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Review

Functions of plastid protein import and the ubiquitin–proteasome system in plastid development☆

Qihua Ling, Paul Jarvis *

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

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ABSTRACT

Plastids, such as chloroplasts, are widely distributed endosymbiotic organelles in plants and algae. Apart from their well-known functions in photosynthesis, they have roles in processes as diverse as signal sensing, fruit ripening, and seed development. As most plastid proteins are produced in the cytosol, plastids have developed dedicated translocon machineries for protein import, comprising the TOC (translocon at the outer envelope membrane of chloroplasts) and TIC (translocon at the inner envelope membrane of chloroplasts) complexes. Multiple lines of evidence reveal that protein import *via* the TOC complex is actively regulated, based on the specific interplay between distinct receptor isoforms and diverse client proteins. In this review, we summarize recent advances in our understanding of protein import regulation, particularly in relation to control by the ubiquitin–proteasome system (UPS), and how such regulation changes plastid development. The diversity of plastid import receptors (and of corresponding preprotein substrates) has a determining role in plastid differentiation and interconversion. The controllable turnover of TOC components by the UPS influences the developmental fate of plastids, which is fundamentally linked to plant development. Understanding the mechanisms by which plastid protein import is controlled is critical to the development of breakthrough approaches to increase the yield, quality and stress tolerance of important crop plants, which are highly dependent on plastid development. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

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1. Introduction: plastids and protein import

Plastids are a group of related organelles existing extensively throughout plants and a variety of algae [1,2]. Among them, chloroplasts in the green tissues of plants and algae attract most attention and are best studied, due to their well-known ability to photosynthetically convert the energy of light into chemical bond energy. Besides, chloroplasts are actually also responsible for many important biosynthetic processes [3,4]. Other plastid types are widely distributed in non-green plant tissues, including the chromoplasts, which are rich in carotenoid pigments and serve to attract animals to fruits and flowers, and the amyloplasts, which are largely made up of starch and play important roles in energy storage in seeds and tubers as well as in plant gravitropism [3,5,6].

One remarkable feature of plastids is their dynamism in relation to morphology and function. In response to developmental or environmental signals, different plastid types can interconvert and such conversion plays an import role in plant development, for example, during fruit ripening (when chloroplasts change to chromoplasts) and senescence (when chloroplasts change to gerontoplasts) [6]. Evidence indicates that such dynamic plastid development is regulated, at least in part, through protein import, particularly at the TOC complex (discussed in detail below), and by the ubiquitin–proteasome system (UPS); these issues will be discussed in this review. In addition, plastids are also well known for their ability to move and redistribute inside the cell [7]. The motility of these organelles is a critical response enabling them to deal with the environmental changes. For example, the movements of chloroplasts and amyloplasts function in strong-light avoidance and gravity sensing, respectively.

Like mitochondria, chloroplasts are endosymbiotic organelles. They are thought to have originated from an ancient photosynthetic prokaryote which is an ancestor of present-day cyanobacteria [8,9]. During evolution, the endogenous gene expression system in the organelle was retained, whereas the size of the organellar genome was largely reduced such that it now expresses only ~100 different proteins [10,11]. Correspondingly, chloroplasts have developed sophisticated mechanisms to import proteins from the cytosol.

Generally speaking, chloroplast import shares many similarities with mitochondrial protein import. Although both organelles contain their own genome, and can express some of their own proteins, the vast majority of the organellar proteins are imported post-translationally from

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* Corresponding author.
E-mail address: paul.jarvis@plants.ox.ac.uk (P. Jarvis).

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the cytosol. For chloroplasts, over 90% of the 3000 organellar proteins are encoded in the nucleus and synthesized by cytosolic ribosomes [12,13]. Similar to mitochondrial proteins, most chloroplast proteins are made as precursors, having a cleavable targeting sequence at the amino-terminal end called a transit peptide. The precursors are imported into chloroplasts through the interaction of the transit peptide with two translocons located in the outer and inner envelope membranes called TOC and TIC (*t*ranslocon at the *o*uter/*i*nner envelope membrane of *c*hloroplasts), respectively [14–21]. Their counterparts in mitochondrial protein import system are the TOM and TIM (*t*ranslocase of the *o*uter/*i*nner *m*itochondrial membrane) complexes [22–24]. Although protein import is broadly similar between chloroplasts and mitochondria, the main constituents of the respective import machineries do not share obvious homology.

The precursor initially interacts with the chloroplast *via* its transit peptide at the TOC complex, and then later it passes through the TIC complex. Upon emergence from the TIC complex, the transit peptide is cleaved and the mature protein domain assumes its native conformation or is further sorted to its destination *via* internal sorting pathways [18,19,25–28]. As mentioned above, recent studies have found that the regulation of protein import through the TOC complex contributes significantly to plastid development. Consequently, here we focus mostly on details pertaining to the TOC complex, and note that the TIC complex and its regulation have been comprehensively reviewed elsewhere [18,29]. The main components of the TOC complexes are discussed in detail in the following sections, while a detailed list of the components is provided in Table 2 of Jarvis [18].

2. Import at the outer membrane

2.1. Overview of components at the outer membrane

Identification of the main envelope components involved in chloroplast protein import occurred about two decades ago. Proteins of the TOC and TIC complexes were identified through extensive biochemical studies conducted using isolated *Pisum sativum* (pea) chloroplasts by researchers from independent laboratories [30–36]. The initially identified proteins included three from the TOC complex and one from the TIC complex, and were Toc34, Toc159, Toc75, and Tic110, named by their molecular weights [37].

All these proteins are integral membrane proteins. The three outer membrane proteins form the core TOC complex, with Toc159 and Toc34 being GTPase-regulated receptors and Toc75 forming a protein-conducting channel. Using artificial lipid vesicles reconstituted with these three proteins *in vitro*, it was shown that a rebuilt TOC complex indeed has the ability to bind precursors and to drive their translocation through the membrane [38].

