

New Insights into the Roles of Ubiquitin Modification in Regulating Plastids and Other Endosymbiotic Organelles

William Broad, Qihua Ling and Paul Jarvis*

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB,
United Kingdom.

*For correspondence: paul.jarvis@plants.ox.ac.uk

Table of Contents

Abstract

1. Introduction

2. The Ubiquitin-Proteasome System

2.1 E3 Ligases

2.2 The ERAD System

3 Regulation of Endosymbiotic Organelles by Ubiquitin

3.1 Mitochondria

3.2 Plastids

3.2.1 Protein Import into Plastids

3.2.2 Actions of the UPS on Plastids

3.2.3 Actions of the UPS on the Plastid Protein Import Machinery

3.2.4 Actions of the UPS on Plastids Under Abiotic Stress

3.2.5 Future Prospects and Unanswered Questions Concerning the Regulation of Plastids by Ubiquitination

3.3 Red Algal Derived Complex Plastids

3.3.1 A Role for Ubiquitin in Complex Plastid Development: The SELMA Translocon

3.3.2 Future Prospects and Unanswered Questions Concerning SELMA

4 Concluding Remarks

Abstract

Recent findings have revealed important and diverse roles for the ubiquitin modification of proteins in the regulation of endosymbiotic organelles, which include the primary plastids of plants as well as complex plastids: the secondary endosymbiotic organelles of cryptophytes, alveolates, stramenopiles and haptophytes. Ubiquitin modifications have a variety of potential consequences, both to the modified protein itself and to cellular regulation. The ubiquitin-proteasome system (UPS) can target individual proteins for selective degradation by the cytosolic 26S proteasome. Ubiquitin modifications can also signal the removal of whole endosymbiotic organelles, for example via autophagy as has been well characterized in mitochondria. As plastids must import >90% of their proteins from the cytosol, the observation that the UPS selectively targets the plastid protein import machinery is particularly significant, as in doing so it may influence the development and interconversions of different plastid types, as well as plastid responses to stress, by reconfiguring the organellar proteome. In complex plastids, the Symbiont-derived ERAD-Like Machinery (SELMA) has co-opted the protein transport capabilities of the Endoplasmic Reticulum Associated Degradation (ERAD) system, whereby misfolded proteins are retrotranslocated from the ER for proteasomal degradation), uncoupling them from proteolysis: SELMA components have been retargeted to the second outermost plastid membrane to mediate protein import. In spite of this wealth of new information, still there remains a large number of unanswered questions and a need to further define the roles of ubiquitin modification in the regulation of plastids.

Key Words: Plastid, Chloroplast, Ubiquitin, Proteasome, SELMA, Endosymbiotic, Stress, Transport.

1. Introduction

Like mitochondria, plastids are organelles of endosymbiotic origin, and, as recent research has shown, dynamic aspects of both organelles are directly affected by the ubiquitin modification of organellar proteins (Ling and Jarvis, 2013). Plastids exist in primary and complex varieties: Primary plastids are the characteristic organelles of plants, the most notable type being the chloroplast, whereas complex plastids are derived from various secondary and tertiary endosymbioses in diverse organisms (Gould et al., 2015).

Endosymbiosis is a phenomenon whereby a single-celled organism resides within another cell as part of a mutually beneficial relationship. For example, in the case of the relationship between rhizobacteria and legumes, the bacteria fix atmospheric nitrogen into a biologically available form within specialized root-nodules of the plant, in exchange for a carbon source and a stable environment (Nelson and Sadowsky, 2015). Endosymbiotic relationships have evolved independently multiple times; even cells themselves containing endosymbionts have become endosymbionts within another organism, resulting in secondary and tertiary endosymbionts (Gould et al., 2015; Zimorski et al., 2014). Mitochondria and plastids originated from prokaryotic endosymbionts respectively related to extant α -proteobacteria (Gray et al., 1999) and cyanobacteria (Gould et al., 2008).

Complex plastids are secondary or tertiary endosymbiotic organelles found in a variety of organisms, including many algae, diatoms and apicomplexa (Zimorski et al., 2014). Such organelles arose from eukaryotes containing either red or green plastids (Zimorski et al., 2014). Those arising from the red algal lineage will be examined in this review.

It is evident that endosymbionts can completely integrate into the host cell lineage to become permanent organelles, provided they can enter the germ-line. In such a scenario,

the functions of the endosymbiont become critical to the host, while a reduction in the endosymbiont's autonomy leads to its reliance on the host cell for its survival. The result is that the two organisms are no longer distinct: neither host nor endosymbiont could complete its lifecycle separately from the other.

During the process of integration, the endosymbionts developed specialized functions, features of which likely originated from their principal contribution to the endosymbiotic relationship. In addition to such adaptation of function, their morphology also changed. For example, mitochondria do not often exist as a single bacterial cell-like organelle, as often depicted, but instead typically form chains and networks, and inside have folded membrane networks called cristae (Okamoto and Shaw, 2005). Plastids also have unique features uncharacteristic of bacteria: their size and shape change dramatically dependent on development, and the chloroplast contains a complex network of folded and interconnected membranes called thylakoids, which are more elaborate than in their cyanobacterial counterparts (Wise and Hooper, 2006).

These changes have been facilitated by the host cell gradually assuming control over its endosymbionts during the course of evolution. This has occurred as a result of the translocation of organellar genes to the nucleus and the concomitant reduction of organellar genomes. While the organelles retain their own genomes, these typically encode fewer than 100 genes, and so most of the genes required for organelle biogenesis and function are located in the cell nucleus (Zimorski et al., 2014). One consequence of this is that the genes have evolved and become regulated differently than they would have been were they retained within the endosymbiont.

Another significant consequence of such gene transfer is that the encoded proteins are synthesized in the cytosol, necessitating protein targeting and import mechanisms for their organellar delivery, and giving the potential for further regulation and control (Richardson et al., 2014; Zimorski et al., 2014). Targeting typically requires a cleavable, N-terminal extension called a presequence (for mitochondrial proteins) or transit peptide (for plastid proteins), and so many organellar proteins are synthesized in precursor, or preprotein, form. Such targeting signals direct preproteins to, and through, their cognate protein transport machineries in the corresponding organellar membranes (Schmidt et al., 2010; Shi and Theg, 2013). These protein targeting and import mechanisms can equally be employed for the delivery of host-derived proteins to the endosymbionts, in addition to endosymbiont-derived proteins (Zimorski et al., 2014), contributing novel functions and leading to further integration. It is estimated that modern plastids of plants may import up to ~3000 different nucleus-encoded proteins from the cytosol, representing ~98% of the organellar proteome (Shi and Theg, 2013).

Regulation of the cellular and organellar proteomes, and of individual proteins and protein complexes, involves a diversity of mechanisms. This can happen at the level of transcription and translation, and through a multitude of post-translational modifications, including ubiquitination. One critical important mechanism of proteome regulation is proteolysis. Proteolysis can serve many purposes, such as quality control, regulation of abundance, and the implementation of rapid proteome changes unachievable by merely altering transcription or translation (van Wijk, 2015). Mitochondria and plastids contain many prokaryote-derived proteases which regulate their internal proteomes, and also cleave the targeting peptides of arriving preproteins (Teixeira and Glaser, 2013; van Wijk, 2015).

Recent studies have revealed that an important and complex host-derived protein degradation system acts upon these endosymbiotic organelles (Ling et al., 2012; Ling and Jarvis, 2013; Livnat-Levanon and Glickman, 2011). The ubiquitin-proteasome system (UPS) has been shown to act upon proteins of chloroplasts and mitochondria, and to directly regulate the development and functions of these organelles. In addition, a subsection of the UPS, called the endoplasmic reticulum associated degradation pathway (ERAD), mediates protein import into complex plastids: the symbiont-derived ERAD-like machinery (SELMA) uses ubiquitin modification as a targeting mechanism, but is uncoupled from proteasomal degradation (Gould et al., 2015; Kalanon et al., 2009). These discoveries have initiated a whole new chapter in the study of these organelles, their functions, and their development.

2. The Ubiquitin-Proteasome System

The UPS can specifically target proteins for degradation. The process involves the modification of a target protein by addition of ubiquitin to signal and mediate its degradation by the cytosolic 26S proteasome, a multi-subunit proteolytic complex. Ubiquitin modification (interchangeably called ubiquitination or ubiquitylation) is a post-translational modification of proteins that involves the ligation of the 76 amino acid protein, ubiquitin, most commonly to a lysine residue of the substrate protein by an iso-peptide bond. Ubiquitin and the UPS are highly conserved and ubiquitous in eukaryotes, play central roles in the regulation and dynamics of the cellular proteome, and are critical to cellular homeostasis and responses to developmental and environmental signals (Kleiger and Mayor, 2014; Vierstra, 2009).

Ubiquitin is bound to its substrate protein through a cascade of three enzymes. An E1 ubiquitin activating enzyme binds and activates ubiquitin in an ATP-dependent process; then, ubiquitin is covalently attached to an E2 ubiquitin conjugating enzyme which delivers ubiquitin to substrate proteins; an E3 ubiquitin ligase acts to provide substrate specificity, allowing the E2 to covalently bind the C-terminal glycine of ubiquitin to the amino side chain of a lysine of the substrate protein (Kleiger and Mayor, 2014; Vierstra, 2009).

Targeting of a substrate protein to the proteasome requires a polyubiquitin chain to be generated on the substrate (Thrower et al., 2000). In this process, successive ubiquitin monomers are ligated to each other, most commonly via lysine-48. It is unclear whether polymerization can be catalysed by the E3 ligase associated with the target protein. It may be that polyubiquitin chains are formed while ubiquitin is still attached to the E2, during the transfer of ubiquitin between E1 and the substrate; alternatively, other factors may be involved (Walsh and Sadanandom, 2014).

Polyubiquitinated proteins are rapidly directed to, and degraded by, the 26S proteasome. This makes identification of ubiquitination targets a challenge, particularly if the target is of low abundance *in vivo*. At the proteasome, the ubiquitinated protein is unfolded and enters the proteolytic complex while the polyubiquitin chain is removed by deubiquitinases (DUBs) on the outside of the proteasome. DUBs generate ubiquitin monomers from polyubiquitin, which are released into the cytosol to be reused. Substrate proteins enter the 20S core proteolytic complex via the 19S regulatory complex. Once inside, the proteins are hydrolysed into short peptides by a variety of proteolytic sites inside the proteasome, which are able to hydrolyse peptide bonds between different residues. Peptides are then released into the cytosol (Kleiger and Mayor, 2014).

