pea appears simple as a sole TOC core complex consisting of three components is known; however, inspection of the available sequenced plant genomes indicates that *Arabidopsis* is not an unusual case, and that the aforementioned TOC receptor diversity is common.

Regardless, exactly how many of each one of these proteins is present in the individual complexes is still a matter of debate. A megadalton complex containing the components has been identified, which suggests that some of them must be present in more than just one copy [39, 107, 180].

In *Arabidopsis*, only Toc75 is encoded by a unique gene (it has homologs but these are either inactive or are not directly implicated in the translocation of transit peptide containing preproteins) [14, 55, 80]. Given the existence of small families of Toc GTPases, a variety of TOC core complexes may exist. Experimental evidence suggests that complexes consisting of atToc159, atToc33 and atToc75 are predominantly present in green, chloroplast-containing tissues and transport mostly photosynthesis-associated genes. Complexes consisting of atToc132/-120, atToc34 and atToc75 are predominantly present in non-green tissues and transport mostly the "house-keeping" proteins [91]. The evidence, however, is not as clear cut as it may appear: atToc34 can complement the absence of atToc33 in *Arabidopsis* suggesting that at least in this case the function of one component can be taken over by another and also replace it in the TOC core complex [50, 95].

Earlier results had shown that non- or slowly-hydrolyzable analogs of GTP inhibit chloroplast protein import in vitro [106, 228]. Therefore, the GTP-binding motifs in the Toc receptor family appeared to be interesting targets for further analysis. Initial experiments analyzed the effects of the GTPase mutants. A triple mutant in the GTP-binding site of atToc159 was non-functional and did not assemble into the TOC complex in planta [20]. But other single mutants that functionally disrupted GTP binding and/or hydrolysis apparently fully complemented the albino *ppi2* mutant, although import activity in some of the mutants was reduced [1, 226]. Similar results were obtained in the case of atToc33 [9, 129]. A particularly interesting result was obtained with the atToc159 A864R mutant that binds GTP strongly but is unable to hydrolyze GTP: this mutant not only rescued the albino phenotype of *ppi2* but increased the preprotein import efficiency in vitro [226]. These results suggest that, unexpectedly, GTP-binding to the TOC receptors is not essential but important for regulation of import activity levels.

9.2.4 Cytosolic Factors and Regulators of Chloroplast Protein Import

Before preproteins destined for the chloroplast reach the TOC complex they have to interact with cytosolic targeting factors (Fig. 9.1) [61, 132]. These include the chaperones Hsp70 and -90 that serve to prevent the newly synthesized preproteins from aggregating, maintaining their import-competent state. It has been shown that Hsp70 interacts with many preproteins immediately after their synthesis. Some preproteins may be phosphorylated at their transit peptide which promotes the binding of a 14-3-3 dimer [58, 148, 153]. Together, with Hsp70, the 14-3-3 dimer forms a so-called guidance complex that accompanies the preprotein to the receptors of the TOC complex and hands over the preprotein to Toc34. A set of cytosolic kinases (STY8-, 14 and -46) have been identified that are implicated in the phosphorylation

of the transit peptide [126, 147]. Yet other preproteins have been shown to form complexes with Hsp90 [58, 166]. Preprotein/Hsp90 complexes interact with Toc64, a tetratricopeptide repeat (TPR)-containing protein, loosely associating with the TOC complex. In addition to Hsp90, Hsp70/Hsp90-organizing protein (Hop) and the immunophilin FKBP73 may be players in the HSP90 pathway [58]. Surprisingly, however, Toc64 is essential neither in *Physcomitrella* nor *Arabidopsis* suggesting that this component of the pathway can be bypassed [8, 76].

Phosphorylation does not only play a role at the level of the transit peptide. It has been shown that Toc34 is a target of phosphorylation, and that in its phosphorylated state it is unable to bind preproteins or GTP and needs to be activated by a phosphatase [7, 96, 200]. Toc159 is highly phosphorylated at its A-domain, and this is most likely due to cytosolic casein kinase II [2]. The role of the phosphorylation is likely of regulatory nature, but exactly how it works still needs to be clarified. The A-domain also exists as a separate, soluble protein. This finding may explain why Toc159 was originally identified as an 86 kD protein lacking the A-domain [31, 40]. Again, the cleavage of the A-domain may have a regulatory purpose, such as activating the protein. More research in this area is required to clarify the role of phosphorylation and other mechanisms in chloroplast protein import.

Interestingly, cytosolic preproteins are rarely observed in living plants. This suggests that preprotein synthesis is tightly coupled to translocation so that very few preproteins remain in the cytosol. In this context, an interesting regulatory mechanism was discovered by which "un-imported" preproteins are degraded by the ubiquitin-proteasome system (UPS) [130]. Thus, both tight coupling of preprotein synthesis and import and the UPS may contribute to efficient accumulation inside the chloroplast and removal of preproteins from the cytosol.

Recently, a second exciting role for the UPS was discovered. A screen for second-site suppressors of the *Arabidopsis ppi1* mutant identified SP1 (*s*uppressor of *p*pi1 locus *1*) [139]. SP1 is a chloroplast outer membrane E3 ubiquitin ligase that directly interacts with components of the TOC core complex. Furthermore, SP1 was shown to ubiquitinate the TOC components in vivo as well as in vitro. The *sp1* mutant showed defects in plastid differentiation; i.e., the etioplast-to-chloroplast and chloroplast-to-gerontoplast (old chloroplasts in aging leaves) transitions. These results suggest that the UPS controls changes in the composition of TOC complexes to accommodate different sets of preproteins according to the needs of the developing plastid type (Fig. 9.1) [139]. Two homologs of SP1 were also identified, and it will be of great interest to see what their respective roles in the chloroplast may be.

9.3 Translocation Across the Inner Membrane

9.3.1 Arrival and Conductance

The Tic22 protein resides in the intermembrane space, and is peripherally associated with the inner membrane [115, 116]. It is perhaps the first TIC component to

be encountered by translocating preproteins, and it may facilitate their passage from TOC to TIC, possibly functioning in association with other intermembrane space components [21, 167]. It might also play a role in the formation of so-called TOC-TIC supercomplexes [3, 116, 158], enabling simultaneous transport across the two membranes. Tic22 homologs exist in cyanobacteria, and so this component was likely acquired with the endosymbiont. Its function is essential in cyanobacteria and apicomplexan parasites (where it is localized in the apicoplast), and structural analyses suggest that it may act as a chaperone [64, 211]. However, Tic22 is not essential in plants, as *Arabidopsis* mutants lacking both canonical Tic22 isoforms exhibit only moderate defects in greening and preprotein import [102, 176].

Tic110 is one of the most abundant TIC components [220]. It is encoded by a single-copy gene in *Arabidopsis*, and is essential [84, 118]. Based on electrophysiological analyses, it was proposed to form a cation-selective, β-barrel channel with a pore diameter of 15-31 Å [66]. However, another study showed that it is composed mainly of α-helices, and that it is anchored in the inner membrane by two N-terminal transmembrane spans [83]. In the latter topology, a large hydrophilic domain is oriented towards the stroma and is thought to recruit stromal chaperones for import propulsion [83, 92, 103]. A later study aimed to resolve these discrepancies, and concluded that the hydrophilic part contains four amphipathic helices that contribute to the channel [15].

Another component that has been proposed to form the TIC channel is Tic20 [116]. This protein possesses four α-helical transmembrane domains, similar to the mitochondrial inner membrane channel components Tim17, Tim22 and Tim23 [98, 101, 116], and interacts with preproteins at a slightly later stage than Tic22 [115, 143]. In *Arabidopsis*, deficiency of the main Tic20 isoform (atTic20-I) causes defects in chloroplast biogenesis and protein import [41], while complete loss causes severe albinism and seedling lethality [71, 101, 108, 203]. In fact, there are four Tic20 genes in *Arabidopsis* that fall into two distinct, evolutionarily-conserved groups: the Group 1 proteins (atTic20-I and atTic20-IV) are demonstrably important for chloroplast biogenesis, whereas the Group 2 proteins are dispensable [101, 206]. The atTic20-I protein seems to be important for the import of photosynthesis-associated preproteins in shoots, while atTic20-IV may deliver mainly non-photosynthetic, "house-keeping" preproteins in roots [71, 108]. Embryos lacking both Group 1 proteins are not viable [71, 101]. It has been suggested that the localization of Tic20 proteins is not restricted to the chloroplast inner envelope membrane [144].

Blue native PAGE analysis indicated that Tic20 exists in a large, 1 MD complex together with Tic21 (see below) and translocating preprotein [108]; Tic21 is only loosely associated with the complex, while Tic20 appears to be a core component. Very recently, the purified complex was found to contain two additional nucleus-encoded proteins (Tic56 and -100) and, surprisingly, the elusive YCF1 protein (Tic214) encoded by the chloroplast genome (Fig. 9.1) [109]. The 1 MD complex reconstituted in a planar lipid bilayer had channel activity, and was therefore proposed to form a general TIC translocon. Electrophysiological analysis also revealed that Tic20 alone is able to form a channel, with cation selectivity and a pore size of 8–14 Å [121]. Notably, Tic110 was absent from the 1 MD complex, and instead was

present in a smaller 200–300 kD complex. Thus, Tic20 may form a large channel complex (including Tic21 and the other components), whereas Tic110 may act later in the import mechanism as part of a distinct motor complex, or in other stromal events such as protein folding (see Sect. 9.3.2) [108, 109]. However, Tic20 protein was reported to be considerably less abundant than other translocon components, and so its candidacy as the main TIC channel has been questioned [121, 220].