The core TOC complex particle was investigated by electron microscopic analysis and estimated to have a height of 10–12 nm and a diameter of 13 nm [39]. The structure was also elucidated to possess a central cavity surrounded by a thick ring wall, and a finger-like structure in the centre which divides the central space into four pore-like domains [39]. It was speculated that one Toc159 molecule formed the central finger-like structure and each pore-like domain is constituted by one Toc34 molecule and one Toc75 molecule; this agreed with the proposed stoichiometry of Toc34, Toc75 and Toc159 as discussed below. Further studies using methods like gel filtration, density gradient centrifugation, and blue native PAGE confirmed that the TOC core complex consists of Toc159, Toc34, and Toc75, and indicated that its size was between 500 kD and 1 MD, in pea and *Arabidopsis* [39–41]. Moreover, it was reported that the stoichiometry of the TOC complex components was 4–5:4:1 [39] or 3:3:1 [41], between Toc34, Toc75 and Toc159, respectively. Differences between these stoichiometric estimates may be due to use of different analytical techniques, the proteolysis of Toc159 (in the ~500 kD complex, Toc159 was present as the 86 kD degraded fragment) [39], dynamic features of complex composition [42], the presence of additional, unidentified TOC components, or the formation of a TOC complex superdimer of 800–1000 kD [41]. Thus, the precise composition of the TOC complex remains elusive.

Pea plants contributed greatly to the identification of the major components of the TOC complex. However, pea is not ideal for studying the *in vivo* functions of individual TOC proteins. The use of *Arabidopsis* as a model plant allowed such studies to be performed [43–45], which gave rise to a more comprehensive understanding of the mechanisms of protein import and their functions in plastid and plant development.

2.2. The receptor proteins

The initial events of chloroplast preprotein import occur at the receptors in the outer membrane, which are the Toc159 and Toc34 proteins. The receptors are both membrane-embedded *via* a C-terminal anchor, and both also contain a homologous GTP-binding domain protruding into the cytosol. Both of them have the ability to recognize and bind precursors directly [30,46,47]. Interestingly, genetic analyses indicate that they are both encoded by a small gene family in higher plants [18,48,49] (see below).

2.2.1. Toc159 gene family

Toc159 and Toc34 were first recognized by their association with precursors in isolated pea chloroplasts [31,32]. Between them, Toc159 is more complex in structure. It contains three domains, including a large acidic (A) domain at the N-terminus and a central GTPase (G) domain, both of which are cytosolic, as well as a large C-terminal membrane (M) domain embedded in the outer membrane. Interestingly, unlike other membrane-spanning protein domains, the Toc159 M-domain is not hydrophobic and lacks a clear transmembrane helix. At first, Toc159 was reported to be able to shuttle between the chloroplast membrane and the cytosol [50,51], which led to the hypothesis that it can serve as a receptor to bind cytosolic precursors and introduce them to the chloroplast envelope. However, later investigation challenged the existence of this soluble form, as it has been reported that under higher-speed centrifugation Toc159 is only found in the membrane fraction but not in the soluble fraction [42], and that the soluble form may in fact represent the free A-domain, as discussed below [52,53].

The A-domain was identified when it was recognized that the initial experiments had described an 86 kD fragment lacking the entire A-domain, which indicated that the A-domain is extremely unstable [33,54]. The significance of the A-domain is unclear. It has been shown that isolated chloroplasts in which Toc159 A-domain has been proteolysed import preproteins less efficiently compared with those with intact Toc159 [54], suggesting that the A-domain plays a role in the import process. However, the Toc159 knockout mutant (*plastid protein import 2*, *ppi2*) phenotype in *Arabidopsis thaliana* can be entirely complemented by truncated protein lacking the A-domain [52,53,55], indicating that the A-domain might have only an accessory function *in vivo*. Recently it has been shown that the A-domain can exist in the cytosol in a highly-phosphorylated form, free from the other Toc159 domains, although the biological significance of this free A-domain has yet to be elucidated [52].

The topology of Toc159 was investigated by protease treatment using isolated chloroplasts, which can digest the protein part exposed in the cytosol. A 52 kD M-domain fragment was identified after such treatment, indicating that the M-domain is rooted in the membrane and that the A- and G-domains are exposed to the cytosol [32,33,56]. It has been suggested that the M-domain is the minimal domain required for protein import. Similar to the A-domain-lacking Toc159 protein, the M-domain alone is capable of complementing the mutant phenotype of *ppi2* plants, albeit only partially [55]. In addition, *in vivo* import assays using transiently expressed protoplasts of *ppi2* plants showed that the import defect was also recovered by M-domain expression. Besides, *in vitro* import experiments using isolated chloroplasts

indicated that preprotein import remained efficient even when just the 52 kD M-domain of Toc159 was retained after proteolysis [56].

The fully-sequenced *Arabidopsis* genome shows that there are four homologs of Toc159, named atToc159, atToc132, atToc120 and atToc90 (note that the "at" prefix denotes species of origin) [44,51,57]. The G- and M-domains of the different isoforms show high similarity, but the A-domains differ largely in sequence. Among them, atToc159 has the biggest A-domain and atToc90 has only a truncated A-domain, while the A-domains of atToc132 and atToc120 are more similar to each other than to that of atToc159 [51]. The functions of the Toc159 family *in vivo* have been studied in detail, confirming that these factors are indeed critical for chloroplast protein import. Moreover, the mutant phenotypes indicate that the receptors can be divided into sub-families functionally, with preferences for different classes of preproteins (discussed in detail in Section 2.4).

In *Arabidopsis*, atToc159 is the predominant isoform of the Toc159 family. Its mutant, *ppi2*, appears albino due to strongly disrupted chloroplast development, and it has the most severe visible phenotype among the single mutants of the Toc159 family [44,57]. In contrast, mutation of atToc132 or atToc120 causes only a weak phenotype or no mutant phenotype at all, respectively. However, double mutants lacking the latter two components exhibit a severe albino phenotype comparable to that of *ppi2*, indicating highly redundant roles of these two proteins in chloroplast biogenesis [51,57]. The overexpression of atToc132 cannot recover the defect of *ppi2* plants, which suggests that the functions of atToc159 and atToc132/120 are divergent [51]; domain swapping assays indicated that the different functions of atToc159 and atToc132 are largely dependent on their A-domains, as discussed in detail in Section 2.4 [58]. By contrast, it seems that atToc90 shares overlapping function with atToc159; whereas the knockout mutant of atToc90 alone does not cause any obvious phenotypes [57,59], *toc90* knockout mutations can slightly accentuate the phenotype of *ppi2*, and the overexpression of atToc90 can partially rescue the phenotype of *ppi2* [59,60]. An interesting question is whether atToc90 also shares such functional similarity with atToc132/120.