Proteasomal degradation is not the only fate of ubiquitinated proteins; a variety of different ubiquitin modifications trigger different fates. For example, in monoubiquitination and multiubiquitination, a single ubiquitin monomer is added to one or multiple lysine residues on the target protein, respectively (Walsh and Sadanandom, 2014). In addition, ubiquitin polymerization via each of ubiquitin's seven lysine residues has been reported (these are: K6, K11, K27, K29, K31, K48 and K63) (Kim et al., 2013), which enables the possibility of multiple, different polyubiquitin types, as well as branched ubiquitin polymers (Walsh and Sadanandom, 2014). Other consequences of ubiquitin modification include directing large protein complexes or cell compartments to the vacuole or lysosome for autophagy (Li et al., 2015), regulation of protein localization or activity (Guerra and Callis, 2012), and regulation or repair of chromatin (Holt et al., 2015).

2.1 E3 Ligases

The E3 ligases are the key determinants of specificity in the ubiquitin proteasome system, enabling the system to target individual proteins and protein complexes with high specificity or in a more general manner. They are single proteins or protein complexes which can associate with target proteins to mediate transfer of ubiquitin. Their activity may be modulated by post-translational modifications such as phosphorylation or ubiquitination, regulatory partner proteins, or adapter proteins that can broaden, restrict or change the specificity and activity of the enzyme (Guerra and Callis, 2012; Sadanandom et al., 2012). Many Really Interesting New Gene (RING) and U-box type E3 ligases (see below) are subject to autoubiquitination and so regulate their own abundance (de Bie and Ciechanover, 2011; Guerra and Callis, 2012). Reflecting their importance in determining specificity, as well as

the breadth and diversity of UPS targets, the model plant *Arabidopsis thaliana* has ~1400 genes encoding E3 ligase components, but only 37 E2 genes and two E1 genes (Vierstra, 2009).

Although extraordinarily diverse, E3 ligases fit into three broad categories: RING, U-Box, and Homologous-to-E6AP-C-Terminus (HECT). The RING and U-Box domains are protein interaction modules which recruit the relevant E2 (Guerra and Callis, 2012). The RING domain comprises a zinc-finger structure, while the U-box is a homologous domain that does not require zinc for its structure to assemble (Guerra and Callis, 2012). The RING group can be subdivided into single-subunit and multi-subunit categories: Single-subunit RING E3s can direct ubiquitination of their substrates alone, whereas multi-subunit E3s are protein complexes (e.g., SCF-type E3 ligases) that comprise additional adapter proteins such as F-Box proteins for substrate interaction (de Bie and Ciechanover, 2011; Guerra and Callis, 2012; Jia et al., 2015; Vierstra, 2009). The HECT E3 ligases are monomeric, and unlike other E3s they covalently bind ubiquitin prior to its ligation to the substrate (de Bie and Ciechanover, 2011; Guerra and Callis, 2012; Vierstra, 2009).

Ubiquitin E3 ligases can exist in the cytosol or the nucleus, or have been predicted or shown to have transmembrane domains that enable their localization to the endomembrane system, to the outer mitochondrial membrane (OMM), or to the plastid outer envelope membrane (OEM) (Deshaies and Joazeiro, 2009). Current evidence suggests that E3s and other component of the UPS do not exist internally within organelles, with the exception of the nucleus (von Mikecz et al., 2008). Therefore, regulation of endosymbiotic organelles by the UPS is mediated by outer membrane components or cytosolic interactors. In the case of the ER, the ERAD system has evolved to retrieve misfolded proteins from the ER lumen, a

process which is coupled to the UPS. In the case of mitochondria, the UPS may be able to extend as far as the intermembrane space (Bragoszewski et al., 2013).

2.2 The ERAD System

As the 26S proteasome is not present inside the ER, the ERAD system is required for the retrotranslocation of misfolded proteins and certain other target proteins from the ER to the cytosol where the proteasome resides (Ruggiano et al., 2014; Vembar and Brodsky, 2008). ERAD is subdivided into three pathways, depending on where the substrate is relative to the ER membrane: ERAD-L, ERAD-M and ERAD-C. ERAD-M targets ER membrane proteins with misfolded transmembrane domains, and ERAD-C targets ER membrane proteins with misfolded cytosolic facing domains (Ruggiano et al., 2014). ERAD-L is the pathway which gave rise to SELMA, and it retrotranslocates soluble ER lumen proteins and ER membrane proteins with misfolded luminal domains (Bolte et al., 2011; Ruggiano et al., 2014). This process requires multiple steps, as summarized below, and multiple components which here are named according to the components characterized in yeast.

While pathways for specific substrates have been identified, the general recognition process for misfolded protein substrates is still unclear, although the Hmg-CoA reductase degradation ligase1 (Hrd1) complex at the ER membrane likely plays a crucial role (Ruggiano et al., 2014). The Hrd1 complex is believed to contain the following ER membrane proteins: Hrd1, an E3 ligase with a cytosolic RING finger domain; Degradation in the ER protein1 (Der1); and Hrd3 which has a lumen-oriented Tetratricopeptide Repet (TPR)-containing domain (Ruggiano et al., 2014). Recent work has shown that Hrd3 plays a crucial role in the recognition of luminal substrates (Mehnert et al., 2015). Once the substrate protein is in association with

the Hrd1 complex at the ER membrane, its retrotranslocation across the membrane can be initiated. The identity of the retrotranslocon is also uncertain, although the Sec61 complex, Hrd1 (which contains six transmembrane spans), and Der1 (which contains four transmembrane spans) have each been proposed to fulfil the role (Bolte et al., 2011; Carvalho et al., 2006; Ruggiano et al., 2014). The substrate is eventually exposed to the cytosol, and is then ubiquitinated through a typical enzymatic cascade involving the E1 Uba1, the E2s Ubc1 and Ubc7, and the aforementioned E3 ligase Hrd1 (Bolte et al., 2011). The ER membrane protein Ubx2 recruits the AAA+ ATPase Cdc48 to the ER membrane along with its cofactors: heterodimers of Npl4 and Ufd1 (Richly et al., 2005; Wolf and Stolz, 2012). Npl4 and Ufd1 are believed to be involved in substrate recruitment by Cdc48, which then provides the motive force to retrotranslocate the substrate into the cytosol where it can be degraded by the 26S proteasome. Although well established in its role, the precise mechanism of translocation by Cdc48 is still unclear (Ruggiano et al., 2014; Wolf and Stolz, 2012).

3. Regulation of Endosymbiotic Organelles by Ubiquitin

3.1 Mitochondria

A typical UPS has not been identified in the prokaryotic ancestors of the endosymbiotic organelles, mitochondria and plastids. Thus, the UPS-mediated control of these organelles may have evolved to ensure full integration into the cellular environment, and perhaps occurred in parallel with their development of new features following endosymbiosis, e.g., their functional and morphological dynamism (Ling and Jarvis, 2013). Collective evidence shows that ubiquitin-mediated protein degradation pathways control a variety of

mitochondrial functions in yeast and mammals, and are associated with several human diseases; this has been extensively reviewed elsewhere (Escobar-Henriques and Langer, 2014; Franz et al., 2015; Mishra and Chan, 2014), and so will only be briefly introduced here. Multiple ubiquitination-related components have been found to have close links to mitochondria, including several E3s, E2s, deubiquitylating enzymes (DUBs), and the AAA+ ATPase Cdc48/p97/VCP (Franz et al., 2015; Pan et al., 2014). In addition to intrinsic OMM E3s, such as March5/Mitol, Mulan/Mapl/Gide/Mul1 and RNF5, the cytosolic E3 ligase Parkin can be recruited to the OMM under stress conditions (Franz et al., 2015). Together, these enzymes control mitochondrial dynamics by mediating the UPS degradation of factors involved in organellar fission/fusion, including the mammalian fission factor Dynamin-related protein1 (Drp1), and the fusion factors Mitofusin 1 and 2 (Mfn1, Mfn2) (Ling and Jarvis, 2013). In such processes, Cdc48 serves as a driving force for substrate translocation from the OMM to the cytosol, paralleling its aforementioned role in the ERAD pathway (Esaki and Ogura, 2012). Parkin and Mulan and other E3s are also involved in mitophagy (i.e., mitochondrial autophagy, which degrades whole mitochondria) (Franz et al., 2015; Livnat-Levanon and Glickman, 2011). Ubiquitination of OMM proteins by these E3 ligases not only provides a signal for proteasomal degradation (which probably also facilitates mitophagy), but it also initiates mitophagy by promoting the reception of the autophagic machinery (Franz et al., 2015). Other post-translational modifications, such as phosphorylation, play critical roles during mitochondrial protein degradation through the UPS and mitophagy. For example, the kinase PINK1 can phosphorylate Mfn2 to promote its interaction with Parkin (Sarraf et al., 2013; Shiba-Fukushima et al., 2012). Most interestingly, it was found for the first time that the protein ubiquitin can be directly phosphorylated by PINK1 and that such phosphorylation is a crucial step in mitophagy, probably by specifically

recruiting autophagic components to trigger mitophagy (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014).

3.2 Plastids

Plastids are the site of important and unique functions in plant cells. These organelles are surrounded by a double-membrane system called the envelope, comprising outer and inner envelope membranes and an intermembrane space, and have a central aqueous matrix called the stroma; additionally, in chloroplasts, there is an internal membrane system called the thylakoids, which encloses an additional space called the thylakoid lumen (Jarvis and Lopez-Juez, 2013).

Different plastid types develop in order to meet changing functional requirements as cells and tissues of the plant differentiate. All plastids develop from proplastids in the embryo; these are small organelles with an undeveloped internal membrane system (Wise and Hooper, 2006). Once developed, plastids continue to sense and respond to their environment and may interconvert upon further cellular differentiation (Jarvis and Lopez-Juez, 2013; Wise and Hooper, 2006). For example, proplastids differentiate into etioplasts in cotyledons in the absence of light, and then into chloroplasts upon illumination, and finally into gerontoplasts when leaves senesce (Jarvis and Lopez-Juez, 2013; Wise and Hooper, 2006). The most well studied plastid is the chloroplast, where photosynthesis takes place. Other important plant plastids include the chromoplasts, named for the carotenoid pigments that provide colour to fruits and flowers, and the amyloplasts which function primarily in starch synthesis and storage (Jarvis and Lopez-Juez, 2013); see Table i for a summary of the different plastid types in plants.

Plant plastids are metabolically and biosynthetically diverse, contributing to the synthesis of amino acids, fatty acids, starch, chlorophylls, carotenoids, signalling molecules, and various precursors, as well as the assimilation of nitrogen and sulphur (Brautigam and Weber, 2009). Another noteworthy functions of plastids is in gravitropism, wherein specialized amyloplasts called statoliths in root and shoot apical meristems enable gravity sensing: The mechanism relies on the high density of these organelles, linked to the starch they contain, which causes them to sediment in the direction of gravity; this information is interpreted by the plant, possibly through interaction of the statoliths with actin (Blancaflor, 2013; Sato et al., 2015). The diversity of functions mediated by plastids means that they have particular importance in cellular homeostasis, and in the perception and responses to signals during development and stress. The latter points is especially noteworthy as photosynthesis is a major source of reactive oxygen species (ROS), which act in signalling but can easily become damaging if not regulated (Shapiguzov et al., 2012; Suzuki et al., 2012).