While Tic110, Tic22 and Tic20 were all identified through biochemical analyses of isolated pea chloroplasts, Tic21 (or CIA5, for *c*hloroplast *i*mport *a*pparatus *5*) was found genetically in *Arabidopsis*, by screening for plants defective in the chloroplast import of a selectable marker [203]. *Arabidopsis* Tic21 knockout mutants are albino, and display similar defects in the import of photosynthetic preproteins to *tic20-I* mutants [108]. Interestingly, *tic21 tic20-I* double mutants do not exhibit phenotypic additivity, supporting the notion that the two proteins function together [203]. It was suggested that Tic20 might act early in plant development, with Tic21 taking over later on [203], but this seems inconsistent with the fact that the two proteins have been found together in the same complex [108].

Tic21 was also reported to act in iron transport, and thus given the alternative name of PIC1 (for *p*ermease *i*n *c*hloroplasts *1*) [54]. *Arabidopsis* PIC1/Tic21 mutants accumulated ferritin (a protein which binds iron to prevent iron loss or oxidative stress caused by free iron ions) in chloroplasts, and displayed up-regulated expression of ferritin and other factors related to iron stress and metabolism, while plants overexpressing PIC1 accumulated free iron ions in the stroma. Moreover, a yeast iron uptake mutation could be complemented using PIC1 [54]. It is conceivable that a block in iron uptake could affect protein import indirectly, accounting for some of the results linking the protein to import. However, genes related to iron homeostasis are also up-regulated in other pale mutants with defects in chloroplast biogenesis [108], and so further work is needed to determine the causal relationship between the iron homeostasis and protein import defects in *pic1*/*tic21* mutants. An alternative possibility is that PIC1/Tic21 has a dual role, acting in both processes [65].

9.3.2 Import Propulsion

The Tic110 C-terminus projects, at least partly, into the stroma and can bind transit peptides upon their emergence from the TIC channel [15, 83, 92]. It also recruits molecular chaperones, and these are believed to consume the ATP that is needed to drive preprotein import, and to assist the folding of newly-imported proteins [3, 61, 103, 158]. In mitochondria, a matrix Hsp70 (mtHsp70) delivers the energy for preprotein import [156], but until recently it has generally been thought that an Hsp100 protein, ClpC/Hsp93, is the principal component of the TIC motor [3, 158]. However, an important role for chloroplast stromal Hsp70 (cpHsc70) in the import mechanism has now been established [186, 197], while a stromal Hsp90 was also recently found to play a role (Fig. 9.1) [90].

Hsp93 (or ClpC) is an Hsp100-type AAA+ATPase. In addition to its function in preprotein import, it also forms part of the Clp protease complex in chloroplasts [61, 184]. It is believed to assemble into hexamers, and to act by threading clients (either importing preproteins or proteins to be degraded) through the resulting axial pore, towards either the stroma or the Clp proteolytic core [93, 179]. Reflecting its different roles, Hsp93 partitions between the envelope and stroma, and recent work identified its N-terminus as an important determinant of envelope association [48]. There are two Hsp93 isoforms in *Arabidopsis*, called atHsp93-V (ClpC1) and atHsp93-III (ClpC2). The former is expressed at much higher levels than the latter, while *hsp93-V* knockouts are pale and exhibit reduced preprotein import efficiency; *hsp93-III* knockout mutants are indistinguishable from wild type [49, 118, 119, 191]. Because double mutants are embryo lethal, and because the mature domains share ~91 % identity [119], the two isoforms are believed to have largely redundant functions.

Tic40 (previously named Com44/Cim44) can be crosslinked to Tic110 via a disulfide bridge under oxidizing conditions [194], and its loss causes a pale phenotype and inefficient chloroplast protein import in *Arabidopsis* [46, 118]. It is anchored in the inner membrane by a single, N-terminal transmembrane span, and it projects a large C-terminal domain into the stroma, much like Tic110 [46, 194]. This stromal region contains a putative TPR domain (whether it is truly a TPR was recently questioned [16]), and a tightly-folded Sti1 domain of the type found in eukaryotic Hip/Hop co-chaperones [24, 46, 47, 100]. Tic40 associates with Tic110 and Hsp93, and these three proteins appear to function at similar times in the import mechanism [46]. It is proposed that Tic40 binds to Tic110 (via its putative TPR domain) when the transit peptide binding site of the latter is occupied [47, 83]. Upon binding of Tic40, the transit peptide is released from Tic110 and passed to Hsp93. The chaperone then draws the preprotein into the stroma at the expense of ATP hydrolysis, which is stimulated by the Tic40 Sti1 domain. Curiously, the Sti1 domain of Tic40 can be functionally replaced with that of mammalian Hip (*H*sp70-*i*nteracting *p*rotein), for which an ATPase-stimulating function was not previously proposed [24].

Early attempts to identify stromal Hsp70 in import complexes failed, seemingly due to the lack of a suitable antibody [3, 158, 197]. However, this issue was recently overcome, while new genetic evidence also supports a role for this chaperone in preprotein import. Two stromal Hsp70 isoforms exist in *Arabidopsis* (cpHsc70-1 and cpHsc70-2), and plants lacking either one exhibit defective preprotein import [196, 197]; double mutants lacking both isoforms are embryo lethal, implying that the proteins share redundant functions [197]. Interestingly, the *cphsc70-1 hsp93-V* and *cphsc70-1 tic40* double mutants are phenotypically more severe than the corresponding single mutants [197], suggesting that cpHsc70's import function acts in parallel to the Tic40/Hsp93 system. The *cphsc70-1 tic40* genotype is lethal, whereas *hsp93-V tic40* causes only a pale phenotype, suggesting that cpHsc70, but not Hsp93, becomes essential and limiting in the *tic40* background [118, 197]. In the moss *Physcomitrella patens*, cpHsc70-deficient mutants similarly display inefficient chloroplast protein import, as do other mutants with a deficiency in the stromal co-chaperone CGE (*c*hloroplast *G*rp*E* homolog) [186]. Related GrpE proteins

promote nucleotide exchange at Hsp70 in prokaryotic systems, and play a well-established role in mitochondrial protein import in conjunction with mtHsp70 [156]. Finally, immunoprecipitation studies in both moss and pea showed that cpHsc70 associates with preproteins and translocon components such as Tic110, Hsp93 and Tic40 [186, 197].

Most recently, a chloroplast Hsp90 protein (Hsp90C) was implicated in import [90]. This chaperone was identified in import intermediates, and was co-purified with translocon components including Tic110, Tic40, Toc75 and Tic22, as well as Hsp93 and cpHsp70. Moreover, an inhibitor of Hsp90 ATPase activity, radicicol, reversibly inhibited the import of several preproteins during inner envelope translocation. Insertion mutations affecting the single Hsp90C gene in *Arabidopsis* are embryo lethal, indicating an essential role for this chaperone, presumably as part of a stromal chaperone complex that facilitates membrane translocation during protein import [90].

Conceivably, the different stromal chaperones implicated in import may act sequentially in the process, or exhibit selectivity towards different preprotein clients. The unusual complexity of the import-associated chaperone network in chloroplasts suggests that the chaperones do not simply function as components of a translocation motor, but perhaps participate in a series of events necessary for efficient import.

9.3.3 Redox Regulation

Chloroplast redox signals inform many important regulatory mechanisms [13], and so it is not surprising that chloroplast protein import is also a proposed target of redox control. The TIC translocon in particular is a proposed target for regulation by chloroplast redox status (Fig. 9.1) [17, 120]. In maize chloroplasts, precursors of different isoforms of ferredoxin and ferredoxin-NADP$^+$reductase (FNR) are imported differentially under light and dark conditions [72]: photosynthetic isoforms are similarly imported by light- and dark-exposed chloroplasts, but non-photosynthetic isoforms are missorted to the intermembrane space in the light. Conceivably, the non-photosynthetic isoforms might interfere with photosynthesis, and so perhaps systems evolved to prevent their import under light conditions.

Two TIC proteins, Tic32 and Tic62, are proposed to modulate preprotein import in response to changing stromal NADP$^+$/NADPH ratios [17, 77, 78, 125]. In fact, their association with the TIC apparatus is dependent on such ratios, with binding and dissociation occurring under oxidizing and reducing conditions, respectively [44, 120, 195]. Both proteins have an N-terminal NADP(H)-binding site and are members of the short-chain dehydrogenase/reductase family, and they associate with Tic110 and other translocon components at the stromal side of the inner membrane [77, 125, 195]. Tic32 also has a binding site for calmodulin, and the inhibition of preprotein import by ophiobolin A and ionomycin, which both disrupt calcium signalling, has been linked to Tic32 [43, 44]. Calmodulin and NADPH binding to Tic32 are mutually exclusive, suggesting that calcium signals are relayed via calmodulin only under oxidizing conditions when Tic32 is associated with the TIC machinery [44].

Like TROL (*t*hylakoid *r*ho*d*anese-*l*ike protein), which tethers FNR to thylakoids for the reduction of $NADP^+$ in photosynthetic electron transport, Tic62 has a C-terminal FNR-binding site [4, 97, 125]. Under reducing conditions, Tic62 breaks its peripheral association with the envelope and moves into the stroma (unlike Tic32, which behaves as an integral membrane protein), thereby increasing its affinity for FNR and enabling its association with the thylakoids [26, 195]. However, the Tic62-bound FNR appears not to be involved in photosynthetic electron transport, even when attached to the thylakoids [26]. Whether Tic62 has different functions dependent on its location (in the envelope, stroma or thylakoids), or the capacity to relay thylakoid signals to the TIC machinery, remains to be seen.