2.2.2. Toc34 gene family

Like Toc159, Toc34 is also regarded as a receptor, but it has a simpler structure. Toc34 is anchored in the outer membrane by a very short, C-terminal transmembrane helix, and the rest of the protein contains a GTPase domain facing the cytosol, which shares about 30% identity with that of Toc159. In general, GTPases can be grouped into two subclasses: conventional GTPases, and GTPases activated by nucleotide-dependent dimerization (GADs) [61]. If the mode of action of Toc34 and Toc159 is similar to conventional GTPase systems, there should be some accessory or regulatory partners, such as GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). These proteins stimulate GTP hydrolysis and replacement of GDP with GTP, respectively, and thus act to switch the activity of GTPase proteins [62]. However, to date, such factors have proved elusive in relation to the chloroplast import system.

Alternatively, Toc34 might belong to the GAD class of GTPases, which includes the signal recognition particle (SRP) and its receptor of the ER translocation system [61,63]. This was suggested by filtration and pull-down assays which indicated that Toc34 can form homodimers through its GTPase domain [64–66]. The possibility was further supported by protein structure analyses using X-ray crystallography performed on *P. sativum* Toc34 and atToc33 (the main Toc34 homologue in *Arabidopsis*) [64,67]. The structural data not only showed that there is a possible precursor-binding cavity inside the Toc34 protein, but also indicated that the GTPase domain can act as an interaction site for homodimerization. These results suggested that each single Toc34 molecule within a dimer might activate the interacting monomer [64].

However, assessments of the consequences of TOC receptor dimerization based on *in vitro* biochemical analysis of an atToc33-R130A mutant protein (intended to abolish dimer formation by disrupting the conserved dimerization motif revealed by the crystal structure analysis), have led to contradictory results [65,66]. Although both Weibel *et al.* [65] and Yeh *et al.* [66] confirmed that the mutation can abrogate dimer formation, the former study did not find that it changed the efficiency of GTP hydrolysis, while the latter study observed significantly reduced GTPase activity. Later, Koenig *et al.* [67] observed a minor GTPase activation upon dimerization, which led to the suggestion that an additional factor such as a co-GAP is needed for homodimerization. However, a more recent report overthrew the previous opinions and suggested that Toc34 homodimerization actually does not stimulate hydrolysis, but instead limits the nucleotide exchange rate. It was found that it is the disruption of the dimer that promotes GDP-GTP exchange, which is triggered by preprotein binding [68,69]. This finding also affects the previous opinion that transit peptides perform a GAP function [70], which was suggested by the finding that preprotein binding can strongly activate GTP hydrolysis [71,72].

Such inconsistencies also happen when assessing the function of dimerization *in vivo*. Although chloroplasts isolated from plants expressing dimerization-defective atToc33 point mutants were shown to be defective in preprotein translocation, specifically at the initial stage [73], such mutations do not obviously influence chloroplast development *in planta* and plant growth [74]. Thus, the exact function of Toc34 dimerization needs to be further investigated. Interestingly, both *in vivo* and *in vitro* experiments also detected the interaction of Toc34 and Toc159, suggesting that these two receptors can form heterodimers, possibly through the interaction of the homologous GTPase domains [74–78]. This suggested the possibility that the existence of one fully-functional GTPase domain in either receptor is sufficient to fulfil the requirement of normal plant development.

In *Arabidopsis*, two Toc34-type proteins exist: atToc33 and atToc34. The sequences of these two proteins are similar, but they do show significant divergence at their C-terminal ends. In terms of expression levels, in general atToc33 is the major isoform. Detailed *in vivo* analysis of Toc34 function has been conducted with *Arabidopsis* mutants, confirming its essential role in plastid import. The analysis of an atToc33 loss-of-function mutant, *ppi1*, for the first time illustrated that a core translocon component is as important *in vivo* as was suggested by biochemical approaches [43]. The *ppi1* mutant shows a clear defect in chloroplast protein import, as indicated by the strikingly reduced chlorophyll level, defective chloroplast ultrastructure, and, more directly, the reduced protein import efficiency *in vitro* [43,79]. By contrast, *ppi3*, the mutant of the minor isoform, atToc34, looks normal in shoot parts, although its root development is compromised [80]. Based on the differences in their mutant phenotypes, it is also proposed that atToc33 is more involved in the import of precursors of the photosynthetic apparatus (so-called photosynthetic preproteins), whereas atToc34 is mainly responsible for non-photosynthetic preprotein import [81] (Fig. 1; discussed in detail in Section 2.4). However, such preference is not absolute, and it is also clear that atToc33 and atToc34 share overlapping functions. This is indicated not only by their high similarity in protein sequence (nearly 80% similarity in amino acids), but also by genetic evidence: that is, that the *ppi1* phenotype can be complemented by the overexpression of atToc34 [43], and that the double knock-out of these two genes causes embryonic lethality [80,82].

2.3. The channel protein and other TOC components

Toc75 is believed to form the channel of the TOC complex [31,83,84], and it also shows ability to bind transit peptides directly [85]. Toc75 is a typical β-barrel protein that is deeply buried within the outer membrane. It belongs to the conserved Omp85 (*o*uter *m*embrane *p*rotein *85*) superfamily of proteins, whose members occur widely in gram-negative bacteria and the mitochondria of eukaryotes [86–88]. There is another Omp85-related protein in the chloroplast outer envelope membrane, termed OEP80 (*o*uter *e*nvelope *p*rotein, 80 kD) [89,90].