The biochemical functions and differentiation state of plastids are defined by the organellar proteome, which is itself influenced by the combined effects of gene expression, protein targeting and import, and anterograde and retrograde signalling. The plastid protein import system is the main known target of the UPS in plastids, and is described in detail in Section 3.2.1 below.

3.2.1 Protein Import into Plastids

It is estimated that up to ~3000 plastid proteins (~98% of the organellar proteome) are synthesized in the cytosol, necessitating mechanisms for the targeting and import of such proteins (Jarvis and Lopez-Juez, 2013; Li and Chiu, 2010). Protein translocation across the

outer and inner envelope membranes is mediated by the TOC and TIC (Translocon at the Outer/Innner membrane of Chloroplasts) complexes, respectively, in what has been called the 'canonical' protein import pathway (Shi and Theg, 2013). Other 'non-canonical' pathways to the plastid, including a co-translational pathway through the endomembrane system (Villarejo et al., 2005), have been described, but these are not well characterized (Jarvis and Lopez-Juez, 2013). Proteins which enter the plastid via the canonical pathway are synthesized as preproteins carrying an N-terminal transit peptide, which is cleaved upon entering the plastid stroma by the stromal processing peptidase (SPP) (Richter and Lamppa, 2003).

The TOC and TIC complexes comprise multiple proteins, and the first of these were identified in the 1990s in pea. Now, there are many known TOC and TIC components, and these are conserved across many plant and algal species (Jarvis and Lopez-Juez, 2013; Shi and Theg, 2013). Only the TOC components will be described here, as only these are known to be targeted by the UPS.

The core components of the TOC apparatus are Toc34, Toc75 and Toc159 – the numbers indicate their respective molecular weights in kDa (Shi and Theg, 2013). The stoichiometry of these components in the TOC complex, in the order Toc34:Toc75:Toc159, has been found to be 4-5:4:1 (Schleiff et al., 2003) or 3:3:1 (Kikuchi et al., 2006). Toc75 forms the protein-conducting channel of the complex. It is an Omp85-related protein (Omp85 is a founding member of a superfamily of β -barrel outer membrane proteins) comprising a conserved β -barrel domain at the C-terminal end, which forms the membrane integral portion of the protein, and an N-terminal Polypeptide Transport Associated (POTRA) domain. There is some experimental evidence to suggest a cytosolic orientation for the POTRA (Sommer et

al., 2011), although it remains conceivable that the orientation of the protein is such that the POTRA domain faces the intermembrane space, paralleling the situation in mitochondria and bacteria where equivalent domains face the intermembrane space and periplasmic space, respectively (Day et al., 2014; Misra et al., 2015).

Toc34 and Toc159 act as preprotein receptors (Richardson et al., 2014). They interact specifically with preproteins as they arrive at the chloroplast surface, and regulate the initial stages of translocation through Toc75 in a GTP-dependent manner (Koenig et al., 2008).

Toc34 and Toc159 are each anchored in the plastid OEM by a C-terminal transmembrane anchor (in Toc159 this domain is unusually large and of unknown configuration, and is termed the M-domain). Both proteins also possess a GTPase domain that protrudes into the cytosol and is often called the G-domain, while Toc159 additionally has an acidic domain at its N-terminus called the A-domain (Shi and Theg, 2013).

Each TOC component typically has multiple isoforms in plants. In Arabidopsis, the relevant families are as follows; isoforms of Toc34: atToc33 and atToc34 (Gutensohn et al., 2000; Jarvis et al., 1998); isoforms of Toc159: atToc159, atToc132, atToc120 and atToc90 (Bauer et al., 2000; Infanger et al., 2010; Ivanova et al., 2004); and isoforms of Toc75: atToc75-III (major isoform), and atToc75-IV (minor isoform) (Baldwin et al., 2005; Huang et al., 2011). Note that the 'at' prefix in each case denotes species of origin, while the Roman numeral suffix indicates the chromosome on which the isoform is encoded.

These different TOC isoforms allow for different TOC arrangements to import distinct groups of proteins, perhaps at different developmental stages or in plastid types (Jarvis and Lopez-Juez, 2013; Li and Teng, 2013; Richardson et al., 2014). For example, atToc33, atToc159 and atToc75-III form the main translocon in chloroplasts, transporting photosynthesis-related

preproteins, often called photosynthetic proteins (Fig. 1) (Jarvis and Lopez-Juez, 2013; Li and Chiu, 2010). By contrast, another translocon containing atToc34, atToc132/120 and atToc75-III is thought to be the main complex in non-photosynthetic plastids, and to conduct other preprotein types such as those required for general organelle functions and maintenance (Fig. 1) (Jarvis and Lopez-Juez, 2013; Li and Chiu, 2010).

A wide variety of observations supports the existence of multiple TOC configurations with different client specificities. Different TOC receptor isoforms have been observed to preferentially associate with each other in distinct groups, and can be pulled down together in those groups (Ivanova et al., 2004). Furthermore, different TOC receptor isoforms have been shown to have differing affinities for different preprotein clients (Dutta et al., 2014; Gutensohn et al., 2000; Ivanova et al., 2004; Jelic et al., 2003). Knockout mutant plants lacking the different receptors also have phenotypes that are consistent with the proposed specificities of the receptors, as summarized below (Bauer et al., 2000; Constan et al., 2004; Kubis et al., 2003; Kubis et al., 2004). It is also noteworthy that some preproteins and transit peptides favour certain plastid types, or plastids of different ages, whereas others are imported independent of plastid type or age (Teng et al., 2012; Yan et al., 2006).

The mutants of TOC components differ in the extent to which they affect the import of different groups of preproteins, or the biogenesis of different plastid types. Mutants of atToc33 (termed *plastid protein import1*, or *ppi1*) and atToc159 (*ppi2*) have pale-yellow and albino phenotypes, respectively, reflecting reduced import of photosynthesis-related proteins and impaired chloroplast biogenesis (Bauer et al., 2000; Jarvis et al., 1998; Kubis et al., 2003). The main Arabidopsis Toc75 isoform, atToc75-III, is expressed at a high level in all tissues, and mutations affecting this component are embryo lethal, indicating its central role

in protein import and showing that plastids are critical to embryo development (Baldwin et al., 2005). The role of atToc75-III has been studied in other tissues by use of a hypomorphic allele and inducible knockdown plants (Huang et al., 2011). Knockdown of atToc75-III caused abnormal development of chloroplasts and non-green plastids, and reduced import of both photosynthesis-related and non-photosynthesis-related preproteins. This indicated that atToc75-III is important, non-specifically for a broad range of plastid functions, and that it performs a general role in protein import. Depletion of atToc75-III also caused reduced abundance of atToc33 and atToc159, consistent with previous reports that Toc75 aids in the biogenesis of TOC components (Huang et al., 2011; Wallas et al., 2003).

Mutations affecting atToc132 or atToc120 do not cause strong phenotypes individually, but corresponding double mutants display severe chlorotic phenotypes owing to plastid biogenesis defects (Ivanova et al., 2004; Kubis et al., 2004). Mutants of atToc34 have less severe phenotypes than *ppi1*, with the only obvious defects occurring in the roots, presumably due to defective development of non-photosynthetic root plastids (Constan et al., 2004). However, *atTOC34* expression can almost fully complement *ppi1* revealing a degree of redundancy, while the double homozygous mutant genotype affecting both atToc33 and atToc34 causes embryo lethality (Gutensohn et al., 2000; Jarvis et al., 1998).

Thus, multiple TOC configurations may give specificity to protein import such that in different plastid types, at different stages of development, or under different conditions, the necessary proteins may be imported into the plastid in the correct abundance. If there were just a single type of TOC complex, competition effects might arise; for example, at the onset of photomorphogenesis, highly-abundant photosynthesis-related proteins could conceivably out-compete other proteins (e.g., those required in lower abundance for more general

organelle functions and maintenance) for entry into the plastid (Jarvis and Lopez-Juez, 2013).

3.2.2 Actions of the UPS on Plastids

The first evidence for a role of the UPS in controlling chloroplasts was the discovery of the interaction of a cytosolic ubiquitin E3 ligase, Arabidopsis C-terminus of HSC70-Interacting Protein (AtCHIP), with plastid preproteins in the cytosol (Lee et al., 2009; Shen et al., 2007).

Related to this, another study found that degradation of two plastid preproteins by the proteasome *in vitro* was dependent on the presence of their transit peptides (Sako et al., 2014). These observations reveal the presence of a quality control pathway for clearing away accumulated or aggregated plastid preproteins in the cytosol to prevent cytotoxic effects.

Proteomic studies aimed at characterising the Arabidopsis 'ubiquitinome' interestingly identified many ubiquitinated chloroplast proteins (Kim et al., 2013; Svozil et al., 2014). However, these results do not necessarily indicate direct regulation of plastids by the UPS as a transit peptide was found to be still attached for some of the proteins (implying modification in the cytosol prior to import), although not for all them. Moreover, many of the detected proteins function inside the plastid, and so it is unlikely that they were ubiquitinated at their final, functional destination. Other identified proteins were atToc159, atToc33 and atToc34, and there is substantial evidence for the ubiquitination of these proteins *in situ* at the chloroplast envelope, as discussed later; these proteomic studies support the ubiquitination of these TOC proteins *in vivo*. Another study specifically attempted to define the chloroplast outer membrane proteome and discovered E3 ligases

(Simm et al., 2013). However, the significance of these results is uncertain as some identifications could potentially have been due to sample contamination.

Other studies do support the existence of E3 ligases in plastids. The first, albeit tentative case was the RING-type E3 ligase, Shoot Gravitropism9 (SGR9) (Nakamura et al., 2011). SGR9 was shown to localize to the amyloplasts in root endodermal cells by analysis of a green fluorescent protein (GFP) fusion of a point mutant form, SGR9^{W244A}, with disabled ubiquitin ligase function. It was not possible to assess localization of the wild-type SGR9-GFP fusion, which was thought to be due to instability of the protein as RING-type E3s often auto-ubiquitinate to regulate their own abundance (de Bie and Ciechanover, 2011; Deshaies and Joazeiro, 2009); inactivation of the ubiquitination function stabilized SGR9 allowing fluorescence imaging. However, there is no further information on the *in vivo* sub-plastidic localization or topology of the SGR9 protein. There is some uncertainty concerning its role due to the fact that it is predicted to have a transit peptide (Ling and Jarvis, 2015a): OEM proteins do not typically possess a transit peptide, and an E3 ligase would be unlikely to function properly inside the plastid due to its isolation from other UPS components. That said, the biogenesis of plastid OEM proteins is not well understood; while most insert without the aid of a transit peptide, the Toc75 protein does possess a transit peptide, and it uses a stop-transfer mechanism for its assembly into the OEM (Day et al., 2014; Inoue et al., 2001; Inoue and Keegstra, 2003).