A third component implicated in the redox-regulation of preprotein import is Tic55, which was identified in a complex with Tic110, translocating preproteins, and other translocon components [36]. Tic55 is anchored in the inner membrane by two C-terminal transmembrane spans, has a Rieske-type iron-sulfur centre and a mononuclear iron-binding site, and is proposed to act via an electron transfer process or as a sensor of oxidative stress [36, 120]. Tic55 can also bind to thioredoxins and contains conserved cysteine residues that have the potential to form disulfide bridges [18]. However, an *Arabidopsis* Tic55 knockout mutant displays neither visible abnormalities nor defects in chloroplast protein import [30]. Doubts over the participation of Tic55 in preprotein import were also raised when two groups failed to detect the protein in import complexes [116, 170].

9.3.4 Transit Peptide Cleavage

Upon emergence from the TIC machinery, the transit peptide of a translocating preprotein is quickly removed by the stromal processing peptidase (SPP) (Fig. 9.1) [174, 201]. SPP is a zinc-binding metalloendopeptidase of the M16 family, which also includes the mitochondrial processing peptidase MPP, the presequence protease PreP (see below), and *Escherichia coli* pitrilysin [171, 217]. A stretch of 10–15 residues near the C-terminus of the transit peptide, where basic residues tend to be concentrated, is recognized by SPP, and cleavage occurs at a loosely-conserved site dependent upon physicochemical properties of the sequence [56, 172, 175, 230]. Interestingly, SPP is encoded by a single gene in *Arabidopsis*, and so the protein must accommodate a wide range of transit peptides with highly variable sequences [34, 171]. Following release of the newly-processed protein, SPP terminates its interaction with the transit peptide by a second cleavage event [172]. The peptide fragments are then degraded by the presequence protease, PreP [151, 172, 173]. The SPP protein is evolutionarily well conserved, as related sequences are found in various algae, apicomplexan parasites, and even cyanobacteria, suggesting that an ancestral activity was probably inherited with the endosymbiont [174].

Suppression of SPP expression in *Arabidopsis* or tobacco plants causes various abnormal phenotypes, ranging from albinism to seedling lethality, and is associated with ultrastructural defects and reduced numbers of chloroplasts [225, 232].

Similarly, a point mutation affecting a conserved glutamate residue of SPP causes chlorosis and small, abnormal chloroplasts in rice [229]. Interestingly, SPP-deficiency leads to reduced chloroplast protein import efficiency [225, 232]; this may reflect the fact that most TIC components, and Toc75, have a transit peptide and so depend on SPP for proper maturation, or indicate that transit peptide cleavage is an integrated step in the import mechanism. *Arabidopsis* SPP knockout mutations are embryo lethal, further emphasizing the importance of this protein for organelle and plant development [212].

9.4 Targeting to the Envelope Membranes

9.4.1 Sorting to the Outer Membrane

Outer envelope membrane proteins typically do not have transit peptides, but instead are targeted by intrinsic, non-cleavable signals. There are several different pathways for outer membrane protein insertion [74], and perhaps the best understood of these is that used by signal-anchored proteins such as OEP7/14 (*o*uter *e*nvelope *p*rotein, *7*/*14* kD) and Toc64/OEP64 [132]. In these proteins, targeting information is linked to the amino-terminal transmembrane domain, which is superficially similar to signal peptides that direct proteins to the ER [74, 127]. Flanking the transmembrane domain there is a positively-charged region that, together with the hydrophobicity of the transmembrane region itself, plays a critical role in ensuring that such proteins are targeted to chloroplasts rather than the ER [127, 131].

Despite early suggestions that signal-anchored proteins insert spontaneously, it is now clear that their targeting involves proteinaceous cofactors and the consumption of nucleoside triphosphates [75, 214]. In fact, competition, cross-linking and reconstitution results indicate that Toc75 is involved, and that in this role it may function without assistance from the TOC receptors, Toc34 and Toc159 [215]. Involvement of Toc75 parallels the situation in mitochondria, where the equivalent import channel, Tom40, is similarly employed [169]. More recently, AKR2A (*ank*yrin *r*epeat-containing protein *2A*) was identified as a cytosolic sorting factor in this pathway [11, 23]. In conjunction with its cofactor, Hsp17.8, a member of the small heat shock protein family, AKR2A is proposed to act as a chaperone, preventing the aggregation of its clients and guiding them to the envelope [110]. Interestingly, AKR2A also mediates protein insertion into the peroxisomal membrane [185], suggesting that it acts in the targeting of a broad class of membrane proteins [231].

Similar intrinsic information directs the targeting of Toc34, but in this case the relevant transmembrane domain lies at the C-terminus (i.e., it is a tail-anchored protein). As with signal-anchored proteins, insertion requires both envelope proteins and an energy source [213]. Indeed, competition results suggest that Toc34, OEP7/14 and Toc64/OEP64 may all follow the same pathway [74, 75, 214]. However, Toc34 insertion was also reported to depend on previously-inserted Toc34, as

well as on membrane lipids, and to follow a different pathway from that used by another tail-anchored protein [51, 165]. Further complexity arises from the fact that some outer membrane proteins are dual-targeted to mitochondria and chloroplasts [199].

Toc159 employs a different targeting mechanism, perhaps due to its large, atypical M-domain. Its insertion is thought to involve a homotypic G-domain interaction with resident Toc34, controlled by guanine nucleotide status, as well as Toc75 [20, 193, 224]. Nonetheless, the M-domain itself seems to possess targeting information [128, 142]. The M-domain has no typical transmembrane spans, and so its insertion most likely depends on the TOC complex. That said, a short hydrophobic segment near the C-terminus may interface with the lipid bilayer [82].

Unusually, Toc75 has a cleavable, bipartite targeting signal at its N-terminus: the N-terminal part is a standard transit peptide, while the second part directs intraorganellar sorting [208, 209]. The latter contains a poly-glycine stretch that enables disengagement from the translocon and membrane integration [86]. The transit peptide is cleaved by SPP, whereas the second domain is removed by an envelope-localized type I signal peptidase (which additionally resides in thylakoids for the maturation of thylakoidal proteins) [79, 88, 188, 189]. How Toc75 becomes integrated into the outer membrane is unclear. In bacteria and mitochondria, the biogenesis of similar β-barrels is assisted by proteins of the Omp85 superfamily [205], and a related protein in chloroplasts, OEP80, was proposed to play a similar role [55, 87]. Supporting this idea, OEP80 is an essential protein in *Arabidopsis* (like Toc75), while its depletion affects Toc75 accumulation in vivo [80, 161]. Phylogenetic data are also consistent with the notion that OEP80 has retained an ancestral function [207].

9.4.2 Sorting to the Intermembrane Space and Inner Membrane

Unlike most outer membrane proteins, those destined for the intermembrane space or inner membrane typically have cleavable, N-terminal targeting information. Sorting to the intermembrane space has been studied for two proteins that follow different pathways: Tic22 and MGD1 (*m*ono*g*alactosyl*d*iacylglycerol synthase *1*) [117, 221]. Both proteins have a targeting sequence, but only that of MGD1 is cleaved by SPP. Along with the energetic requirements for its import, this indicates that MGD1 partially enters the stroma. In contrast, Tic22 is processed by an unknown protease in the intermembrane space, suggesting that it does not enter the TIC channel. There is also uncertainty over the participation of the TOC apparatus in Tic22 sorting.

Inner membrane proteins typically follow one of two routes: the stop-transfer and post-import pathways. The requirement for a transit peptide in both cases implies involvement of the TOC/TIC apparatus [114, 141, 194]. In the stop transfer pathway, a hydrophobic transmembrane domain arrests preprotein transport in the channel, enabling lateral exit into the membrane [32, 60, 114, 210]. This pathway may be particularly important for hydrophobic or polytopic proteins that are prone to aggregation. Recent work on the APG1 (*a*lbino or *p*ale *g*reen mutant *1*) protein, a stop-transfer client, revealed that membrane targeting information lies in the

transmembrane domain, which is sufficient to direct stop-transfer insertion even in the context of heterologous passenger proteins [218].

In the post-import pathway, proteins undergo complete translocation into the stroma, where they form soluble intermediates, prior to membrane insertion [137, 141, 210]. Tic40 and Tic110 are clients of this pathway, and both are anchored in the inner membrane by N-terminal helices and have large stromal domains. Interference with their membrane integration leads to the accumulation of SPP-processed forms in the stroma [24, 84]. Tic40 possesses a bipartite targeting sequence, but the role of the second domain is unclear as a serine/proline-rich region of the mature sequence and the adjacent transmembrane domain control insertion [137, 210]. The latter two may cooperate to form a membrane insertion loop, while in Tic110 the two transmembrane domains may create an equivalent structure. Efficacy of Tic40's targeting information is influenced by context within the protein sequence, implying that post-import signals are complex, which might be necessary to avoid stop-transfer insertion and an incorrect topology [218]. Stromal events in the post-import pathway may involve Hsp93 [222], while integration depends on proteinaceous membrane components [137].