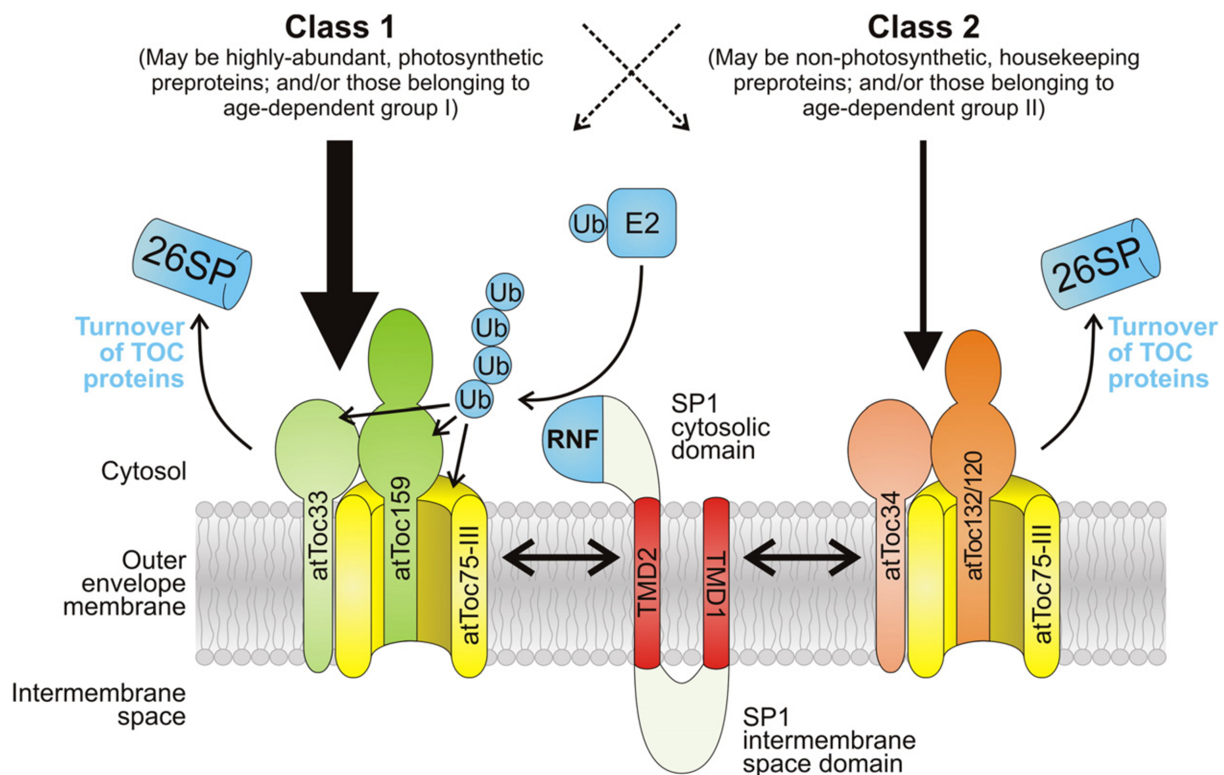

Fig. 1. Model for the operation of substrate-specific plastid protein import pathways and their regulation by the SP1 ubiquitin E3 ligase. In *Arabidopsis* (and indeed in other plants where genome sequence information is available), components of the TOC apparatus occur in different isoforms (*e.g.*, atToc33 and atToc34), enabling the formation of different TOC complexes with non-identical specificities for precursor protein substrates. The main TOC component isoforms (those that predominate in chloroplasts) form complexes with preference for precursors of the photosynthetic apparatus, or preproteins belonging to age-dependent group I [116]; whereas other TOC isoforms form complexes with preference for housekeeping precursors, or preproteins belonging to age-dependent group II [116]. Dotted crossing arrows indicate that these client preferences may not be strict. The balance between these import pathways controls the composition of the organellar proteome, and in turn this controls the developmental fate and functions of the organelle. The SP1 E3 ligase is inserted in the plastid outer envelope membrane by two transmembrane domains (TMD1, TMD2). Its RING finger (RNF) domain faces the cytosol where it recruits E2 ubiquitin (Ub) conjugating enzyme in order to mediate ubiquitination of TOC components, which are recognized by the SP1 intermembrane-space domain. Ubiquitinated TOC proteins are then degraded by the cytosolic 26S proteasome (26SP). The SP1 protein plays a critical role in controlling the balance between the different substrate-specific protein import pathways, by facilitating the turnover of TOC components and their replacement with alternative isoforms, leading to the reorganization of the import machinery. This figure is adapted from Ling *et al.* [112].

Both of these chloroplast proteins, as typical Omp85 superfamily members, contain two parts in structure: an N-terminal domain with three POTRA (*po*lypeptide *tr*ansport *a*ssociated) repeats, and a C-terminal β-barrel domain as described previously [86,87,91,92]. While the β-barrel domain of Toc75 acts in channel formation [85,86], the POTRA domain may be responsible for the binding of TOC receptors and/or preproteins [86]. On the other hand, OEP80 is not part of the TOC complex, and may be responsible for the biogenesis of β-barrel proteins (*e.g.*, Toc75) in the outer membrane, with a similar function to its bacterial and mitochondrial counterparts, BamA and Sam50/Tob55 [89,93–95]. However, the precise acting mechanism of OEP80 still remains to be determined.

Unlike the receptors, the channel protein Toc75 in *Arabidopsis* is encoded by a single gene: *atTOC75-III*. Knockout mutations affecting *atTOC75-III* result in a severe embryo lethality phenotype in *Arabidopsis*, as plastid import is critical for early embryo development [82,96]. The significance of Toc75 in chloroplast development was demonstrated by the knockdown of *atTOC75-III* expression by RNA interference (RNAi) and a hypomorphic *toc75-III* mutant allele, both of which lead to reduced chlorophyll content in plants [94,97].

In *Arabidopsis*, OEP80 is also encoded by a single gene. And its necessity *in planta* is revealed by *oep80* knockout mutants, which are embryo-lethal [98]. Similar to the *atTOC75-III* RNAi lines, equivalent knockdown lines of *AtOEP80* showed a chlorotic phenotype, albeit with reduced severity by comparison with the *atTOC75-III* lines [94]. Notably, it was shown that the protein level of Toc75 is specifically down-regulated in the AtOEP80 RNAi plants, which agrees with OEP80's proposed function in the insertion of Toc75 protein into the outer membrane.

Two other proteins, Toc12 and Toc64, were later identified as putative new components of the TOC complex. Toc12 was at first described as a DnaJ-like protein docked in the outer membrane with a large soluble part located in the intermembrane space [99]. However, its existence in the envelope is disputed after the finding that the originally identified Toc12 protein in pea is actually a truncated stromal DnaJ-J8 protein with function that is not involved in protein import [100].

Toc64 was first identified in the isolated pea TOC complex, and was assumed to be loosely associated with other TOC components [39,101]. A structurally similar protein called Tom70 is found in mitochondria, and is known to be involved in mitochondrial protein import [102]. However, the role of Toc64 in chloroplast protein import is debatable. *In vitro* biochemical studies indicated that Toc64 might act as a receptor for preproteins delivered by cytosolic factors, and might also assist preprotein translocation [103]. However, the significance of Toc64 in protein import is not evidenced by genetic experiments, as mutations affecting *Arabidopsis* Toc64 homologues do not lead to any obvious defects, under various conditions [104], strongly suggesting that Toc64 is not essential for protein import *in vivo*. Similar results were also observed for Toc64 knockout mutations in moss [105]. A possible scenario is that Toc64 plays an accessory role in chloroplast import and only shows significance under certain circumstances.