The *SGR9* gene was identified in a mutagenesis screen for gravitropism mutants: *sgr9* plants were found to have defects in amyloplast sedimentation, a phenomenon that is thought to enable gravity sensing by the cell. Nakamura et al. (2011) found that an actin filament mutant, *fiz1*, which induces fragmentation of actin, suppressed the gravitropism defect of

sgr9. They hypothesized that SGR9 facilitates amyloplast detachment from actin filaments to allow their sedimentation, although the targets of SGR9 are currently unknown.

Interestingly, another study found that atToc75 and atToc132 (which are thought to be ubiquitinated as a part of a different regulatory mechanism; see later) have important roles in gravitropism (Stanga et al., 2009). There is also evidence to indicate that the TOC machinery interacts with actin (Jouhet and Gray, 2009), and so it is tempting to speculate that translocon components are targets of SGR9. However, it is equally possible that SGR9 operates through a different mechanism entirely.

3.2.3 Actions of the UPS on the Plastid Protein Import Machinery

The first plastid E3 ligase to be identified with clearly established localization and topology was Suppressor of *ppi1* locus 1 (SP1) (Ling et al., 2012). SP1 was discovered in a mutagenesis screen designed to identify novel regulators of plastid protein import; i.e., second-site suppressors of *ppi1* (the atToc33 mutant) were sought. As mentioned earlier, *ppi1* has reduced ability to import photosynthetic proteins and a distinctive pale-yellow phenotype, but it is still able to complete its life-cycle (Jarvis et al., 1998). The *sp1* mutation was found to partially recover *ppi1* by allowing increased protein import, resulting in better developed chloroplasts and a greener plant. SP1 was predicted to have two transmembrane domains, and biochemical analysis showed it to be an integral component of the plastid outer membrane, with a C-terminal, cytosol-facing RING domain and an intermembrane space domain connecting the two transmembrane domains (Fig. 1). The cytosolic orientation of the RING domain gives SP1 access to other components of the UPS. Substitution of a critical residue in the RING domain interfered with ubiquitination of SP1 *in vitro* and *in vivo*, which

led to the conclusion that SP1 is regulated by auto-ubiquitination like other E3 ligases (de Bie and Ciechanover, 2011; Ling et al., 2012). The intermembrane space domain of SP1 was shown to be important for interaction with target proteins.

The identification and characterization of SP1 revealed for the first time that the UPS acts directly upon plastids to regulate their development, which it does by targeting components of the TOC machinery for proteasomal degradation (Fig. 1) (Ling et al., 2012). Analysis of *sp1* mutant and *SP1* overexpressor *Arabidopsis* plants showed that *SP1* expression levels correlate inversely with the abundance of TOC proteins *in vivo*. Co-immunoprecipitation and *in vitro* pull-down results showed that SP1 can interact with many *Arabidopsis* TOC proteins (atToc159, atToc132, atToc120, atToc34, atToc33 and atToc75), and as noted earlier such interaction was mediated primarily by the SP1 intermembrane space domain.

The SP1 protein was shown to mediate ubiquitination of atToc159, atToc75 and atToc33 *in vitro*, while the extent to which these components were ubiquitinated *in vivo* was dependent on the expression level of SP1 (Ling et al., 2012). Evidence that ubiquitinated TOC components are directed to the proteasome for degradation was provided by the observation that polyubiquitinated atToc33 is stabilized upon addition of the proteasome inhibitor MG132. The link between protein import and the proteasome was further supported by the observation that the phenotypes of *ppi1* and an atToc75-III hypomorphic mutant allele, *toc75-III-3*, are suppressed by the proteasome mutations *pbe1* or *rpn8a*, essentially phenocopying the suppression mediated by *sp1* (Ling et al., 2012).

The data described above, when considered alongside hypotheses discussed earlier concerning the roles of different TOC component isoforms in client-specific import pathways, suggested that SP1 could be a part of a mechanism that regulates TOC

component abundance during important developmental transitions (e.g., during etioplast-to-chloroplast conversion in de-etiolation, or chloroplast-to-gerontoplast conversion in leaf senescence): with the assistance of SP1, TOC complexes might rearrange to contain different component isoforms, allowing different proteins to be imported as required (Fig. 1). Evidence for this hypothesis was gained by carefully analysing de-etiolation and leaf senescence in *sp1* mutant and SP1 overexpressor plants: in *sp1* the rates of transition were slower than in wild type, whereas in the overexpressor plants the transitions happened more quickly (Ling et al., 2012). During de-etiolation, the abundance of TOC components was found to change rapidly in an SP1-dependent manner; most notably, the ratio of atToc159 to atToc132/120 increased markedly in the wild type, but hardly at all in the *sp1* mutant, possibly to enable the biogenesis of photosynthetic proteins which rapidly accumulate during de-etiolation (Ling et al., 2012).

3.2.4 Actions of the UPS on Plastids Under Abiotic Stress

A recent paper has given further insight into the function of SP1, and revealed an important role for plastid protein import regulation in plant responses to abiotic stress. Ling and Jarvis (2015b) observed that SP1 overexpressor plants display greater tolerance of osmotic, salinity and oxidative stresses, as determined by measuring seedling greening, development or survival. The observed stress tolerance was found to be related to the production of ROS (Fig. 2A), which can over-accumulate in chloroplasts under conditions which cause imbalances in photosynthetic electron transport, such as the aforementioned abiotic stress conditions. In this case, the abundance of the H₂O₂ (a particularly stable ROS) was negatively correlated with SP1 abundance under the stress conditions tested (Ling and Jarvis, 2015b).

To further support this connection, *sp1* was crossed to two mutants related to a ROS scavenging pathway, *sos2* and *enh1*. The *sp1 sos2* and *sp1 enh1* double mutants were found to display synergistically enhanced sensitivity when subjected to mild oxidative stress, a result that was interpreted to indicate that SP1 co-acts in ROS regulation along with SOS2 and ENH1; as SOS2 and ENH1 are known to operate in ROS scavenging, SP1 was proposed to operate in ROS avoidance (Ling and Jarvis, 2015b).

The accumulation of ROS is due to the overproduction of, and/or inability to use, energized electrons within the photosystems. ROS quenching pathways exist in the chloroplast, but their capacity can be exceeded under stress conditions (Shapiguzov et al., 2012; Suzuki et al., 2012). Ling and Jarvis (2015b) hypothesized that SP1 helps to control ROS accumulation by limiting the import of photosynthetic preproteins into chloroplasts, by targeting TOC components for removal under stress conditions, thereby limiting the maintenance and biogenesis of the photosystems and reducing photosynthetic activity (Fig. 2A). This notion was supported by the observation that TOC components are rapidly and specifically depleted in *Arabidopsis* plants during stress treatments, and that such changes are dependent on SP1: TOC components reached an even lower level in SP1 overexpressor plants following stress, whereas in *sp1* mutant plants such changes were very much reduced (Ling and Jarvis, 2015b). These effects on TOC component abundance were correlated with changes in protein import capacity, which further supported the hypothesis (Ling and Jarvis, 2015b).

A complementary pathway of chloroplast maintenance under oxidative stress involving ubiquitination was revealed by Woodson et al. (2015), who identified a cytosolic ubiquitin E3 ligase, Plant U-Box4 (PUB4), and proposed its involvement in the selective, vacuolar

degradation of whole, ROS-damaged chloroplasts by an unknown mechanism (Fig. 2B) As mentioned earlier, mitochondrial autophagy is closely associated with ubiquitination, which provides a targeting signal for the autophagic machinery. Damaged chloroplasts, or chloroplasts in dark-treated, senescing tissue, are known to be subject to recycling by autophagy in the vacuole. However, information on the autophagy of whole organelles is limited, and it is better known that components of chloroplasts undergo autophagy via Rubisco-containing bodies (RCBs) or other vesicles called ATI-PS bodies (named for their association with the autophagy-related protein ATG8-Interacting1 [ATI1], and with plastids) (Izumi et al., 2015; Li and Vierstra, 2012; Xie et al., 2015). A third pathway of chloroplast vacuolar degradation is not associated with autophagy, and is triggered by both senescence and abiotic stress. This pathway is dependent upon the Chloroplast Vesiculation (CV) protein, and involves the formation of CV-Containing Vesicles (CCVs) which mediate the transfer of stromal proteins, as well as thylakoid membrane and thylakoid lumen proteins, to the vacuole (Xie et al., 2015). Senescence Associated Vacuoles (SAVs) are a fourth pathway of chloroplast degradation, which exhibit independent proteolytic activity and are also independent of autophagy (Xie et al., 2015).

The *Arabidopsis pub4* mutant was identified as a suppressor of the *ferrochelatase 2 (fc2)* mutant, which affects the plastid-localized ferrochelatase 2 enzyme, a component of the tetrapyrrole biosynthetic pathway that differentiates the heme branch from the chlorophyll branch and synthesizes heme from protoporphyrin IX (Woodson et al., 2015).

Protoporphyrin IX accumulates in *fc2* and is a potent photosensitizing molecule, generating the ROS singlet oxygen (Shao et al., 2007). Under short-day conditions (i.e., 8 or fewer hours of light per 24 hour period), such production of singlet oxygen leads to chloroplast damage and subsequent chloroplast degradation, as observed by electron microscopy, resulting in a

failure to green and, ultimately, death of *fc2* mutants within three days (Woodson et al., 2015). In the *pub4 fc2* double mutant, plants are partially recovered and chloroplast number is increased, and such changes were attributed to the disabling of the selective degradation of damaged chloroplasts. Evidence for the PUB4-dependent ubiquitination of chloroplasts under stress was presented, but the *pub4* mutant does not share characteristics typical of autophagy-related mutants, indicating that PUB4 may operate via an alternative, unknown mechanism, or be a part of a highly specialized autophagic pathway. Possible effects of PUB4 on the abundance of known targets of ubiquitination, or indeed of any plastid proteins, have not been assessed, nor is there any indication as to what its targets might be in chloroplasts; PUB4 has not yet been shown to associate with plastid OEM proteins. Nonetheless, Woodson et al. (2015) present an interesting evidence for a new mechanism of ubiquitin-dependent regulation of chloroplast dynamics.