In mitochondria, sorting to the inner membrane employs stop-transfer and conservative sorting pathways [157]. The latter is similar to the post-import pathway of chloroplasts and, as its name suggests, it is at least partly of prokaryotic origin. Bearing this in mind, it is intriguing that a second Sec translocase (in addition to the well-known thylakoidal system) was recently identified in chloroplast envelopes [192]. There is also evidence that resident Tic40 (and possibly Tic110) acts in the integration of other proteins into the membrane [45, 84].

9.5 Alternative Protein Import Pathways

9.5.1 Dual-Targeting

Although most chloroplast proteins are targeted specifically to plastids, a significant number (>100) are transported to more than one location [37, 145, 190]. Transport to chloroplasts and mitochondria is the most common form of dual-targeting, but there are also proteins that exist in the nucleus, ER or peroxisomes as well as in chloroplasts [122, 135, 177]. Such multi-destination transport implies that protein targeting is rather flexible, and is supportive of a model for the relocation of organellar genes to the nucleus that depends on the "minor mistargeting" of many proteins to multiple locations [146]. Dual-targeted proteins tend to have highly-conserved functions that are easily shared, including nucleic acid and protein synthesis or processing, and cellular stress response [37, 145]. A particularly striking example occurs amongst the aminoacyl-tRNA synthetases, where 17 of the 24 organellar proteins in *Arabidopsis* are targeted to both chloroplasts and mitochondria [53]; some are even targeted to all three of the compartments that possess translational machinery.

Dual-targeting to chloroplasts and mitochondria typically involves one of two mechanisms [162]. In the first of these, alternative splicing and/or differential transcriptional or translational initiation leads to the production of protein variants with different N-terminal leader sequences and distinct targeting properties. In the second mechanism, a single protein is produced that possesses an ambiguous leader, competent for sorting to both chloroplasts and mitochondria. Alternatively, dual-targeting information may be, either wholly or partly, an intrinsic feature of the mature protein [12, 216].

Ambiguous transit peptides for dual-targeting to endosymbiotic organelles have been scrutinized, and in general they have properties intermediate between those that target either organelle specifically [27, 28, 162, 164]. In the N-terminal region, serine content is more similar to that of chloroplast transit peptides, while arginine content is more similar to that of mitochondrial presequences. They show enrichment of phenylalanine and leucine residues and, while certain segments are more important for transport to one or the other organelle in some cases, they do not share a common functional-domain architecture [27, 28]. Dual-targeting is also influenced by the mature domain of the preprotein, and by developmental factors [38, 145]. Software for the predication of ambiguous targeting peptides suggests that as many as ~400–500 proteins may be dual-targeted to chloroplasts and mitochondria [150]. Competition data indicate that dual-targeted proteins utilize the same import machineries as organelle-specific proteins [28].

9.5.2 Non-canonical Protein Transport

In recent years it has become apparent that transit peptide-dependent import is not the only sorting pathway to the chloroplast interior [94, 112, 168]. One study estimated that more than 10% of plastid proteins lack a typical transit peptide [6]. The ceQORH (*c*hloroplast *e*nvelope *q*uinone *o*xido*r*eductase *h*omolog) protein was identified through proteomics, and found to associate with the inner envelope membrane even though it lacks a transit peptide. An internal sequence of ~40 residues controls its localization, and while its import does require proteinaceous machinery and ATP, the TOC/TIC apparatus is not involved [149]. Another inner membrane protein, Tic32/IEP32 (*i*nner *e*nvelope *p*rotein, *32* kD), similarly lacks a transit peptide, and it too localizes independently of the TOC translocon [152]. Competition results imply that ceQORH and Tic32 utilize different import pathways [149].

Proteomic analysis also led to the identification of a large number of chloroplast proteins with predicted signal peptides for ER translocation [112]. Chloroplast protein traffic through the endomembrane system is well documented in organisms that have complex plastids with more than two bounding membranes, such as algae and apicomplexan parasites [155], but was not thought to occur in plants. That said, physical and functional links between the ER and the outer envelope membrane have long been known [5, 25, 227], while glycoproteins and proteins with apparent

signal peptides were detected in plastids [10, 42, 62]. The breakthrough came from analyses of *Arabidopsis* CAH1 (*c*arbonic *a*n*h*ydrase *1*) [219]. This stromal protein has a signal peptide and is imported and processed by ER microsomes, but not by chloroplasts. Glycosylated forms of CAH1 and other proteins are present in chloroplasts, while brefeldin A (a chemical that disrupts traffic through the Golgi) interferes with their localization, indicating passage through the Golgi en route to chloroplasts [154, 219]. Other data suggest that this sorting pathway, and the glycosylation it enables, are functionally important [35, 81].

Exactly how proteins are directed through the endomembrane system to chloroplasts remains unclear. Some data suggest that the signal peptide provides the necessary sorting information [42], while others argue that surface characteristics of the mature protein are important [111]. The proteins may be released into the intermembrane space upon vesicle fusion with the outer membrane, before entering an unknown translocon, the TIC apparatus, or vesicles that pinch off from the inner membrane [168].

9.6 Concluding Remarks

Our understanding of chloroplast protein import, and of the molecular events that underlie the process, has improved significantly in recent years. Nonetheless, important unanswered questions remain, while several inconsistencies in the literature need to be resolved. Even though our knowledge concerning TOC receptor GTPase function has expanded, the precise mode-of-action of these receptors is hotly debated, and a consensus model is lacking. It is generally accepted that client-specific protein import pathways operate in chloroplasts, but the molecular basis for TOC receptor (and possibly also TIC channel) selectivity requires further work. Chloroplast protein import must be tightly regulated, and indeed recent research has unveiled direct control of the TOC machinery by the ubiquitin-proteasome system, while other work suggests redox-regulation at the TIC apparatus. Nonetheless, the mechanistic details behind such regulatory systems are largely unknown. We have learnt much about the functions of putative inner envelope channel components, but there is now a need to reconcile the different hypotheses that have been proposed. Identification of a large TIC complex containing Tic20, but excluding Tic110 and Tic40, indicates that a more comprehensive framework is required that integrates the roles of these proteins. Furthermore, the confounding complexity of stromal chaperone complexes involved in chloroplast protein import needs to be unravelled. We expect that future research focusing on these fascinating questions will bring us closer to a full understanding of this essential process.

Acknowledgements Research in the authors' laboratories is supported by the Biotechnology and Biological Sciences Research Council (BBSRC) and the Gatsby Charitable Foundation (to P.J.), and the University of Neuchâtel and the Swiss National Science Foundation (to F.K.). We thank Jocelyn Bédard for helpful comments on the manuscript.

References

1. Agne B et al (2009) A Toc159 import receptor mutant, defective in hydrolysis of GTP, supports preprotein import into chloroplasts. J Biol Chem 284:8670–8679
2. Agne B et al (2010) The acidic A-domain of Arabidopsis Toc159 occurs as a hyperphosphorylated protein. Plant Physiol 153:1016–1030
3. Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136:983–994
4. Alte F, Stengel A, Benz JP, Petersen E, Soll J, Groll M, Bolter B (2010) Ferredoxin: NADPH oxidoreductase is recruited to thylakoids by binding to a polyproline type II helix in a pH-dependent manner. Proc Natl Acad Sci U S A 107:19260–19265
5. Andersson MX, Goksör M, Sandelius AS (2007) Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. J Biol Chem 282:1170–1174
6. Armbruster U et al (2009) Chloroplast proteins without cleavable transit peptides: rare exceptions or a major constituent of the chloroplast proteome? Mol Plant 2:1325–35
7. Aronsson H, Combe J, Patel R, Jarvis P (2006) In vivo assessment of the significance of phosphorylation of the *Arabidopsis* chloroplast protein import receptor, atToc33. FEBS Lett 580:649–655
8. Aronsson H, Boij P, Patel R, Wardle A, Töpel M, Jarvis P (2007) Toc64/OEP64 is not essential for the efficient import of proteins into chloroplasts in *Arabidopsis thaliana*. Plant J 52:53–68
9. Aronsson H, Combe J, Patel R, Agne B, Martin M, Kessler F, Jarvis P (2010) Nucleotide binding and dimerization at the chloroplast pre-protein import receptor, atToc33, are not essential in vivo but do increase import efficiency. Plant J 63:297–311
10. Asatsuma S, Sawada C, Itoh K, Okito M, Kitajima A, Mitsui T (2005) Involvement of alpha-amylase I-1 in starch degradation in rice chloroplasts. Plant Cell Physiol 46:858–869
11. Bae W, Lee YJ, Kim DH, Lee J, Kim S, Sohn EJ, Hwang I (2008) AKR2A-mediated import of chloroplast outer membrane proteins is essential for chloroplast biogenesis. Nat Cell Biol 10:220–227
12. Bahaji A et al (2011) Dual targeting to mitochondria and plastids of AtBT1 and ZmBT1, two members of the mitochondrial carrier family. Plant Cell Physiol 52:597–609
13. Baier M, Dietz KJ (2005) Chloroplasts as source and target of cellular redox regulation: a discussion on chloroplast redox signals in the context of plant physiology. J Exp Bot 56: 1449–1462
14. Baldwin A, Wardle A, Patel R, Dudley P, Park SK, Twell D, Inoue K, Jarvis P (2005) A molecular-genetic study of the Arabidopsis Toc75 gene family. Plant Physiol 138:715–733
15. Balsera M, Goetze TA, Kovács-Bogdán E, Schürmann P, Wagner R, Buchanan BB, Soll J, Bölter B (2009) Characterization of Tic110, a channel-forming protein at the inner envelope membrane of chloroplasts, unveils a response to $Ca^{2+}$and a stromal regulatory disulfide bridge. J Biol Chem 284:2603–2616
16. Balsera M, Soll J, Buchanan BB (2009) Protein import in chloroplasts: an emerging regulatory role for redox. Adv Bot Res 52:277–332
17. Balsera M, Soll J, Buchanan BB (2010) Redox extends its regulatory reach to chloroplast protein import. Trends Plant Sci 15:515–521
18. Bartsch S, Monnet J, Selbach K, Quigley F, Gray J, von Wettstein D, Reinbothe S, Reinbothe C (2008) Three thioredoxin targets in the inner envelope membrane of chloroplasts function in protein import and chlorophyll metabolism. Proc Natl Acad Sci U S A 105:4933–4938
19. Bauer J, Chen K, Hiltbrunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203–207
20. Bauer J, Hiltbrunner A, Weibel P, Vidi PA, Alvarez-Huerta M, Smith MD, Schnell DJ, Kessler F (2002) Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. J Cell Biol 159:845–854