2.4. Diverse receptors and preproteins in plastid development

As stated previously (in Section 2.2), one of the outstanding features of the TOC receptors is their diversity, which is in contrast to the Toc75 channel protein and most TIC components. Correspondingly, preproteins

also show clear diversity, ranging from photosynthetic preproteins to non-photosynthetic or housekeeping preproteins, with the former being much more important in chloroplasts than other types of plastids. Therefore, when different receptors were recognized initially, it was hypothesised that such diversity may contribute to the different protein import requirements of different types of plastids [43]. Such specificity was realized following the discovery that photosynthetic proteins are preferentially imported into chloroplasts, whereas chloroplasts and non-green plastids are similarly receptive to non-photosynthetic proteins [106,107]. Another potential explanation for the diversity of receptors is the need to accommodate the differing import requirements of plastids at different stages of plant development (see Section 4) [116]. The relevant study also showed that preproteins which follow a common pathway can compete with each other, and so receptor diversity may be a mechanism to avoid such inefficiencies. Overall, a consensus exists that the different TOC receptors enable an important regulatory mechanism, mediated through the import of specific preprotein substrates, and this is supported by an accumulating amount of evidence.

Genetic evidence from *Arabidopsis* first established the concept of the existence of two groups of receptors: one including atToc159 and atToc33, responsible for the import of photosynthetic preproteins; and another including atToc132/120 and atToc34, responsible for the import of non-photosynthetic, housekeeping preproteins [43,44,51,57,80,81]. This is clearly illustrated by the mutant phenotypes: *ppi2* (*toc159*) and *ppi1* (*toc33*) mutants exhibit strong defects in chloroplasts as shown by albino or chlorotic leaf phenotypes [43,57,81,108]; however, they only have mild defects in root plastids, whereas the root developmental defects are more pronounced in *toc132 toc120* and *ppi3* (*toc34*) mutants [57,80,108]. The finding that atToc132 plays a role in root gravitropism further emphasizes its importance in root plastid development [97].

Such specificity of the receptors was also demonstrated more directly by *in vitro* import assays that assessed the uptake efficiencies of different substrates by chloroplasts of the TOC receptor mutants. For example, *in vitro* import assays using isolated *ppi1* chloroplasts showed that import rates of photosynthetic preproteins are specifically reduced compared with those of a non-photosynthetic preprotein [81]. In addition, *in vivo* import studies through protoplast transient expression similarly indicated the selective targeting of preproteins *via* atToc159 [47]. Such receptor-specific import consequently influences the proteome and transcriptome in the corresponding receptor mutants. In general, the expression of photosynthetic genes/proteins is markedly reduced in *ppi2* (*toc159*) and *ppi1* (*toc33*), while the expression of non-photosynthetic genes/proteins is relatively stable [44,57,81,109]. The reduced protein levels could be directly due to the defective import of photosynthetic proteins in the mutants, while the changed proteome may further prevent the futile expression of genes (which might otherwise exceed the import capacity of the plastids) through plastid-to-nucleus signalling pathways. By contrast, the channel protein of the TOC complex, Toc75, does not show specificity in the import of photosynthetic or non-photosynthetic preproteins, as judged by different aspects of its mutant analyses [94, 97].

Receptor specificity is also suggested by the corresponding gene expression patterns. Although atToc159 and atToc33 are overall the dominant isoforms in their families, their expression proportions are variable in different tissues. For example, the expression levels of atToc159 and atToc33 are strikingly high in leaves, while atToc132, atToc120 and atToc34 tend to be expressed more in roots [81,110]. These tissue-specific expression patterns cope well with the hypothesis that the functions of receptors are substrate specific, and also signify that there is a huge requirement for the import of highly-abundant photosynthetic proteins in leaf chloroplasts. Furthermore, the tissue specific organization of receptors might actually reflect plastid type specificity. Because the whole set of plastid-encoded genes used for establishing all plastids is the same in different plastids within a particular organism, it is believed that imported proteins are responsible for controlling the functions and developmental fate of each organelle [111]. The decisive role of distinct receptors in plastid differentiation was underlined by an investigation of relative receptor levels during plastid transitions; more specifically, the *in vivo* importance of the reorganisation of Toc159 family proteins for the transition from etioplasts to chloroplasts during de-etiolation, in order to facilitate the import of photosynthetic proteins, was described [112,113] (discussed in detail in Section 3.3).

A range of molecular and biochemical experiments also provided direct evidence of the specificity of the receptors. The binding of different isoforms of Toc34 and Toc159 to different classes of precursors proved to be specific in *in vitro* pull-down experiments [47,71]. This was recently confirmed by a split-ubiquitin yeast two-hybrid analysis which tested a variety of precursors [114]. In addition, co-immunoprecipitation assays indicated that atToc159 tends to be associated with atToc33, whereas atToc132/120 preferentially interacts with atToc34 [51]. This analysis also suggested that atToc159 and atToc132/120 exist in different complexes *in vivo*, supporting a model in which two distinct TOC complexes exist (one incorporating atToc159 and atToc33, and another incorporating atToc132/120 and atToc34) and are involved in the import of different subsets of proteins (Fig. 1). However, further work is needed to verify the existence of such distinct complexes. Moreover, recent evidence has suggested that the classification of substrates in this model needs refinement, as discussed in detail in Section 3.4 [115,116].

Some evidence suggests that receptor-preprotein specificity is determined by the interaction between the transit peptide and the receptors [47,107], although the molecular mechanism is not clear yet. By comparing the sequences of the transit peptides of two precursor groups, it is difficult to identify the general defining elements [57,110]. However, a clue was discovered by analysing the Rubisco small subunit precursor (pSSU), which indicated that some specific motifs in the transit peptide help the selection of the atToc159-dependent import pathway [117]. On the other hand, the specificity-determining domain of the receptors has been revealed by a recent study: a significant role of the Toc159 A-domain in controlling specific interactions with distinct substrates was shown by analysing multiple Toc159 family A-domain variants [58]. The responsibility of the A-domain for selectivity was suggested by the fact that the presence of the atToc132 A-domain obstructs partial complementation of the phenotype of *ppi2* (*toc159*) *Arabidopsis* plants, implying that the A-domain acts to confine interactions to a subset of preproteins. In addition, both split-ubiquitin yeast two-hybrid and *in vitro* binding assays demonstrated that the atToc132 A-domain has a negative effect on the binding of photosynthetic proteins [114]. Interestingly, spectroscopic analysis characterised the Toc159 A-domains as being intrinsically disordered. Proteins with such characteristics are usually involved in highly dynamic protein–protein interactions, suggesting they might be ideal for precursor binding [118]. However, the physiological significance of the Toc159 A–domain remains puzzling, as its deletion does not influence plant development [52,53,55].