3.2.5 Future Prospects and Unanswered Questions Concerning the Regulation of Plastids by Ubiquitination

SP1 has two homologues in Arabidopsis, *SP1-like1* (*SPL1*) and *SPL2*, which share sequence and topological similarity with *SP1* and have been shown to localize to the chloroplast OEM by fluorescent protein fusion analysis (Ling et al., 2012). Interestingly, overexpression of *SPL1* was unable to complement *sp1 ppi1* (Ling et al., 2012), indicating that this gene is likely to have divergent functions, and therefore that there may be further, as yet unknown, roles for the UPS in the regulation of plastids. Further research on these homologues should prove to be very informative. Other research on the *SP1* family, by Basnayake et al. (2011), suggested that these proteins are involved in programmed cell death in response to biotic

stress. However, it should be noted that, although these proteins were selected by the authors as putative Arabidopsis homologues of Drosophila inhibitor of apoptosis 1 (DIAP1), the SP1 family of proteins do not actually share homology with DIAP1 outside of the highly-conserved RING domain (Basnayake et al., 2011). Nonetheless, the link to biotic stress presents another exciting avenue for research on the UPS regulation of plastids.

Experimental evidence suggests that apoptosis in response to biotic stress may include the autophagy of chloroplasts (Dong and Chen, 2013), and the implication of the PUB4 E3 ligase in chloroplast degradation suggests that ubiquitination signals could play a role. Although PUB4 is thought to be regulated by ROS stress to mediate the selective degradation of chloroplasts (Woodson et al., 2015), exactly where and how it fits into the chloroplast degradation pathway remains uncertain. Loss of PUB4 was shown to negatively affect the abundance of polyubiquitinated proteins in the *fc2* mutant background, but interestingly this was not the case in the wild-type *FC2* background which may indicate that the activity of PUB4 at chloroplasts is specific to certain conditions or to those chloroplasts which are damaged (Woodson et al., 2015).

A notable missing piece in the model for SP1- and UPS-mediated regulation of plastids is how SP1 activity is controlled. Is it differentially (de)stabilized under different conditions to mediate the ubiquitination of its target proteins at the correct moments, and/or is its target specificity regulated? Are there other factors involved in either of these processes, and if so what are they? Moreover, plastid OEM proteins like Toc75 are deeply embedded in the membrane and so may require energy for their removal prior to proteasomal degradation. In some cases, components of the proteasome's regulatory particle may be sufficient (Ruggiano et al., 2014), but in others it is conceivable that additional factors, such as specialized chaperone proteins, are required for substrate extraction.

3.3 Red Algal Derived Complex Plastids

Organisms containing complex plastids of red algal origin include cryptophytes, alveolates, stramenopiles and haptophytes; the so-called CASH lineages (Gould et al., 2015). The functions of the complex plastids in these species are diverse. Many of them retain phototrophy, but others do not. For example, some alveolates, a group which includes the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum*, contain plastids whose essential functions may be restricted to the production of certain isoprenoids and fatty acids (Seeber and Soldati-Favre, 2010).

Red complex plastids typically have four membranes (Gould et al., 2015; Zimorski et al., 2014), which may be counted from the outermost membrane to the innermost plastid membrane as follows: Membrane 1 is, with the exception of alveolates, contiguous with the host ER, reflecting the recently proposed origin of the two outermost plastid membranes (i.e., 1 and 2) as a host ER-derived envelope. Membrane 2 is the periplastidial membrane (PPM). The PPM encloses the periplastidial compartment (PPC) which contains the remnant of the algal nucleus, known as the nucleomorph, but has lost its other features like mitochondria (Gould et al., 2015; Zimorski et al., 2014). Membranes 3 and 4 are respectively equivalent to the outer and inner envelope membranes of primary chloroplasts.

As with primary plastids the majority of secondary plastid proteins are encoded in the host cell nucleus, with a minority encoded by the nucleomorph or the plastid itself. Thus, many cytosolically synthesized preproteins have several membranes to cross, and this requires complex mechanisms. Protein transport into complex plastids begins with the Sec61 complex, which co-translationally transports proteins into the lumen enclosed by the ER-

derived outermost membrane. Plastid-localized proteins of alveolates may enter vesicles of the secretory pathway to cross membrane 1, or via another mechanism involving direct contact between the ER and membrane 1; and these pathways also begin with Sec61-mediated co-translational transport into the ER (Gould et al., 2015; Zimorski et al., 2014). The transport system operating at membrane 2 of the complex plastid is known as SELMA (Fig. 3), and is of particular interest because it is a derivative of the ERAD system (Bolte et al., 2011; Gould et al., 2015; Stork et al., 2012). Transport across membranes 3 and 4 employs TOC and TIC complexes, respectively, as in primary plastids (Gould et al., 2015). In spite of the diversity of the organism groups containing them, complex red plastids are arguably of monophyletic origin: The existence of the SELMA translocon in all such lineages, having arisen through neofunctionalization of ERAD components, is cited as evidence for this, in much the same way that the ubiquity of the TOC/TIC machinery is taken as evidence for a single origin of all primary plastids (Zimorski et al., 2014).

3.3.1 A Role for Ubiquitin in Complex Plastid Development: The SELMA Translocon

Although its components are homologous to those of the ERAD system, SELMA is uncoupled from protein degradation at the proteasome (Fig. 3) (Stork et al., 2012). Thus, it appears that the ERAD system has been co-opted for its protein transport capability, and that SELMA is not a part of the UPS. Nonetheless, SELMA represents an interesting case of neofunctionalization of ERAD and UPS components, wherein ubiquitin modification plays a critical role in the translocation of proteins across the PPM, and so it is relevant to the discussion here. Further requirements for UPS-associated components in SELMA pertain to the removal of ubiquitin in the PPC, before preproteins can assume their functions or

engage onward transport through the TOC and TIC translocons (Agrawal et al., 2013; Hempel et al., 2010).

The first indication of the existence of an ERAD-like system operating at the PPM was the discovery of genes encoding homologues of the ERAD components Der1, Hrd1, Ufd1 and Cdc48 in the nucleomorph genome of *Guillardia theta* (Sommer et al., 2007). This study demonstrated that the nucleomorph *Der1* gene can partially complement a yeast $\Delta der1$ mutant (confirming its ability to encode functional Der1). Sommer et al. (2007) also discovered duplicated ERAD component genes in other organisms of the red algal lineage, but these were nucleus-encoded and contained a plastid targeting signal. The lack of an endomembrane system within the PPC, where an ERAD system might operate, led the authors to hypothesize that the symbiont-specific Der1 is involved in preprotein translocation across the PPM. Supporting this hypothesis, Agrawal et al. (2009) revealed a plastid protein import defect in an apicoplast *der1* mutant of *T. gondii*, which was described as being similar to that of a *T. gondii tic20* mutant, a mutant of an inner envelope (innermost membrane) translocon component (van Dooren et al., 2008). Further information on SELMA Der1 arose from studies on the diatom *Phaeodactylum tricornutum*, which has two symbiont-specific Der1 homologues, sDer1-1 and sDer1-2, that both localize to the PPM and can form homo- and heteromeric complexes, and may be able to interact with certain plastid preproteins (Hempel et al., 2009). In the apicomplexan *P. falciparum*, duplicated genes predicted to encode ERAD components with plastid targeting signals were identified, and these included Der1-1 and Der1-2, Cdc48 and its co-factor Ufd1, ubiquitin, an E2 ubiquitin conjugating enzyme, Ubc, and two E1 ubiquitin activating enzymes, Uba1 and Uba2; moreover, plastid localization was shown for the Der1-1 and Uba1 homologues (Spork et al., 2009). A symbiont-localized E3 was later found in *P. falciparum*, while a

deubiquitinase was also found in *P. tricornutum*, providing evidence (although not yet within the same organism) of a near complete repertoire of components which would be required for SELMA function (Hempel et al., 2010). Subsequently, molecular phylogenetic studies identified the presence of duplicated ERAD components with PPC targeting signals in multiple CASH organisms (Stork et al., 2012).

Mechanistic details are now beginning to emerge, as components believed to be necessary for SELMA function are characterized. It was previously shown that a Cdc48 homologue is localized to the apicoplast in *T. gondii*, while more recent work identified and confirmed the *in vivo* localizations, and the *in vitro* functions, of the PPC E1, E2 and E3 ubiquitination components in *P. falciparum* (Agrawal et al., 2013; Agrawal et al., 2009). However, it should be noted that the symbiont E3s in the apicomplexa may not be related to Hrd1, and that the predicted symbiont Hrd1 genes in other lineages appear to lack some of the transmembrane domains present in the canonical ERAD component (Stork et al., 2012). The *in vivo* role of ubiquitination in PPM protein transport was supported by studies on a *T. gondii* inducible knockdown mutant of an apicoplast E2 (Agrawal et al., 2013): when the E2 was knocked down, parasite growth was severely impaired, and this was linked to an apicoplast protein import defect. The function of the apicoplast E2 was also shown to be dependent on a cysteine residue at the putative active site, indicating conserved function of the protein (Agrawal et al., 2013). Overall, these findings regarding plastid-localized ubiquitination system proteins validate a critical mechanistic component of the SELMA model. Very recently, a link between the ubiquitination components and the preproteins themselves has been established, as amino-terminal lysine residues have been found to be critical for the translocation of preproteins across the PPM (Lau et al., 2015). This is significant, as ubiquitin is attached almost exclusively to lysine residues of substrate proteins.

3.3.2 Future Prospects and Unanswered Questions Concerning SELMA

The discovery of the SELMA translocon reveals an interesting evolutionary story and solution to the problem of targeting proteins across the second outermost membrane of complex plastids of the red lineage. However, the pathway and each of the mechanisms which comprise SELMA have yet to be fully characterized. Some areas in which we lack understanding are very broad, for instance regarding the mechanism and substrate interaction of the Cdc48 complex which is involved in many aspects of cell regulation; others may be applicable to ERAD as well as SELMA, most notably regarding the identity and structure of the (retro)translocon; and others relate specifically to SELMA. Although many core ERAD-like components thought to comprise the SELMA translocon have been identified, it does not appear that all putative components have been characterized in a single organism. Moreover, key features remain elusive. For example, chaperone or targeting factors that might direct the preproteins to the translocon have not been discovered. It also appears that some components that are essential to ERAD, which one might expect to be just as critical to SELMA, have not been identified; one such example is Npl4, which is thought to form a heterodimer with Ufd1 to act as a cofactor for Cdc48 (Wolf and Stolz, 2012). Such components may have eluded identification thus far, but it is also possible that other proteins fulfil equivalent roles in SELMA, or that these factors are simply not necessary for translocon function.

4. Concluding Remarks

Ubiquitin modifications have a well-established role in the regulation of mitochondrial dynamics, but their influence over plastids is only just beginning to be uncovered.