21. Becker T, Hritz J, Vogel M, Caliebe A, Bukau B, Soll J, Schleiff E (2004) Toc12, a novel subunit of the intermembrane space preprotein translocon of chloroplasts. Mol Biol Cell 15:5130–5144
22. Becker T, Jelic M, Vojta A, Radunz A, Soll J, Schleiff E (2004) Preprotein recognition by the Toc complex. EMBO J 23:520–530
23. Bédard J, Jarvis P (2008) Green light for chloroplast outer-membrane proteins. Nat Cell Biol 10:120–122
24. Bédard J, Kubis S, Bimanadham S, Jarvis P (2007) Functional similarity between the chloroplast translocon component, Tic40, and the human co-chaperone, Hsp70-interacting protein (Hip). J Biol Chem 282:21404–21414
25. Benning C (2009) Mechanisms of lipid transport involved in organelle biogenesis in plant cells. Annu Rev Cell Dev Biol 25:71–91
26. Benz JP et al (2009) *Arabidopsis* Tic62 and ferredoxin-NADP(H) oxidoreductase form light-regulated complexes that are integrated into the chloroplast redox poise. Plant Cell 21: 3965–3983
27. Berglund AK, Pujol C, Duchene AM, Glaser E (2009) Defining the determinants for dual targeting of amino acyl-tRNA synthetases to mitochondria and chloroplasts. J Mol Biol 393:803–814
28. Berglund AK, Spanning E, Biverstahl H, Maddalo G, Tellgren-Roth C, Maler L, Glaser E (2009) Dual targeting to mitochondria and chloroplasts: characterization of Thr-tRNA synthetase targeting peptide. Mol Plant 2:1298–1309
29. Bischof S et al (2011) Plastid proteome assembly without Toc159: photosynthetic protein import and accumulation of N-acetylated plastid precursor proteins. Plant Cell 23:3911–3928
30. Boij P, Patel R, Garcia C, Jarvis P, Aronsson H (2009) In vivo studies on the roles of Tic55-related proteins in chloroplast protein import in *Arabidopsis thaliana*. Mol Plant 2:1397–1409
31. Bölter B, May T, Soll J (1998) A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. FEBS Lett 441:59–62
32. Brink S, Fischer K, Klösgen RB, Flügge UI (1995) Sorting of nuclear-encoded chloroplast membrane proteins to the envelope and the thylakoid membrane. J Biol Chem 270: 20808–20815
33. Bruce BD (2000) Chloroplast transit peptides: structure, function and evolution. Trends Cell Biol 10:440–447
34. Bruce BD (2001) The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. Biochim Biophys Acta 1541:2–21
35. Burén S et al (2011) Importance of post-translational modifications for functionality of a chloroplast-localized carbonic anhydrase (CAH1) in *Arabidopsis thaliana*. PLoS ONE 6:e21021
36. Caliebe A, Grimm R, Kaiser G, Lubeck J, Soll J, Heins L (1997) The chloroplastic protein import machinery contains a Rieske-type iron- sulfur cluster and a mononuclear iron-binding protein. EMBO J 16:7342–7350
37. Carrie C, Small I (2013) A reevaluation of dual-targeting of proteins to mitochondria and chloroplasts. 1833: 253-259
38. Carrie C, Giraud E, Whelan J (2009) Protein transport in organelles: dual targeting of proteins to mitochondria and chloroplasts. FEBS J 276:1187–1195
39. Chen KY, Li HM (2007) Precursor binding to an 880-kDa Toc complex as an early step during active import of protein into chloroplasts. Plant J 49:149–158
40. Chen K, Chen X, Schnell DJ (2000) Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiol 122:813–822
41. Chen X, Smith MD, Fitzpatrick L, Schnell DJ (2002) In vivo analysis of the role of atTic20 in protein import into chloroplasts. Plant Cell 14:641–654
42. Chen MH, Huang LF, Li HM, Chen YR, Yu SM (2004) Signal peptide-dependent targeting of a rice alpha-amylase and cargo proteins to plastids and extracellular compartments of plant cells. Plant Physiol 135:1367–1377
43. Chigri F, Soll J, Vothknecht UC (2005) Calcium regulation of chloroplast protein import. Plant J 42:821–831

44. Chigri F, Hörmann F, Stamp A, Stammers DK, Bölter B, Soll J, Vothknecht UC (2006) Calcium regulation of chloroplast protein translocation is mediated by calmodulin binding to Tic32. Proc Natl Acad Sci U S A 103:16051–16056
45. Chiu CC, Li HM (2008) Tic40 is important for reinsertion of proteins from the chloroplast stroma into the inner membrane. Plant J 56:793–801
46. Chou ML, Fitzpatrick LM, Tu SL, Budziszewski G, Potter-Lewis S, Akita M, Levin JZ, Keegstra K, Li HM (2003) Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. EMBO J 22:2970–2980
47. Chou ML, Chu CC, Chen LJ, Akita M, Li HM (2006) Stimulation of transit-peptide release and ATP hydrolysis by a cochaperone during protein import into chloroplasts. J Cell Biol 175:893–900
48. Chu CC, Li HM (2012) The amino-terminal domain of chloroplast Hsp93 is important for its membrane association and functions in vivo. Plant Physiol 158:1656–1665
49. Constan D, Froehlich JE, Rangarajan S, Keegstra K (2004) A stromal Hsp100 protein is required for normal chloroplast development and function in Arabidopsis. Plant Physiol 136:3605–3615
50. Constan D, Patel R, Keegstra K, Jarvis P (2004) An outer envelope membrane component of the plastid protein import apparatus plays an essential role in *Arabidopsis*. Plant J 38:93–106
51. Dhanoa PK, Richardson LG, Smith MD, Gidda SK, Henderson MP, Andrews DW, Mullen RT (2010) Distinct pathways mediate the sorting of tail-anchored proteins to the plastid outer envelope. PLoS ONE 5:e10098
52. Dobberstein B, Blobel G, Chua NH (1977) In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci U S A 74:1082–1085
53. Duchêne AM, Giritch A, Hoffmann B, Cognat V, Lancelin D, Peeters NM, Zaepfel M, Marechal-Drouard L, Small ID (2005) Dual targeting is the rule for organellar aminoacyl-tRNA synthetases in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 102:16484–16489
54. Duy D, Wanner G, Meda AR, von Wiren N, Soll J, Philippar K (2007) PIC1, an ancient permease in *Arabidopsis* chloroplasts, mediates iron transport. Plant Cell 19:986–1006
55. Eckart K, Eichacker L, Sohrt K, Schleiff E, Heins L, Soll J (2002) A Toc75-like protein import channel is abundant in chloroplasts. EMBO Rep 3:557–562
56. Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8:978–984
57. Ertel F, Mirus O, Bredemeier R, Moslavac S, Becker T, Schleiff E (2005) The evolutionarily related beta-barrel polypeptide transporters from *Pisum sativum* and *Nostoc* PCC7120 contain two distinct functional domains. J Biol Chem 280:28281–28289
58. Fellerer C, Schweiger R, Schongruber K, Soll J, Schwenkert S (2011) Cytosolic HSP90 cochaperones HOP and FKBP interact with freshly synthesized chloroplast preproteins of *Arabidopsis*. Mol Plant 4:1133–1145
59. Ferro M et al (2010) AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol Cell Proteomics 9:1063–1084
60. Firlej-Kwoka E, Strittmatter P, Soll J, Bolter B (2008) Import of preproteins into the chloroplast inner envelope membrane. Plant Mol Biol 68:505–519
61. Flores-Pérez U, Jarvis P (2013) Molecular chaperone involvement in chloroplast protein import. Biochim Biophys Acta 1833:332–340
62. Gaikwad A, Tewari KK, Kumar D, Chen W, Mukherjee SK (1999) Isolation and characterisation of the cDNA encoding a glycosylated accessory protein of pea chloroplast DNA polymerase. Nucleic Acids Res 27:3120–3129
63. Gentle I, Gabriel K, Beech P, Waller R, Lithgow T (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. J Cell Biol 164:19–24
64. Glaser S, van Dooren GG, Agrawal S, Brooks CF, McFadden GI, Striepen B, Higgins MK (2012) Tic22 is an essential chaperone required for protein import into the apicoplast. J Biol Chem 287:39505–39512