As mentioned previously, different types of plastids can interconvert during certain developmental stages. As different identities of plastids have diverse proteomes, one can easily imagine that the types of TOC receptors will need to be altered to facilitate the import of the required proteins [119–121]. Recent evidence indeed shows that the relative abundances of the receptors are subject to change during plastid transitions, through the action of an important protein degradation pathway: the ubiquitin–proteasome system (UPS) [112,113].

3. The UPS and plastid dynamics

3.1. The ubiquitin–proteasome system (UPS)

Because of their importance, protein turnover systems are highly developed in plastids, and can be divided into three major pathways. Plastids contain intrinsic proteases inherited from their bacterial ancestors which serve in the quality control of organellar proteins [122]. Autophagy is another pathway which mainly contributes to nutrient recycling by the bulk removal of whole or partial plastids through

their delivery to the plant vacuole [123]. Finally, the UPS recently emerged as an important protein turnover pathway affecting plastid proteins.

The UPS is a major proteolytic system in eukaryotes [124]. It acts by tagging targets with ubiquitin protein(s) through a cascade of reactions involving three key enzymes: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes. Then, the ubiquitinated target protein is usually received by the 26S proteasome for degradation. Substrate specificity for ubiquitination is determined mainly by the E3 ligases, which accounts for their dominance in number (there are ~1400 in *Arabidopsis*, but only ~40 E2s and two E1s) [124]. There are four classes of E3 ligases in plants, of which three comprise a single protein with a distinct conserved domain; these are HECT (*h*omologous to the *E*6-AP *c*arboxyl *t*erminus), U-box, and RING (*r*eally *i*nteresting *n*ew *g*ene) E3 ligases. Another class is composed of a protein complex and are called cullin-RING ligases (CRLs) [124]. In *Arabidopsis*, there are nearly 500 proteins merely for a single class, the RING-type E3 ligases. The exceptional diversity of E3 ligases means that they can specifically recognize, and thus regulate, a wide variety of substrates, and so their roles extend to almost all aspects of plant development, including plant morphology, hormone signalling, abiotic and biotic stress defence, transcriptional regulation, chromatin remodelling and epigenetics.

The proteasome is a huge protein complex comprising two subcomplexes: the 19S regulatory particle and the 20S core particle, which are responsible for the recognition and degradation of ubiquitinated substrates, respectively [125]. The UPS operates in different cellular compartments, not only in the nucleus and cytosol where the proteasome and free ubiquitin are easily accessible, but also in membrane-confined organelles like the endoplasmic reticulum (ER). The cytosolic UPS machinery is central to the protein quality control in the ER, particularly because it does not contain internal proteases like the plastids. To eliminate physical obstruction of the ER membrane, the ER-associated degradation (ERAD) pathway adopts special components, such as the AAA+ ATPase CDC48 and several integral membrane ubiquitination enzymes, in order to extract specific substrates to be degraded by the cytosolic proteasome [126]. Recent findings have turned a new page in the field of UPS action: the endosymbiotic organelles [127]. The role of the UPS in mitochondria has been extensively investigated in animal and yeast in the past a few years. It turns out that UPS control is largely concerned with mitochondrial dynamics, including fusion, fission, mitophagy and mobility. Similar to ERAD, mitochondria employ cytosolic UPS components as well as some special constituents of the outer membrane of mitochondria (OMM). Several E3 ligases have been shown to act at the OMM, including resident components such as MARCH5/MITOL, MULAN/MAPL/GIDE/Mul1 and RNF, and the cytosolic E3 ligase Parkin which can translocate to the OMM in response to stress. These E3 ligases enable the UPS to control diverse targets in the OMM under different cellular conditions.

3.2. The UPS and chloroplast precursor degradation

Although the UPS has been found to be extensively active in many cellular compartments, knowledge on its role (if any) in controlling plant plastids was lacking for a long time. The UPS was initially linked to chloroplasts through the characterisation of a role for the cytosolic E3 ligase CHIP in targeting two chloroplast precursor proteins (ClpP4 and FtsH1) for degradation under high-light conditions [128]. It became clear from later work that CHIP (which is assisted by the Hsc70-4 chaperone) plays a more general role in the removal of un-imported chloroplast precursors by the proteasome [129]. It is proposed that this regulation might serve as a general quality control pathway to clear accumulating cytosolic plastid precursors. As such precursors are thought to be unfolded, accumulation of them in the cytosol might cause aggregation, which would be toxic to the cells. Involvement of the proteasome was also evidenced by a recent proteomic study that sought novel proteasome-interacting proteins [130]. This work confirmed that certain chloroplast precursors can interact with the proteasome both *in vivo* and *in vitro*. Notably, transit peptides of those proteins are essential for such interaction, which might be important to ensure that the degradation is confined to un-imported precursors. Notwithstanding these plastid-related proteasome functions in the cytosol, the direct action of the UPS on chloroplasts was not revealed until recently.

3.3. The UPS and chloroplast resident proteins

The identification of a chloroplast resident ubiquitin E3 ligase for the first time connected the UPS directly to chloroplasts. It is a RING-type E3 ligase, encoded by *SUPPRESSOR OF PPI1 LOCUS1* (*SP1*), whose name indicates that it was found through a genetic screen for second-site suppressors of *ppi1* [112]. The screen aimed to elucidate the regulation of protein import acting at the TOC complex, which was not well studied. It demonstrated that the mutation of *SP1* can specifically recover the protein import defect of the *ppi1* mutant, to improve chloroplast development and allow *ppi1* mutant plants to grow greener and larger. Interestingly, the activity of SP1 seems to be specific to the TOC components, as *sp1* can also recover the mutant phenotype of a *toc75-III* mutant but not TIC mutants. The E3 ligase activity of SP1 protein was verified and shown to be dependent on its RING domain by *in vitro* and *in vivo* data [112,131]. Florescent protein fusion and biochemical assays revealed that SP1 is anchored in the outer envelope membrane (OEM) by two transmembrane domains, thereby presenting a C-terminal RING domain to the cytosol and a central domain in the intermembrane space. Such topology is highly suitable for the control of OEM targets: a cytosol-exposed RING domain is typically adopted by organellar membrane-embedded E3 ligases, enabling access to the cytosolic UPS components, while the intermembrane-space domain may contribute to interactions with substrates in the OEM. Intriguingly, the sequence and topology of SP1 are reminiscent of that of another E3 ligase, MULAN, of mammalian mitochondria.