Nonetheless, currently available information on ubiquitin-related regulation of plastids already reveals considerable diversity of function. The discovery of SP1 revealed a role for the UPS in plant plastid interconversions, most notably during chloroplast development.

More recently, both SP1 and the PUB4 E3 ligase have been shown to regulate plastids under ROS stress, albeit via different mechanisms: SP1 acting to enable a ROS avoidance strategy, and PUB4 acting to selectively-degrade chloroplasts which are already damaged. A role for ubiquitination directly related to protein import in complex plastids has also been established, with the discovery of SELMA. In this system, ERAD components and the ubiquitination, uncoupled from proteasomal degradation, have been co-opted for their ability to mediate protein targeting across membranes. These recent insights have raised further interesting questions concerning the roles played by ubiquitin modification in the biogenesis and homeostasis of endosymbiotic organelles, and have provided great opportunities to further our understanding of these essential organelles.

Acknowledgements

We are grateful to Robert Sowden for assistance in the preparation of the figures. Work in the authors' laboratory was supported by the Biotechnology and Biological Sciences Research Council (BBSRC; grant numbers BB/J017256, BB/J009369 and BB/K018442/1).

Figures and Tables

Figure 1. Regulation of Protein Import Into Plastids by the Ubiquitin E3 Ligase SP1.

The figure shows a schematic representation of two outer membrane translocon types:

Type I consists of atToc33, atToc75-III and atToc159, and is believed to preferentially transport highly-abundant photosynthesis-related preproteins; Type II consists of atToc34, atToc75-III, and atToc132 and/or atToc120, and is believed to have preference for preproteins of other metabolic pathways and general functions of the plastid, such as plastid maintenance. At the onset of chloroplast biogenesis, the ubiquitin E3 ligase SP1 ubiquitinates components of the Type II TOC translocon to signal their proteasomal degradation, possibly to facilitate the assembly of components comprising the Type I translocon in the outer membrane. SP1 also ubiquitinates the Type I TOC complex during reactive oxygen species (ROS) stress, as part of a ROS avoidance strategy. Finally, SP1 may ubiquitinate TOC components to enable the transformation of chloroplasts into other plastid types, for example during senescence, and plastid type interconversions more generally. RING, Really Interesting New Gene, an E3 domain which interacts with ubiquitin E2 conjugating enzymes. OEM, Outer Envelope Membrane of the plastid. IMS, Intermembrane Space.

Figure 2. The Regulation of Chloroplasts Under Stress Conditions by Ubiquitin Modification.

A. A mechanism for ROS avoidance is implemented under moderate abiotic stress (e.g., salinity or osmotic stresses). 1. Under normal conditions, proteins are imported into the chloroplast through the TOC translocon. 2. These imported proteins are required for the

biogenesis and maintenance of the photosynthetic apparatus, as well as other metabolic functions. 3. Under such conditions, the OEM E3 ligase SP1 regulates its abundance by autoubiquitination. 4. Under moderate abiotic stress, chloroplast functions are affected. 5. Damaging reactive oxygen species (ROS) accumulate as a result of imbalances in photosynthesis. 6. In response, SP1 becomes stabilized via an unknown pathway. 7. SP1 is then able to target TOC components and so reduce protein import into the chloroplast. 8. Reduced protein import reduces the biogenesis and maintenance of the photosynthetic apparatus, and so photosynthesis is attenuated leading to reduced ROS accumulation. 9. When ROS accumulation is reduced, or the abiotic stress is alleviated, the system can return to normal.

B. A separate, quality-control mechanism selectively degrades damaged chloroplasts under more severe stress. 1. Some chloroplasts accumulate and are severely damaged by ROS under strongly photo-oxidizing conditions. 2. Through an unknown mechanism, certain plastid proteins (presumably in the outer envelope membrane) may become selectively ubiquitinated by the cytosolic E3 ligase PUB4. 3. This leads to the selective removal of these damaged chloroplasts through an unknown mechanism, possibly involving the vacuole. Ub, Ubiquitin.

Figure 3. The SELMA Translocation Pathway in Complex Plastids of the Red Lineage.

1. Plastid preproteins are co-translationally transported across Membrane 1 (or across the ER membrane) via the Sec61 complex. 2. Once in the adjacent lumenal space, preproteins are targeted through an unknown mechanism to the translocon of the periplastidial membrane (PPM; Membrane 2), and are transported across the membrane by symbiont-

specific sDer1-1 and sDer1-2, and possibly an sE3 ligase. 3. Ubiquitin is activated by the sE1 in an ATP-dependent manner, and is transferred to an sE2; the PPM-localized sE3 then mediates the ubiquitination of preproteins as they become exposed to the periplastidial compartment. 4. The sCdc48 complex, in conjunction with the adapter protein sUfd1, binds to ubiquitinated preproteins; sCdc48 then uses the energy of ATP hydrolysis to drive the translocation of preproteins into the periplastidial compartment. 5. Ubiquitin conjugated to preproteins is removed by a symbiont-localized deubiquitinase (sDUB) in the periplastidial compartment, allowing the preproteins to assume their functions or be further transported. Note that the lower case 's' prefix indicates symbiont localization for each of the relevant ERAD-related proteins. Ub, Ubiquitin.

References

- Agrawal, S., Chung, D.W., Ponts, N., van Dooren, G.G., Prudhomme, J., Brooks, C.F., Rodrigues, E.M., Tan, J.C., Ferdig, M.T., Striepen, B., Le Roch, K.G., 2013. An apicoplast localized ubiquitylation system is required for the import of nuclear-encoded plastid proteins. *PLoS Pathog.* 9, e1003426.
- Agrawal, S., van Dooren, G.G., Beatty, W.L., Striepen, B., 2009. Genetic evidence that an endosymbiont-derived endoplasmic reticulum-associated protein degradation (ERAD) system functions in import of apicoplast proteins. *J. Biol. Chem.* 284, 33683-33691.
- Baldwin, A., Wardle, A., Patel, R., Dudley, P., Park, S.K., Twell, D., Inoue, K., Jarvis, P., 2005. A molecular-genetic study of the *Arabidopsis* Toc75 gene family. *Plant Physiol.* 138, 715-733.
- Basnayake, B.M., Li, D., Zhang, H., Li, G., Virk, N., Song, F., 2011. *Arabidopsis* DAL1 and DAL2, two RING finger proteins homologous to *Drosophila* DIAP1, are involved in regulation of programmed cell death. *Plant Cell Rep.* 30, 37-48.
- 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.
- Blancaflor, E.B., 2013. Regulation of plant gravity sensing and signaling by the actin cytoskeleton. *Am. J. Bot.* 100, 143-152.
- Bolte, K., Gruenheit, N., Felsner, G., Sommer, M.S., Maier, U.G., Hempel, F., 2011. Making new out of old: recycling and modification of an ancient protein translocation system during eukaryotic evolution. Mechanistic comparison and phylogenetic analysis of ERAD, SELMA and the peroxisomal importomer. *BioEssays* 33, 368-376.
- Bragoszewski, P., Gornicka, A., Sztolsztener, M.E., Chacinska, A., 2013. The ubiquitin-proteasome system regulates mitochondrial intermembrane space proteins. *Mol. Cell. Biol.* 33, 2136-2148.

- Brautigam, A., Weber, A.P., 2009. Proteomic analysis of the proplastid envelope membrane provides novel insights into small molecule and protein transport across proplastid membranes. *Mol. Plant* 2, 1247-1261.
- Carvalho, P., Goder, V., Rapoport, T.A., 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126, 361-373.
- 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.
- Day, P.M., Potter, D., Inoue, K., 2014. Evolution and targeting of Omp85 homologs in the chloroplast outer envelope membrane. *Front. Plant Sci.* 5, 535.
- de Bie, P., Ciechanover, A., 2011. Ubiquitination of E3 ligases: self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. *Cell Death Differ.* 18, 1393-1402.
- Deshaies, R.J., Joazeiro, C.A., 2009. RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* 78, 399-434.
- Dong, J., Chen, W., 2013. The role of autophagy in chloroplast degradation and chlorophagy in immune defenses during *Pst DC3000 (AvrRps4)* infection. *PLoS ONE* 8, e73091.
- Dutta, S., Teresinski, H.J., Smith, M.D., 2014. A split-ubiquitin yeast two-hybrid screen to examine the substrate specificity of atToc159 and atToc132, two *Arabidopsis* chloroplast preprotein import receptors. *PLoS ONE* 9, e95026.
- Esaki, M., Ogura, T., 2012. Cdc48p/p97-mediated regulation of mitochondrial morphology is Vms1p-independent. *J. Struct. Biol.* 179, 112-120.
- Escobar-Henriques, M., Langer, T., 2014. Dynamic survey of mitochondria by ubiquitin. *EMBO Rep.* 15, 231-243.
- Franz, A., Kevei, E., Hoppe, T., 2015. Double-edged alliance: mitochondrial surveillance by the UPS and autophagy. *Curr. Opin. Cell Biol.* 37, 18-27.
- Gould, S.B., Maier, U.G., Martin, W.F., 2015. Protein import and the origin of red complex plastids. *Curr. Biol.* 25, R515-R521.

- Gould, S.B., Waller, R.F., McFadden, G.I., 2008. Plastid evolution. *Annu. Rev. Plant Biol.* 59, 491-517.
- Gray, M.W., Burger, G., Lang, B.F., 1999. Mitochondrial evolution. *Science* 283, 1476-1481.
- Guerra, D.D., Callis, J., 2012. Ubiquitin on the move: the ubiquitin modification system plays diverse roles in the regulation of endoplasmic reticulum- and plasma membrane-localized proteins. *Plant Physiol.* 160, 56-64.
- Gutensohn, M., Schulz, B., Nicolay, P., Flügge, U.I., 2000. Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. *Plant J.* 23, 771-783.
- Hempel, F., Bullmann, L., Lau, J., Zauner, S., Maier, U.G., 2009. ERAD-derived preprotein transport across the second outermost plastid membrane of diatoms. *Mol. Biol. Evol.* 26, 1781-1790.
- Hempel, F., Felsner, G., Maier, U.G., 2010. New mechanistic insights into pre-protein transport across the second outermost plastid membrane of diatoms. *Mol. Microbiol.* 76, 793-801.
- Holt, M.T., David, Y., Pollock, S., Tang, Z., Jeon, J., Kim, J., Roeder, R.G., Muir, T.W., 2015. Identification of a functional hotspot on ubiquitin required for stimulation of methyltransferase activity on chromatin. *Proc. Natl. Acad. Sci. USA* 112, 10365-10370.
- 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.
- Infanger, S., Bischof, S., Hiltbrunner, A., Agne, B., Baginsky, S., Kessler, F., 2010. The chloroplast import receptor Toc90 partially restores the accumulation of Toc159 client proteins in the *Arabidopsis thaliana* *ppi2* mutant. *Mol. Plant* 4, 252-263.
- Inoue, K., Demel, R., de Kruijff, B., Keegstra, K., 2001. The N-terminal portion of the preToc75 transit peptide interacts with membrane lipids and inhibits binding and import of precursor proteins into isolated chloroplasts. *Eur. J. Biochem.* 268, 4036-4043.