65. Gross J, Bhattacharya D (2009) Revaluating the evolution of the Toc and Tic protein translocons. Trends Plant Sci 14:13–20
66. Heins L, Mehrle A, Hemmler R, Wagner R, Küchler M, Hörmann F, Sveshnikov D, Soll J (2002) The preprotein conducting channel at the inner envelope membrane of plastids. EMBO J 21:2616–2625
67. Highfield PE, Ellis RJ (1978) Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. Nature 271:420–424
68. Hiltbrunner A, Bauer J, Alvarez-Huerta M, Kessler F (2001) Protein translocon at the *Arabidopsis* outer chloroplast membrane. Biochem Cell Biol 79:629–635
69. Hiltbrunner A, Grunig K, Alvarez-Huerta M, Infanger S, Bauer J, Kessler F (2004) AtToc90, a new GTP-binding component of the *Arabidopsis* chloroplast protein import machinery. Plant Mol Biol 54:427–440
70. Hinnah SC, Wagner R, Sveshnikova N, Harrer R, Soll J (2002) The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. Biophys J 83:899–911
71. Hirabayashi Y, Kikuchi S, Oishi M, Nakai M (2011) *In vivo* studies on the roles of two closely related *Arabidopsis* Tic20 proteins, AtTic20-I and AtTic20-IV. Plant Cell Physiol 52:469–478
72. Hirohashi T, Hase T, Nakai M (2001) Maize non-photosynthetic ferredoxin precursor is missorted to the intermembrane space of chloroplasts in the presence of light. Plant Physiol 125:2154–2163
73. Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266:1989–1992
74. Hofmann NR, Theg SM (2005) Chloroplast outer membrane protein targeting and insertion. Trends Plant Sci 10:450–457
75. Hofmann NR, Theg SM (2005) Protein- and energy-mediated targeting of chloroplast outer envelope membrane proteins. Plant J 44:917–927
76. Hofmann NR, Theg SM (2005) Toc64 is not required for import of proteins into chloroplasts in the moss *Physcomitrella patens*. Plant J 43:675–687
77. Hörmann F, Küchler M, Sveshnikov D, Oppermann U, Li Y, Soll J (2004) Tic32, an essential component in chloroplast biogenesis. J Biol Chem 279:34756–34762
78. Hörmann F, Kuchler M, Sveshnikov D, Oppermann U, Li Y, Soll J (2009) Tic32, an essential component in chloroplast biogenesis (correction). J Biol Chem 284:29240
79. Hsu SC, Endow JK, Ruppel NJ, Roston RL, Baldwin AJ, Inoue K (2011) Functional diversification of thylakoidal processing peptidases in *Arabidopsis thaliana*. PLoS ONE 6:e27258
80. Huang W, Ling Q, Bédard J, Lilley K, Jarvis P (2011) In vivo analyses of the roles of essential Omp85-related proteins in the chloroplast outer envelope membrane. Plant Physiol 157:147–159
81. Hummel E, Osterrieder A, Robinson DG, Hawes C (2010) Inhibition of Golgi function causes plastid starch accumulation. J Exp Bot 61:2603–2614
82. Inaba T, Schnell DJ (2008) Protein trafficking to plastids: one theme, many variations. Biochem J 413:15–28
83. Inaba T, Li M, Alvarez-Huerta M, Kessler F, Schnell DJ (2003) atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. J Biol Chem 278:38617–38627
84. Inaba T, Alvarez-Huerta M, Li M, Bauer J, Ewers C, Kessler F, Schnell DJ (2005) *Arabidopsis* Tic110 is essential for the assembly and function of the protein import machinery of plastids. Plant Cell 17:1482–1496
85. Inoue H, Akita M (2008) Three sets of translocation intermediates are formed during the early stage of protein import into chloroplasts. J Biol Chem 283:7491–7502
86. Inoue K, Keegstra K (2003) A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. Plant J 34:661–669
87. Inoue K, Potter D (2004) The chloroplastic protein translocation channel Toc75 and its paralog OEP80 represent two distinct protein families and are targeted to the chloroplastic outer envelope by different mechanisms. Plant J 39:354–365

88. Inoue K, Baldwin AJ, Shipman RL, Matsui K, Theg SM, Ohme-Takagi M (2005) Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. J Cell Biol 171:425–430
89. Inoue H, Rounds C, Schnell DJ (2010) The molecular basis for distinct pathways for protein import into *Arabidopsis* chloroplasts. Plant Cell 22:1947–1960
90. Inoue H, Li M, Schnell DJ (2013) An essential role for chloroplast heat shock protein 90 (Hsp90C) in protein import into chloroplasts. Proc Natl Acad Sci U S A 110:3173–3178
91. Ivanova Y, Smith MD, Chen K, Schnell DJ (2004) Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. Mol Biol Cell 15: 3379–3392
92. Jackson DT, Froehlich JE, Keegstra K (1998) The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment. J Biol Chem 273:16583–16588
93. Jackson-Constan D, Akita M, Keegstra K (2001) Molecular chaperones involved in chloroplast protein import. Biochim Biophys Acta 1541:102–113
94. Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants (Tansley Review). New Phytol 179:257–285
95. Jarvis P, Chen LJ, Li H, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. Science 282:100–103
96. Jelic M, Soll J, Schleiff E (2003) Two Toc34 homologues with different properties. Biochemistry 42:5906–5916
97. Juric S et al (2009) Tethering of ferredoxin:NADP$^+$ oxidoreductase to thylakoid membranes is mediated by novel chloroplast protein TROL. Plant J 60:783–794
98. Kalanon M, McFadden GI (2008) The chloroplast protein translocation complexes of *Chlamydomonas reinhardtii*: a bioinformatic comparison of Toc and Tic components in plants, green algae and red algae. Genetics 179:95–112
99. Kaneko T et al (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3:109–136
100. Kao YF, Lou YC, Yeh YH, Hsiao CD, Chen C (2012) Solution structure of the C-terminal NP-repeat domain of Tic40, a co-chaperone during protein import into chloroplasts. J Biochem 152:443–451
101. Kasmati AR, Töpel M, Patel R, Murtaza G, Jarvis P (2011) Molecular and genetic analyses of Tic20 homologues in *Arabidopsis thaliana* chloroplasts. Plant J 66:877–889
102. Kasmati AR, Töpel M, Khan NZ, Patel R, Ling Q, Karim S, Aronsson H, Jarvis P (2013) Evolutionary, molecular and genetic analyses of Tic22 homologues in *Arabidopsis thaliana* chloroplasts. PLoS ONE 8:e63863
103. Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries of the chloroplast. Proc Natl Acad Sci U S A 93:7684–7689
104. Kessler F, Schnell DJ (2004) Chloroplast protein import: solve the GTPase riddle for entry. Trends Cell Biol 14:334–338
105. Kessler F, Schnell DJ (2006) The function and diversity of plastid protein import pathways: a multilane GTPase highway into plastids. Traffic 7:248–257
106. Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science 266:1035–1039
107. Kikuchi S, Hirohashi T, Nakai M (2006) Characterization of the preprotein translocon at the outer envelope membrane of chloroplasts by blue native PAGE. Plant Cell Physiol 47:363–71
108. Kikuchi S, Oishi M, Hirabayashi Y, Lee DW, Hwang I, Nakai M (2009) A 1-megadalton translocation complex containing Tic20 and Tic21 mediates chloroplast protein import at the inner envelope membrane. Plant Cell 21:1781–1797
109. Kikuchi S, Bédard J, Hirano M, Hirabayashi Y, Oishi M, Imai M, Takase M, Ide T, Nakai M (2013) Uncovering the protein translocon at the chloroplast inner envelope membrane. Science 339:571–574

110. Kim DH, Xu ZY, Na YJ, Yoo YJ, Lee J, Sohn EJ, Hwang I (2011) Small heat shock protein Hsp17.8 functions as an AKR2A cofactor in the targeting of chloroplast outer membrane proteins in *Arabidopsis*. Plant Physiol 157:132–146
111. Kitajima A et al (2009) The rice alpha-amylase glycoprotein is targeted from the Golgi apparatus through the secretory pathway to the plastids. Plant Cell 21:2844–2858
112. Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjolander K, Gruissem W, Baginsky S (2004) The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol 14:354–362
113. Kleffmann T, Hirsch-Hoffmann M, Gruissem W, Baginsky S (2006) plprot: a comprehensive proteome database for different plastid types. Plant Cell Physiol 47:432–436
114. Knight JS, Gray JC (1995) The N-terminal hydrophobic region of the mature phosphate translocator is sufficient for targeting to the chloroplast inner envelope membrane. Plant Cell 7:1421–1432
115. Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J Cell Biol 139:1677–1685
116. Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143:991–1002
117. Kouranov A, Wang H, Schnell DJ (1999) Tic22 is targeted to the intermembrane space of chloroplasts by a novel pathway. J Biol Chem 274:25181–25186
118. Kovacheva S, Bédard J, Patel R, Dudley P, Twell D, Ríos G, Koncz C, Jarvis P (2005) *In vivo* studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import. Plant J 41:412–428
119. Kovacheva S, Bédard J, Wardle A, Patel R, Jarvis P (2007) Further *in vivo* studies on the role of the molecular chaperone, Hsp93, in plastid protein import. Plant J 50:364–379
120. Kovacs-Bogdan E, Soll J, Bölter B (2010) Protein import into chloroplasts: the Tic complex and its regulation. Biochim Biophys Acta 1803:740–747
121. Kovacs-Bogdan E, Benz JP, Soll J, Bolter B (2011) Tic20 forms a channel independent of Tic110 in chloroplasts. BMC Plant Biol 11:133
122. Krause K, Krupinska K (2009) Nuclear regulators with a second home in organelles. Trends Plant Sci 14:194–199
123. Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D, Jarvis P (2003) The *Arabidopsis ppi1* mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. Plant Cell 15:1859–1871
124. Kubis S et al (2004) Functional specialization amongst the *Arabidopsis* Toc159 family of chloroplast protein import receptors. Plant Cell 16:2059–2077
125. Küchler M, Decker S, Hörmann F, Soll J, Heins L (2002) Protein import into chloroplasts involves redox-regulated proteins. EMBO J 21:6136–6145
126. Lamberti G, Gugel IL, Meurer J, Soll J, Schwenkert S (2011) The cytosolic kinases STY8, STY17, and STY46 are involved in chloroplast differentiation in *Arabidopsis*. Plant Physiol 157:70–85
127. Lee YJ, Kim DH, Kim YW, Hwang I (2001) Identification of a signal that distinguishes between the chloroplast outer envelope membrane and the endomembrane system in vivo. Plant Cell 13:2175–2190
128. Lee KH, Kim SJ, Lee YJ, Jin JB, Hwang I (2003) The M domain of atToc159 plays an essential role in the import of proteins into chloroplasts and chloroplast biogenesis. J Biol Chem 278:36794–36805
129. Lee J, Wang F, Schnell DJ (2009) Toc receptor dimerization participates in the initiation of membrane translocation during protein import into chloroplasts. J Biol Chem 284: 31130–31141
130. Lee S, Lee DW, Lee Y, Mayer U, Stierhof YD, Jurgens G, Hwang I (2009) Heat shock protein cognate 70-4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in *Arabidopsis*. Plant Cell 21:3984–4001

