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Review

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

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ABSTRACT

Background: Plastids, such as chloroplasts, are widely distributed endosymbiotic organelles in plants and algae. Apart from their well-known function 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 the TIC (translocon at the inner envelope membrane of chloroplasts) complexes.
Scope of review: 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 how protein import is regulated, and how this regulation changes plastid development, particularly in relation to control by the ubiquitin–proteasome system (UPS).
Major conclusions: The diversification 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 the plant development.
General significance: 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.

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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the cytosol. For chloroplasts, over 90% of the 3000 organellar proteins are encoded in the nucleus and translated by cytosolic ribosomes [12,13]. Similar to mitochondrial proteins, most chloroplast proteins are synthesized 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 the cytosolic precursors and introduce them onto 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 had 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

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