- 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.
- Ivanova, Y., Smith, M.D., Chen, K., Schnell, D.J., 2004. Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. *Mol. Biol. Cell* 15, 3379-3392.
- Izumi, M., Hidema, J., Ishida, H., 2015. From *Arabidopsis* to cereal crops: Conservation of chloroplast protein degradation by autophagy indicates its fundamental role in plant productivity. *Plant Signal. Behav.*, doi:10.1080/15592324.2015.1101199.
- Jarvis, P., Chen, L.J., Li, H., Peto, C.A., Fankhauser, C., Chory, J., 1998. An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* 282, 100-103.
- Jarvis, P., Lopez-Juez, E., 2013. Biogenesis and homeostasis of chloroplasts and other plastids. *Nat. Rev. Mol. Cell Biol.* 14, 787-802.
- Jelic, M., Soll, J., Schleiff, E., 2003. Two Toc34 homologues with different properties. *Biochemistry* 42, 5906-5916.
- Jia, F., Wang, C., Huang, J., Yang, G., Wu, C., Zheng, C., 2015. SCF E3 ligase PP2-B11 plays a positive role in response to salt stress in *Arabidopsis*. *J. Exp. Bot.* 66, 4683-4697.
- Jouhet, J., Gray, J.C., 2009. Interaction of actin and the chloroplast protein import apparatus. *J. Biol. Chem.* 284, 19132-19141.
- Kalanon, M., Tonkin, C.J., McFadden, G.I., 2009. Characterization of two putative protein translocation components in the apicoplast of *Plasmodium falciparum*. *Eukaryot. Cell* 8, 1146-1154.
- Kane, L.A., Lazarou, M., Fogel, A.I., Li, Y., Yamano, K., Sarraf, S.A., Banerjee, S., Youle, R.J., 2014. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *J. Cell Biol.* 205, 143-153.

- Kazlauskaitė, A., Kondapalli, C., Gourlay, R., Campbell, D.G., Ritorto, M.S., Hofmann, K., Alessi, D.R., Knebel, A., Trost, M., Muqit, M.M., 2014. Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. *Biochem. J.* 460, 127-139.
- 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, 363-371.
- Kim, D.Y., Scalf, M., Smith, L.M., Vierstra, R.D., 2013. Advanced proteomic analyses yield a deep catalog of ubiquitylation targets in *Arabidopsis*. *Plant Cell* 25, 1523-1540.
- Kleiger, G., Mayor, T., 2014. Perilous journey: a tour of the ubiquitin-proteasome system. *Trends Cell Biol.* 24, 352-359.
- Koenig, P., Oreb, M., Hofle, A., Kaltofen, S., Rippe, K., Sinning, I., Schleiff, E., Tews, I., 2008. The GTPase cycle of the chloroplast import receptors Toc33/Toc34: implications from monomeric and dimeric structures. *Structure* 16, 585-596.
- Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H., Yoshihara, H., Hirokawa, T., Endo, T., Fon, E.A., Trempe, J.F., Saeki, Y., Tanaka, K., Matsuda, N., 2014. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* 510, 162-166.
- 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.
- Kubis, S., Patel, R., Combe, J., Bédard, J., Kovacheva, S., Lilley, K., Biehl, A., Leister, D., Ríos, G., Koncz, C., Jarvis, P., 2004. Functional specialization amongst the *Arabidopsis* Toc159 family of chloroplast protein import receptors. *Plant Cell* 16, 2059-2077.
- Lau, J.B., Stork, S., Moog, D., Sommer, M.S., Maier, U.G., 2015. N-terminal lysines are essential for protein translocation via a modified ERAD system in complex plastids. *Mol. Microbiol.* 96, 609-620.

- Lee, S., Lee, D.W., Lee, Y., Mayer, U., Stierhof, Y.D., 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.
- Li, F., Vierstra, R.D., 2012. Autophagy: a multifaceted intracellular system for bulk and selective recycling. *Trends Plant Sci.* 17, 526-537.
- Li, H.M., Chiu, C.C., 2010. Protein transport into chloroplasts. *Annu. Rev. Plant Biol.* 61, 157-180.
- Li, H.M., Teng, Y.S., 2013. Transit peptide design and plastid import regulation. *Trends Plant Sci.* 18, 360-366.
- Li, M., Rong, Y., Chuang, Y.S., Peng, D., Emr, S.D., 2015. Ubiquitin-dependent lysosomal membrane protein sorting and degradation. *Mol. Cell* 57, 467-478.
- 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.
- Ling, Q., Jarvis, P., 2013. Dynamic regulation of endosymbiotic organelles by ubiquitination. *Trends Cell Biol.* 23, 399-408.
- Ling, Q., Jarvis, P., 2015a. Functions of plastid protein import and the ubiquitin-proteasome system in plastid development. *Biochim. Biophys. Acta* 1847, 939-948.
- Ling, Q., Jarvis, P., 2015b. Regulation of chloroplast protein import by the ubiquitin E3 Ligase SP1 is important for stress tolerance in plants. *Curr. Biol.* 25, 2527-2534.
- Livnat-Levanon, N., Glickman, M.H., 2011. Ubiquitin-proteasome system and mitochondria - reciprocity. *Biochim. Biophys. Acta* 1809, 80-87.
- Mehnert, M., Sommermeyer, F., Berger, M., Kumar Lakshmipathy, S., Gauss, R., Aebi, M., Jarosch, E., Sommer, T., 2015. The interplay of Hrd3 and the molecular chaperone system ensures efficient degradation of malformed secretory proteins. *Mol. Biol. Cell* 26, 185-194.
- Mishra, P., Chan, D.C., 2014. Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat. Rev. Mol. Cell Biol.* 15, 634-646.

- Misra, R., Stikeleather, R., Gabriele, R., 2015. In vivo roles of BamA, BamB and BamD in the biogenesis of BamA, a core protein of the beta-barrel assembly machine of *Escherichia coli*. J. Mol. Biol. 427, 1061-1074.
- Nakamura, M., Toyota, M., Tasaka, M., Morita, M.T., 2011. An *Arabidopsis* E3 ligase, SHOOT GRAVITROPISM9, modulates the interaction between statoliths and F-actin in gravity sensing. Plant Cell 23, 1830-1848.
- Nelson, M.S., Sadowsky, M.J., 2015. Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. Front. Plant Sci. 6, 491.
- Okamoto, K., Shaw, J.M., 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu. Rev. Genet. 39, 503-536.
- Pan, R., Kaur, N., Hu, J., 2014. The *Arabidopsis* mitochondrial membrane-bound ubiquitin protease UBP27 contributes to mitochondrial morphogenesis. Plant J. 78, 1047-1059.
- Richardson, L.G.L., Paila, Y.D., Siman, S.R., Chen, Y., Smith, M.D., Schnell, D.J., 2014. Targeting and assembly of components of the TOC protein import complex at the chloroplast outer envelope membrane. Front. Plant Sci. 5, 1-14.
- Richly, H., Rape, M., Braun, S., Rumpf, S., Hoegel, C., Jentsch, S., 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell 120, 73-84.
- Richter, S., Lamppa, G.K., 2003. Structural properties of the chloroplast stromal processing peptidase required for its function in transit peptide removal. J. Biol. Chem. 278, 39497-39502.
- Ruggiano, A., Foresti, O., Carvalho, P., 2014. Quality control: ER-associated degradation: protein quality control and beyond. J. Cell Biol. 204, 869-879.
- Sadanandom, A., Bailey, M., Ewan, R., Lee, J., Nelis, S., 2012. The ubiquitin-proteasome system: central modifier of plant signalling. New Phytol. 196, 13-28.

- Sako, K., Yanagawa, Y., Kanai, T., Sato, T., Seki, M., Fujiwara, M., Fukao, Y., Yamaguchi, J., 2014. Proteomic analysis of the 26S proteasome reveals its direct interaction with transit peptides of plastid protein precursors for their degradation. *J. Proteome Res.* 13, 3223-3230.
- Sarraf, S.A., Raman, M., Guarani-Pereira, V., Sowa, M.E., Huttlin, E.L., Gygi, S.P., Harper, J.W., 2013. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature* 496, 372-376.
- Sato, E.M., Hijazi, H., Bennett, M.J., Vissenberg, K., Swarup, R., 2015. New insights into root gravitropic signalling. *J. Exp. Bot.* 66, 2155-2165.
- Schleiff, E., Soll, J., K  chler, M., Kuhlbrandt, W., Harrer, R., 2003. Characterization of the translocon of the outer envelope of chloroplasts. *J. Cell Biol.* 160, 541-551.
- Schmidt, O., Pfanner, N., Meisinger, C., 2010. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* 11, 655-667.
- Seeber, F., Soldati-Favre, D., 2010. Metabolic pathways in the apicoplast of apicomplexa. *Int. Rev. Cell Mol. Biol.* 281, 161-228.
- Shao, N., Krieger-Liszkay, A., Schroda, M., Beck, C.F., 2007. A reporter system for the individual detection of hydrogen peroxide and singlet oxygen: its use for the assay of reactive oxygen species produced in vivo. *Plant J.* 50, 475-487.
- Shapiguzov, A., Vainonen, J.P., Wrzaczek, M., Kangasjarvi, J., 2012. ROS-talk - how the apoplast, the chloroplast, and the nucleus get the message through. *Front. Plant Sci.* 3, 292.
- Shen, G., Adam, Z., Zhang, H., 2007. The E3 ligase AtCHIP ubiquitylates FtsH1, a component of the chloroplast FtsH protease, and affects protein degradation in chloroplasts. *Plant J.* 52, 309-321.
- Shi, L.X., Theg, S.M., 2013. The chloroplast protein import system: from algae to trees. *Biochim. Biophys. Acta* 1833, 314-331.