131. Lee J, Lee H, Kim J, Lee S, Kim DH, Kim S, Hwang I (2011) Both the hydrophobicity and a positively charged region flanking the C-terminal region of the transmembrane domain of signal-anchored proteins play critical roles in determining their targeting specificity to the endoplasmic reticulum or endosymbiotic organelles in *Arabidopsis* cells. Plant Cell 23:1588–1607
132. Lee DW, Jung C, Hwang I (2013) Cytosolic events involved in chloroplast protein targeting. Biochim Biophys Acta 1833:245–252
133. Leheny EA, Theg SM (1994) Apparent inhibition of chloroplast protein import by cold temperatures is due to energetic considerations not membrane fluidity. Plant Cell 6:427–437
134. Leipe DD, Wolf YI, Koonin EV, Aravind L (2002) Classification and evolution of P-loop GTPases and related ATPases. J Mol Biol 317:41–72
135. Levitan A, Trebitsh T, Kiss V, Pereg Y, Dangoor I, Danon A (2005) Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. Proc Natl Acad Sci U S A 102:6225–6230
136. Li HM, Chiu CC (2010) Protein transport into chloroplasts. Annu Rev Plant Biol 61:157–180
137. Li M, Schnell DJ (2006) Reconstitution of protein targeting to the inner envelope membrane of chloroplasts. J Cell Biol 175:249–259
138. Li HM, Kesavulu MM, Su PH, Yeh YH, Hsiao CD (2007) Toc GTPases. J Biomed Sci 14:505–508
139. Ling Q, Huang W, Baldwin A, Jarvis P (2012) Chloroplast biogenesis is regulated by direct action of the ubiquitin-proteasome system. Science 338:655–659
140. Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15:4230–4238
141. Lübeck J, Heins L, Soll J (1997) A nuclear-coded chloroplastic inner envelope membrane protein uses a soluble sorting intermediate upon import into the organelle. J Cell Biol 137:1279–1286
142. Lung SC, Chuong SD (2012) A transit peptide-like sorting signal at the C terminus directs the *Bienertia sinuspersici* preprotein receptor Toc159 to the chloroplast outer membrane. Plant Cell 24:1560–1578
143. Ma Y, Kouranov A, LaSala SE, Schnell DJ (1996) Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. J Cell Biol 134:315–327
144. Machettira AB, Gross LE, Sommer MS, Weis BL, Englich G, Tripp J, Schleiff E (2011) The localization of Tic20 proteins in *Arabidopsis thaliana* is not restricted to the inner envelope membrane of chloroplasts. Plant Mol Biol 77:381–390
145. Mackenzie SA (2005) Plant organellar protein targeting: a traffic plan still under construction. Trends Cell Biol 15:548–554
146. Martin W (2010) Evolutionary origins of metabolic compartmentalization in eukaryotes. Phil Trans R Soc Lond B 365:847–855
147. Martin T, Sharma R, Sippel C, Waegemann K, Soll J, Vothknecht UC (2006) A protein kinase family in *Arabidopsis* phosphorylates chloroplast precursor proteins. J Biol Chem 281:40216–40223
148. May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12:53–64
149. Miras S, Salvi D, Piette L, Seigneurin-Berny D, Grunwald D, Reinbothe C, Joyard J, Reinbothe S, Rolland N (2007) Toc159- and Toc75-independent import of a transit sequence-less precursor into the inner envelope of chloroplasts. J Biol Chem 282:29482–29492
150. Mitschke J, Fuss J, Blum T, Hoglund A, Reski R, Kohlbacher O, Rensing SA (2009) Prediction of dual protein targeting to plant organelles. New Phytol 183:224–235
151. Moberg P, Stahl A, Bhushan S, Wright SJ, Eriksson A, Bruce BD, Glaser E (2003) Characterization of a novel zinc metalloprotease involved in degrading targeting peptides in mitochondria and chloroplasts. Plant J 36:616–628

152. Nada A, Soll J (2004) Inner envelope protein 32 is imported into chloroplasts by a novel pathway. J Cell Sci 117:3975–3982
153. Nakrieko KA, Mould RM, Smith AG (2004) Fidelity of targeting to chloroplasts is not affected by removal of the phosphorylation site from the transit peptide. Eur J Biochem 271:509–516
154. Nanjo Y et al (2006) Rice plastidial *N*-glycosylated nucleotide pyrophosphatase/phosphodiesterase is transported from the ER-golgi to the chloroplast through the secretory pathway. Plant Cell 18:2582–2592
155. Nassoury N, Morse D (2005) Protein targeting to the chloroplasts of photosynthetic eukaryotes: getting there is half the fun. Biochim Biophys Acta 1743:5–19
156. Neupert W, Brunner M (2002) The protein import motor of mitochondria. Nat Rev Mol Cell Biol 3:555–565
157. Neupert W, Herrmann JM (2007) Translocation of proteins into mitochondria. Annu Rev Biochem 76:723–749
158. Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16:935–946
159. Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J Biol Chem 267:433–439
160. Pain D, Blobel G (1987) Protein import into chloroplasts requires a chloroplast ATPase. Proc Natl Acad Sci U S A 84:3288–3292
161. Patel R, Hsu S, Bédard J, Inoue K, Jarvis P (2008) The Omp85-related chloroplast outer envelope protein OEP80 is essential for viability in *Arabidopsis*. Plant Physiol 148:235–245
162. Peeters N, Small I (2001) Dual targeting to mitochondria and chloroplasts. Biochim Biophys Acta 1541:54–63
163. Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6:93–105
164. Pujol C, Marechal-Drouard L, Duchene AM (2007) How can organellar protein N-terminal sequences be dual targeting signals? In silico analysis and mutagenesis approach. J Mol Biol 369:356–367
165. Qbadou S, Tien R, Soll J, Schleiff E (2003) Membrane insertion of the chloroplast outer envelope protein, Toc34: constrains for insertion and topology. J Cell Sci 116:837–846
166. Qbadou S, Becker T, Mirus O, Tews I, Soll J, Schleiff E (2006) The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. EMBO J 25:1836–1847
167. Qbadou S, Becker T, Bionda T, Reger K, Ruprecht M, Soll J, Schleiff E (2007) Toc64- a preprotein-receptor at the outer membrane with bipartide function. J Mol Biol 367:1330–1346
168. Radhamony RN, Theg SM (2006) Evidence for an ER to Golgi to chloroplast protein transport pathway. Trends Cell Biol 16:385–387
169. Rapaport D (2005) How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane? J Cell Biol 171:419–423
170. Reumann S, Keegstra K (1999) The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. Trends Plant Sci 4:302–307
171. Richter S, Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. Proc Natl Acad Sci U S A 95:7463–7468
172. Richter S, Lamppa GK (2002) Determinants for removal and degradation of transit peptides of chloroplast precursor proteins. J Biol Chem 277:43888–43894
173. Richter S, Lamppa GK (2003) Structural properties of the chloroplast stromal processing peptidase required for its function in transit peptide removal. J Biol Chem 278: 39497–39502
174. Richter S, Zhong R, Lamppa G (2005) Function of the stromal processing peptidase in the chloroplast import pathway. Physiol Plant 123:362–368
175. Rudhe C, Clifton R, Chew O, Zemam K, Richter S, Lamppa G, Whelan J, Glaser E (2004) Processing of the dual targeted precursor protein of glutathione reductase in mitochondria and chloroplasts. J Mol Biol 343:639–647