The specific suppression of *ppi1* and *toc75-III* mutants mediated by *sp1* can be attributed to the increased accumulation of other TOC components, which may partially compensate for the TOC mutations. This conversely suggests the function of SP1 is to promote the degradation of TOC proteins through the UPS, which was demonstrated by interaction and ubiquitination assays both *in vivo* and *in vitro* [112]. As mentioned earlier, the TOC receptors may participate in determining the developmental fate of plastids. Accordingly, when plastids interconvert during processes such as de-etiolation or senescence, the relative proportions of each receptor change correspondingly. Control by SP1 was found to be important during plastid transitions, as *sp1* mutations inhibit developmental processes requiring such transitions [112]. Remarkably, SP1 rapidly degrades atToc132 and atToc120 during de-etiolation and thus enhances the proportion of atToc159, presumably in order to facilitate the switch of TOC complexes to the form more suitable for chloroplasts. Such TOC reorganization is required for changes in the plastid proteome, as indicated by the fact that the rapid accumulation of photosynthetic proteins during de-etiolation is dependent upon SP1. This suggests a possible broader role of SP1 in other plastid transitions, some of which may have agricultural applications, such as the transformation of chloroplasts to chromoplasts during fruit ripening in crops like tomato and citrus. Interestingly, there are two SP1 homologues, SPL1 and SPL2, which share with SP1 similar sequence and topology in the OEM, but their functions are likely to be divergent as revealed by genetic data, perhaps to target different OEM components [112].

Another role of the UPS in plastids might be to control their motility. The redistribution of amyloplasts influenced by gravity is involved in gravitropism, which is the perception of gravity by plant organs for directional growth in order to efficiently receive light (in shoots), water and nutrients (in roots) [7]. It was reported that the *Arabidopsis* E3 ligase SGR9 (*s*hoot *g*ravitropism *9*) plays an important role in gravitropism through the control of amyloplast dynamics [132]. This

was attributed to the ability of SGR9 to regulate dynamic interactions between amyloplasts and actin filaments by sensing the gravity, which is disrupted by the *sgr9* mutation. Biochemical assays showed that SGR9 has ubiquitin E3 ligase activity *in vitro*, suggesting that it acts through the UPS [132]. However, one puzzle is the localization of SGR9. Although fluorescent protein fusion analysis suggested amyloplast localization, detailed localization information is lacking. As SGR9 is predicted to have a cleavable transit peptide [133], it seems unlikely that it is localized in the OEM [134]. And if SGR9 instead resides inside the plastid, it would be isolated from the other UPS components in the cytosol. Thus, identification of it substrate(s) and clarification of its suborganellar localization will help to elucidate the molecular mechanism of SGR9. Intriguingly, some TOC mutants also have altered gravitropic responses [97], while biochemical studies indicated that TOC proteins might be responsible for interactions between actin and plastids [135]. This implies a direct interplay between the TOC import machinery and the regulation of gravitropism, which might be SGR9 dependent.

As there is no ubiquitin or proteasome inside the plastid, the direct UPS-mediated control of internal plastid proteins would appear difficult to achieve. Therefore, the regulation of the TOC import machinery is an ideal agent to mediate proteome changes affecting the whole organelle. Since TOC proteins face the cytosol, they can rapidly respond to cellular or environmental stimuli mediated by the UPS, ultimately leading to adjusted plastid protein levels. This might be particularly important during short transition processes, such as de-etiolation, where the SP1-dependent degradation of the atToc132/120 receptors is so dramatic that it could not be accomplished by transcriptional down-regulation (actually, the transcript profiles of Toc159 family genes are relatively stable during de-etiolation, as shown by public microarray data) [112,136].

Rapidity of action of such UPS control might be delivered by its regulation at several levels. For example, the accumulation level of SP1 can be regulated both transcriptionally and post-translationally. The expression profile of SP1 from public microarray data indicates that it is expressed most highly during senescence, which is reflected by the mutant phenotype [112,136]. In addition, SP1 (and SPL1) transcripts were also reported to be up-regulated by pathogen infection [137]. Autoubiquitination also influences SP1 and controls its protein level, as revealed by the detection of SP1 polyubiquitination *in vivo*, which is dependent on its intact RING domain [112]. Such autoubiquitination may lead to proteasomal degradation, as SP1 protein accumulated following proteasome inhibitor treatment [112]. This may explain why proteomic analyses of the chloroplast envelope have not uncovered SP1 [138,139]. Autoubiquitination is also likely to occur at SGR9, as it was shown that SGR9 protein can be stabilized *in vivo* by mutation of its RING domain causing loss of E3 ligase activity [132]. Such autoubiquitination and degradation can ensure the abundance of the E3s themselves are tightly controlled, in order to avoid possible adverse effects if they are over-produced. However, the molecular basis of plastid E3 regulation is still unclear, and it will be of interest to reveal its regulators such as transcription factors, partners and accessory proteins.

Advances in UPS research pertaining to mitochondria have revealed a wide range of resident mitochondrial proteins as substrates, even including proteins inside mitochondria [127]. Interestingly, a recent proteomic study on purified chloroplast envelopes identified ubiquitin and some ubiquitin ligases [139]. However, contamination cannot be ruled out because of their high abundance. Moreover, modified proteomic studies for the identification of ubiquitination targets have revealed many chloroplast proteins to be ubiquitinated *in vivo* [140,141]; a surprisingly high proportion of the ubiquitinated proteins identified is predicted to be targeted into chloroplasts, including atToc159, atToc33 and atToc34 which had been identified previously *in vivo* [112,141]. For many of the proteins identified, a peptide from the transit peptide sequence was detected, indicating that the corresponding proteins are precursors localized in the cytosol, further confirming that the ubiquitination of precursors occurs generally. However, many chloroplast proteins were only identified after denaturation (reportedly to disrupt membranes), but not in native conditions, which implies that at least some of them are indeed chloroplast-localized ubiquitin conjugates. It will be interesting to investigate which of them are truly ubiquitinated chloroplast resident proteins and what the significance of such ubiquitination is.