- Shiba-Fukushima, K., Imai, Y., Yoshida, S., Ishihama, Y., Kanao, T., Sato, S., Hattori, N., 2012. PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci. Rep.* 2, 1002.
- Simm, S., Papasotiriou, D.G., Ibrahim, M., Leisegang, M.S., Muller, B., Schorge, T., Karas, M., Mirus, O., Sommer, M.S., Schleiff, E., 2013. Defining the core proteome of the chloroplast envelope membranes. *Front. Plant Sci.* 4, 11.
- Sommer, M.S., Daum, B., Gross, L.E., Weis, B.L., Mirus, O., Abram, L., Maier, U.G., Kuhlbrandt, W., Schleiff, E., 2011. Chloroplast Omp85 proteins change orientation during evolution. *Proc. Natl. Acad. Sci. USA* 108, 13841-13846.
- Sommer, M.S., Gould, S.B., Lehmann, P., Gruber, A., Przyborski, J.M., Maier, U.G., 2007. Der1-mediated preprotein import into the periplastid compartment of chromalveolates? *Mol. Biol. Evol.* 24, 918-928.
- Spork, S., Hiss, J.A., Mandel, K., Sommer, M., Kooij, T.W., Chu, T., Schneider, G., Maier, U.G., Przyborski, J.M., 2009. An unusual ERAD-like complex is targeted to the apicoplast of *Plasmodium falciparum*. *Eukaryot. Cell* 8, 1134-1145.
- Stanga, J.P., Boonsirichai, K., Sedbrook, J.C., Otegui, M.S., Masson, P.H., 2009. A role for the TOC complex in *Arabidopsis* root gravitropism. *Plant Physiol.* 149, 1896-1905.
- Stork, S., Moog, D., Przyborski, J.M., Wilhelmi, I., Zauner, S., Maier, U.G., 2012. Distribution of the SELMA translocon in secondary plastids of red algal origin and predicted uncoupling of ubiquitin-dependent translocation from degradation. *Eukaryot. Cell* 11, 1472-1481.
- Suzuki, N., Koussevitzky, S., Mittler, R., Miller, G., 2012. ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ.* 35, 259-270.
- Svozil, J., Hirsch-Hoffmann, M., Dudler, R., Gruissem, W., Baerenfaller, K., 2014. Protein abundance changes and ubiquitylation targets identified after inhibition of the proteasome with Syngolin A. *Mol. Cell. Proteomics* 13, 1523-1536.

- Teixeira, P.F., Glaser, E., 2013. Processing peptidases in mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1833, 360-370.
- Teng, Y.S., Chan, P.T., Li, H.M., 2012. Differential age-dependent import regulation by signal peptides. *PLoS Biol.* 10, e1001416.
- Thrower, J.S., Hoffman, L., Rechsteiner, M., Pickart, C.M., 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94-102.
- van Dooren, G.G., Tomova, C., Agrawal, S., Humbel, B.M., Striepen, B., 2008. *Toxoplasma gondii* Tic20 is essential for apicoplast protein import. *Proc. Natl. Acad. Sci. USA* 105, 13574-13579.
- van Wijk, K.J., 2015. Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. *Annu. Rev. Plant Biol.* 66, 75-111.
- Vembar, S.S., Brodsky, J.L., 2008. One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* 9, 944-957.
- Vierstra, R.D., 2009. The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.* 10, 385-397.
- Villarejo, A., Buren, S., Larsson, S., Dejardin, A., Monne, M., Rudhe, C., Karlsson, J., Jansson, S., Lerouge, P., Rolland, N., von Heijne, G., Grebe, M., Bako, L., Samuelsson, G., 2005. Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. *Nat. Cell Biol.* 7, 1124-1131.
- von Mikecz, A., Chen, M., Rockel, T., Scharf, A., 2008. The nuclear ubiquitin-proteasome system: visualization of proteasomes, protein aggregates, and proteolysis in the cell nucleus. *Methods Mol. Biol.* 463, 191-202.
- Wallas, T.R., Smith, M.D., Sanchez-Nieto, S., Schnell, D.J., 2003. The roles of Toc34 and Toc75 in targeting the Toc159 preprotein receptor to chloroplasts. *J. Biol. Chem.* 278, 44289-44297.
- Walsh, C.K., Sadanandom, A., 2014. Ubiquitin chain topology in plant cell signaling: a new facet to an evergreen story. *Front. Plant Sci.* 5.

- Wise, R.R., Hooper, J.K., 2006. The structure and function of plastids. In *Advances in Photosynthesis and Respiration*, 1st ed, Vol. 23, pp. 3-26. Springer Netherlands, Dordrecht.
- Wolf, D.H., Stolz, A., 2012. The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochim. Biophys. Acta* 1823, 117-124.
- Woodson, J.D., Joens, M.S., Sinson, A.B., Gilkerson, J., Salome, P.A., Weigel, D., Fitzpatrick, J.A., Chory, J., 2015. Ubiquitin facilitates a quality-control pathway that removes damaged chloroplasts. *Science* 350, 450-454.
- Xie, Q., Michaeli, S., Peled-Zehavi, H., Galili, G., 2015. Chloroplast degradation: one organelle, multiple degradation pathways. *Trends Plant Sci.* 20, 264-265.
- Yan, X., Khan, S., Hase, T., Emes, M.J., Bowsher, C.G., 2006. Differential uptake of photosynthetic and non-photosynthetic proteins by pea root plastids. *FEBS Lett.* 580, 6509-6512.
- Zimorski, V., Ku, C., Martin, W.F., Gould, S.B., 2014. Endosymbiotic theory for organelle origins. *Curr. Opin. Microbiol.* 22, 38-48.

Figure 1

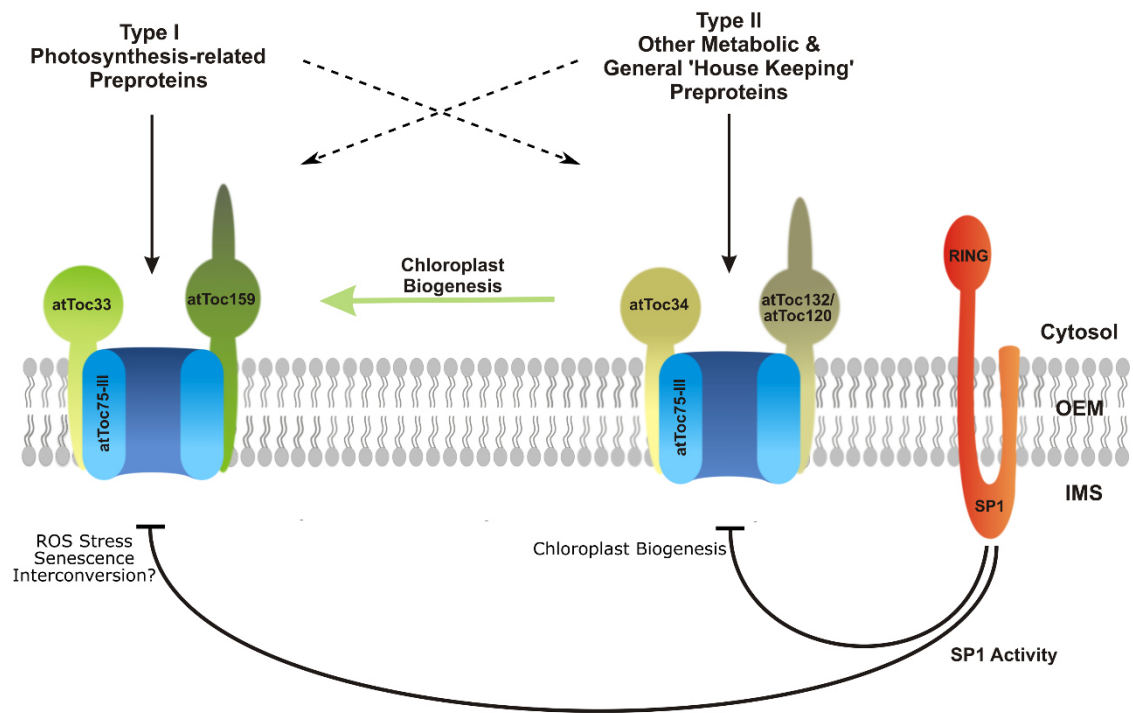


Figure 2

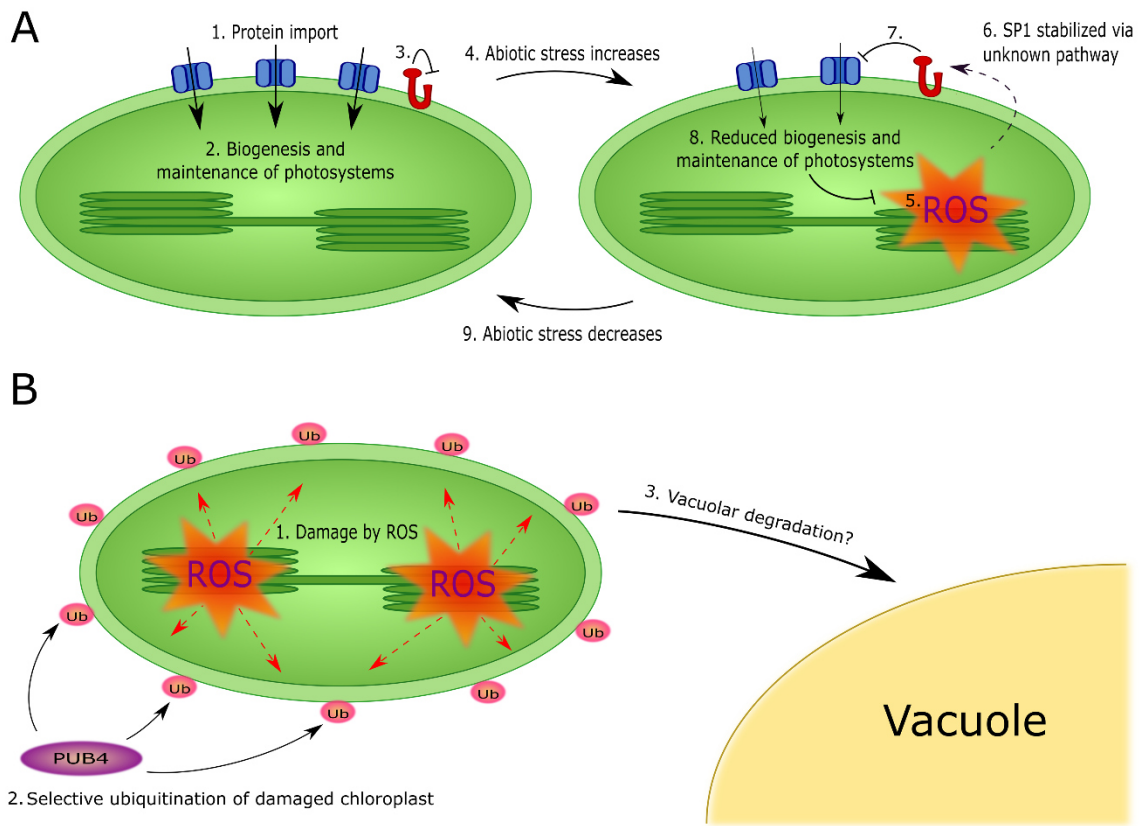


Figure 3