176. Rudolf M et al (2012) In vivo function of Tic22, a protein import component of the intermembrane space of chloroplasts. Mol Plant 6:817–829
177. Sapir-Mir M, Mett A, Belausov E, Tal-Meshulam S, Frydman A, Gidoni D, Eyal Y (2008) Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. Plant Physiol 148:1219–1228
178. Sato S, Nakamura Y, Kaneko T, Asamizu E, Tabata S (1999) Complete structure of the chloroplast genome of *Arabidopsis thaliana*. DNA Res 6:283–290
179. Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21:289–296
180. Schleiff E, Jelic M, Soll J (2003) A GTP-driven motor moves proteins across the outer envelope of chloroplasts. Proc Natl Acad Sci U S A 100:4604–4609
181. Schleiff E, Soll J, Küchler M, Kuhlbrandt W, Harrer R (2003) Characterization of the translocon of the outer envelope of chloroplasts. J Cell Biol 160:541–551
182. Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266:1007–1012
183. Schnell DJ, Blobel G, Keegstra K, Kessler F, Ko K, Soll J (1997) A consensus nomenclature for the protein-import components of the chloroplast envelope. Trends Cell Biol 7:303–304
184. Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. Plant Cell 7:1713–1722
185. Shen G, Kuppu S, Venkataramani S, Wang J, Yan J, Qiu X, Zhang H (2010) ANKYRIN REPEAT-CONTAINING PROTEIN 2A is an essential molecular chaperone for peroxisomal membrane-bound ASCORBATE PEROXIDASE3 in *Arabidopsis*. Plant Cell 22:811–831
186. Shi LX, Theg SM (2010) A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella patens*. Plant Cell 22:205–220
187. Shi LX, Theg SM (2013) The chloroplast protein import system: from algae to trees. Biochim Biophys Acta 1833: 314–331
188. Shipman RL, Inoue K (2009) Suborganellar localization of plastidic type I signal peptidase 1 depends on chloroplast development. FEBS Lett 583:938–942
189. Shipman-Roston RL, Ruppel NJ, Damoc C, Phinney BS, Inoue K (2010) The significance of protein maturation by plastidic type I signal peptidase 1 for thylakoid development in *Arabidopsis* chloroplasts. Plant Physiol 152:1297–1308
190. Silva-Filho MC (2003) One ticket for multiple destinations: dual targeting of proteins to distinct subcellular locations. Curr Opin Plant Biol 6:589–595
191. Sjögren LL, MacDonald TM, Sutinen S, Clarke AK (2004) Inactivation of the *clpC1* gene encoding a chloroplast Hsp100 molecular chaperone causes growth retardation, leaf chlorosis, lower photosynthetic activity, and a specific reduction in photosystem content. Plant Physiol 136:4114–4126
192. Skalitzky CA, Martin JR, Harwood JH, Beirne JJ, Adamczyk BJ, Heck GR, Cline K, Fernandez DE (2011) Plastids contain a second sec translocase system with essential functions. Plant Physiol 155:354–369
193. Smith MD, Hiltbrunner A, Kessler F, Schnell DJ (2002) The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP. J Cell Biol 159:833–843
194. Stahl T, Glockmann C, Soll J, Heins L (1999) Tic40, a new "old" subunit of the chloroplast protein import translocon. J Biol Chem 274:37467–37472
195. Stengel A, Benz P, Balsera M, Soll J, Bölter B (2008) Tic62- redox-regulated translocon composition and dynamics. J Biol Chem 283:6656–6667
196. Su PH, Li HM (2008) *Arabidopsis* stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. Plant Physiol 146:1231–1241
197. Su PH, Li HM (2010) Stromal Hsp70 is important for protein translocation into pea and *Arabidopsis* chloroplasts. Plant Cell 22:1516–1531

198. Sun Q, Zybailov B, Majeran W, Friso G, Olinares PD, van Wijk KJ (2009) PPDB, the plant proteomics database at Cornell. Nucleic Acids Res 37:D969–D974
199. Sun F, Carrie C, Law S, Murcha MW, Zhang R, Law YS, Suen PK, Whelan J, Lim BL (2012) AtPAP2 is a tail-anchored protein in the outer membrane of chloroplasts and mitochondria. Plant Signal Behav 7:927–932
200. Sveshnikova N, Soll J, Schleiff E (2000) Toc34 is a preprotein receptor regulated by GTP and phosphorylation. Proc Natl Acad Sci U S A 97:4973–4978
201. Teixeira PF, Glaser E (2013) Processing peptidases in mitochondria and chloroplasts. Biochim Biophys Acta 1833:360–370
202. Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J Biol Chem 264:6730–6736
203. Teng YS, Su YS, Chen LJ, Lee YJ, Hwang I, Li HM (2006) Tic21 is an essential translocon component for protein translocation across the chloroplast inner envelope membrane. Plant Cell 18:2247–2257
204. Teng YS, Chan PT, Li HM (2012) Differential age-dependent import regulation by signal peptides. PLoS Biol 10:e1001416
205. Tommassen J (2010) Assembly of outer-membrane proteins in bacteria and mitochondria. Microbiology 156:2587–2596
206. Töpel M, Jarvis P (2011) The Tic20 gene family: phylogenetic analysis and evolutionary considerations. Plant Signal Behav 6:1046–1048
207. Töpel M, Ling Q, Jarvis P (2012) Neofunctionalization within the Omp85 protein superfamily during chloroplast evolution. Plant Signal Behav 7:161–164
208. Tranel PJ, Keegstra K (1996) A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell 8:2093–2104
209. Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14:2436–2446
210. Tripp J, Inoue K, Keegstra K, Froehlich JE (2007) A novel serine/proline-rich domain in combination with a transmembrane domain is required for the insertion of AtTic40 into the inner envelope membrane of chloroplasts. Plant J 52:824–838
211. Tripp J et al (2012) Structure and conservation of the periplasmic targeting factor Tic22 protein from plants and cyanobacteria. J Biol Chem 287:24164–24173
212. Trösch R, Jarvis P (2011) The stromal processing peptidase of chloroplasts is essential in *Arabidopsis*, with knockout mutations causing embryo arrest after the 16-cell stage. PLoS ONE 6:e23039
213. Tsai LY, Tu SL, Li HM (1999) Insertion of atToc34 into the chloroplastic outer membrane is assisted by at least two proteinaceous components in the import system. J Biol Chem 274:18735–18740
214. Tu SL, Li HM (2000) Insertion of OEP14 into the outer envelope membrane is mediated by proteinaceous components of chloroplasts. Plant Cell 12:1951–1960
215. Tu SL, Chen LJ, Smith MD, Su YS, Schnell DJ, Li HM (2004) Import pathways of chloroplast interior proteins and the outer-membrane protein OEP14 converge at Toc75. Plant Cell 16:2078–2088
216. Ueda M, Nishikawa T, Fujimoto M, Takanashi H, Arimura S, Tsutsumi N, Kadowaki K (2008) Substitution of the gene for chloroplast RPS16 was assisted by generation of a dual targeting signal. Mol Biol Evol 25:1566–1575
217. VanderVere PS, Bennett TM, Oblong JE, Lamppa GK (1995) A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. Proc Natl Acad Sci U S A 92:7177–7181
218. Viana AA, Li M, Schnell DJ (2010) Determinants for stop-transfer and post-import pathways for protein targeting to the chloroplast inner envelope membrane. J Biol Chem 285:12948–12960
219. Villarejo A et al (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nat Cell Biol 7:1124–1131

220. Vojta A, Alavi M, Becker T, Hörmann F, Küchler M, Soll J, Thomson R, Schleiff E (2004) The protein translocon of the plastid envelopes. J Biol Chem 279:21401–21405
221. Vojta L, Soll J, Bölter B (2007) Protein transport in chloroplasts—targeting to the intermembrane space. FEBS J 274:5043–5054
222. Vojta L, Soll J, Bölter B (2007) Requirements for a conservative protein translocation pathway in chloroplasts. FEBS Lett 581:2621–2624
223. von Heijne G, Nishikawa K (1991) Chloroplast transit peptides. The perfect random coil? FEBS Lett 278:1–3
224. Wallas TR, Smith MD, Sanchez-Nieto S, Schnell DJ (2003) The roles of Toc34 and Toc75 in targeting the Toc159 preprotein receptor to chloroplasts. J Biol Chem 278:44289–44297
225. Wan J, Bringloe D, Lamppa GK (1998) Disruption of chloroplast biogenesis and plant development upon down-regulation of a chloroplast processing enzyme involved in the import pathway. Plant J 15:459–468
226. Wang F, Agne B, Kessler F, Schnell DJ (2008) The role of GTP binding and hydrolysis at the atToc159 preprotein receptor during protein import into chloroplasts. J Cell Biol 183:87–99
227. Whatley JM, McLean B, Juniper BE (1991) Continuity of chloroplast and endoplasmic reticulum membranes in *Phaseolus vulgaris*. New Phytol 117:209–217
228. Young ME, Keegstra K, Froehlich JE (1999) GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. Plant Physiol 121:237–244
229. Yue R, Wang X, Chen J, Ma X, Zhang H, Mao C, Wu P (2010) A rice stromal processing peptidase regulates chloroplast and root development. Plant Cell Physiol 51:475–485
230. Zhang XP, Glaser E (2002) Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. Trends Plant Sci 7:14–21
231. Zhang H, Li X, Zhang Y, Kuppu S, Shen G (2010) Is AKR2A an essential molecular chaperone for a class of membrane-bound proteins in plants? Plant Signal Behav 5:1520–1522
232. Zhong R, Wan J, Jin R, Lamppa G (2003) A pea antisense gene for the chloroplast stromal processing peptidase yields seedling lethals in *Arabidopsis*: survivors show defective GFP import *in vivo*. Plant J 34:802–812