3.4. Ubiquitination and protein import into complex plastids

Another newly-discovered active site of ubiquitination is in the complex plastids, where it acts to control protein import. Unlike plant chloroplasts, the complex plastids of different algal and parasite species (for example, apicoplasts in apicomplexan parasites) evolved from secondary endosymbioses and possess up to four membranes, and consequently have more complex protein targeting pathways [142]. Plastid proteins cross the outermost plastid membrane through fusion of endosomal vesicles derived from endoplasmic reticulum, and then gain access to the inner membranes through different translocons. The innermost two membranes are similar to chloroplast membranes, and contain translocons homologous to the TOC and TIC machines. A third translocon serving to transport proteins across the second outermost membrane (the periplastid membrane) is believed to employ components similar to the ERAD machinery; for example, the core component, CDC48 [143,144]. Surprisingly, the ubiquitination normally involved in protein degradation in ERAD is retained in this import system, including a whole series of E1, E2 and E3 ubiquitin enzymes and a de-ubiquitination enzyme [145,146]. Importantly, biochemical and genetic analyses indicate that such enzymes are indeed functional and critical for the protein import system in complex plastids [146]. In contrast to the ubiquitination which happens in primary plastids, like plant chloroplasts, which plays a role in protein degradation [112], the function of ubiquitination in complex plastids is proteasome independent. Its precise role in protein import still remains to be characterised.

4. Other regulation of plastid protein import

Other regulatory mechanisms act to control the TOC components in order to meet changing demands for protein import during different developmental stages and under different growth conditions. For example, it has long been known that the expression levels of atToc33 and pea Toc75 are much higher during early developmental stages [43,83], presumably to fulfil the massive requirement for protein import at such times when chloroplast biogenesis activity is intense. Indeed, import rates of several precursor proteins were highest in juvenile chloroplasts and gradually declined during the ageing of chloroplasts [147]. However, whether this was a general effect for all precursors or just for some specific proteins was not clear, as only a few precursors were analysed. A recent study using a more extensive number of chloroplast preproteins depicted a more complex scenario of age-dependent regulation of protein import [116]. In this study, it was confirmed that in higher plants the import of different chloroplast preproteins is specifically regulated in accordance with developmental stages. The data showed that chloroplast precursors can be divided into three groups which display distinct preferences with regard to import into chloroplasts of different ages: group I precursors were more efficiently imported into young chloroplasts, and so may be more important for chloroplasts in rapidly dividing and expanding cells in young leaves; group II precursors did not display preference in respect of chloroplast age, suggesting that they are “housekeeping” proteins which maintain basic chloroplast functions throughout development; group III precursors were more prone to be imported into old chloroplasts, which is controversial in relation to previous thinking [147]. It was also shown that such selection is dependent on the transit peptide. Notably, the replacement of the transit peptide of a group III precursor with that of a group I precursor resulted in a growth defect of the plants, suggesting

that such age-related import control plays an important role in chloroplast development.

By considering earlier proteomic analyses of *Arabidopsis* TOC receptor mutants, it was proposed that atToc159 is the receptor for group I precursors, and that atToc132 is the receptor for group II precursors [116]; clear indications as to the identity of the group III receptor were not forthcoming. Thus, such development-based groupings of precursors might complement the previous functional classifications, in which "photosynthetic" and "housekeeping" preproteins correspond to specific TOC receptors (Fig. 1). Indeed, recent investigations indicated that the situation might not be that simple. A large-scale proteomic study implied that the substrates of atToc159 may not be strictly photosynthetic-related preproteins [115]. In addition, binding affinity studies indicated that the Toc159 family proteins have different but overlapping preferences for preproteins [114]. The new information on age-dependent import suggests that the specific interactions of substrate precursors and TOC receptors are rather complex, which might reflect differing proteome requirements of different plastid types or specific developmental stages of plastids.

Chloroplast protein import is also influenced by environmental cues. It has been reported that import rates are negatively influenced by temperature-stresses, accompanied by declining RNA and protein levels of TOC/TIC components [148], but whether this is due to active regulation or merely damaging effects of stress is unclear. If it is specific regulation, it may function to inhibit photosynthesis in order to avoid production of excessive reactive oxygen species which might do harm to the plant [149, 150]. Although the regulatory mechanism is not clear, transcriptional control is one possibility. To date, CIA2 (*c*hloroplast *i*mport *a*pparatus 2) is the only transcription factor found to control chloroplast protein import as well as the chloroplast translation system [151,152]. It is suggested to regulate the expression of certain TOC components, and thus to adjust import activity throughout development. Nonetheless, direct protein level control is also possible, for example *via* the aforementioned UPS or autophagy degradation pathways. Clearly, it will be of considerable interest to identify more regulators involved in chloroplast protein import in the future.

5. Concluding remarks

Recent advances have given rise to a more profound understanding of the molecular mechanisms underlying protein import into chloroplasts, particularly in relation to substrate-receptor specificity at the TOC machinery and its role in plastid type differentiation. However, most of our knowledge on protein import is about the chloroplast, and our understanding with respect to other non-green plastids is rather meagre. This might be due to the fact that most studies on protein import were done in *Arabidopsis*, which limits the investigation of other plastid types. With the prevalence of next generation genomic sequencing, we now have the opportunity to study the molecular basis of protein import in other important plastids, for example, chromoplasts. In addition, models about substrate-receptor specificity can be verified and generalized in different species. Currently, although such specificity has been acknowledged, there is still discrepancy concerning the classification of different precursor proteins. By investigating more precursors, a consensus may emerge. In addition, more and more evidence indicates that protein import can be finely regulated in response to developmental and environmental cues. The UPS is one mechanism of such regulation, and it is particularly important during plastid transitions. However, compared with its role in mitochondria, our understanding of UPS control in plastids is limited. Recent proteomic studies have found many ubiquitinated chloroplast proteins, implying broader functions of the UPS in plastids. Identification of further relevant UPS components will greatly enhance our understanding of the roles of the UPS in plastids. One interesting area to investigate will be its role in stress. Plants have a particularly high number of E3s, possibly to enable more refined responses to stress conditions necessitated by their sessile nature. Rapid action by the UPS may be an ideal regulatory pathway to allow plants to adapt to the changing environment with regard to their plastids. In addition, emerging studies have uncovered ubiquitin-like modifiers which can act synergistically or antagonistically with ubiquitination in plants [153], and their roles in plastids are unknown and need to be discovered.

Conflict of interest

No conflict of interest.

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