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Post-translational modifications of TOC159 at early developmental stages in *Arabidopsis thaliana*

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“Post-translational modifications of TOC-159 at early developmental stages in *Arabidopsis thaliana*”

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Le Doyen, Prof. P. Felber

P. Fel

A tutti i nonni del mondo.

Abstract

Plant cells are eukaryotic and contain chloroplasts, a double-membrane-bound organelle specialized in photosynthesis. Non-photosynthetic proplastids convert into photosynthetically active chloroplasts during germination or when dark-grown seedlings are transferred to the light. Chloroplast biogenesis requires the import of different precursor proteins from the cytoplasm. Precursor proteins are synthesized in the cytosol with a cleavable amino-terminal targeting sequence called transit peptide. They are imported post-translationally into the chloroplast through the TOC-TIC protein complex (Translocon of the Outer and Inner membranes of the Chloroplast, respectively). The TOC complex is composed of two homologous receptor GTPases, Toc33 and Toc159, and a protein-conducting channel, Toc75. Together these proteins form the core of the TOC complex. Additional factors direct the preproteins from the cytosol to the TOC complex: the so-called guidance complex binds to TOC receptors in a GTP-dependent manner to deliver precursor proteins. Chloroplast biogenesis requires gibberellic acid (GA), whereas DELLA proteins act as negative regulators of GA signaling, known to inhibit seed germination, seed growth, and other GA-dependent pathways. My results show that Toc159 binds directly to DELLA and is targeted for degradation by the ubiquitin-proteasome pathway. Accumulation of Toc159 depends on gibberellic acid during early stages of plant development, as it leads to the degradation of DELLA thereby releasing TOC159 from proteasome-mediated degradation to promote chloroplast biogenesis. Apart from ubiquitination, Toc159 is also known to be post-translationally modified by phosphorylation although the functional significance of this is not understood.

An important part of my thesis is dedicated to a third type of post-translational modification, SUMOylation. TOC159 both binds the SUMO3 isoform at its GTP-binding (G-) domain and is covalently modified by SUMO3 at its membrane (M-) domain. The results show that covalent SUMOylation increases the stability of Toc159 under low gibberellic acid conditions. While it is not clear what the role of non-covalent SUMO-binding is, I hypothesize that covalent SUMOylation at the M-domain finetunes Toc159 stability and concentrations depending on internal and external conditions.

Keywords: Chloroplast biogenesis; TOC159; SUMOylation; *Arabidopsis thaliana*.

Résumé de thèse

Les cellules végétales sont eucaryotes et contiennent des chloroplastes, un organite à double membrane spécialisé dans la photosynthèse. Les proplastides non photosynthétiques se transforment en chloroplastes photosynthétiquement actifs pendant la germination ou lorsque des semis cultivés à l'obscurité sont transférés à la lumière. La biogenèse des chloroplastes nécessite l'importation de différentes protéines précurseurs du cytoplasme. Les protéines précurseurs sont synthétisées dans le cytosol avec une séquence de ciblage amino-terminale clivable appelée peptide de transit. Elles sont importées post-traductionnellement dans le chloroplaste par le complexe protéique TOC-TIC (Translocon des membranes externe et interne du chloroplaste, respectivement). Le complexe TOC est composé de deux récepteurs homologues des GTPases, Toc33 et Toc159, et d'un canal conducteur de protéines, Toc75. Ensemble, ces protéines forment le noyau du complexe TOC. Des facteurs supplémentaires dirigent les préprotéines du cytosol vers le complexe TOC: le complexe dit de guidage se lie aux récepteurs TOC de manière dépendante des GTP pour délivrer les protéines précurseurs. La biogenèse des chloroplastes nécessite de l'acide gibbérellique (AG), tandis que les protéines DELLA agissent comme des régulateurs négatifs de la signalisation de l'AG, connus pour inhiber la germination des graines, la croissance des graines et d'autres voies dépendantes de l'AG. Mes résultats montrent que Toc159 se lie directement à DELLA et est ciblé pour la dégradation par la voie ubiquitine-protéasome. L'accumulation de Toc159 dépend de l'acide gibbérellique pendant les premiers stades du développement de la plante, car elle conduit à la dégradation de DELLA, libérant ainsi le TOC159 de la dégradation médiée par le protéasome pour favoriser la biogenèse du chloroplaste. Outre l'ubiquitination, Toc159 est également connu pour être modifié post-traductionnellement par phosphorylation, bien que l'on ne comprenne pas la signification fonctionnelle de ce phénomène.

Une partie importante de ma thèse est dédiée à un troisième type de modification post-traductionnelle, la SUMOylation. Le TOC159 se lie à l'isoforme SUMO3 dans son domaine de liaison au GTP (G-) et est modifié de manière covalente par SUMO3 dans son domaine membranaire (M-). Les résultats montrent que la SUMOylation covalente augmente la stabilité de Toc159 dans des conditions de faible teneur en acide gibbérellique. Bien que le rôle de la liaison non covalente de SUMO ne soit pas clair, je suppose que la SUMOylation covalente au niveau du domaine M affine la stabilité et les concentrations de Toc159 en fonction des conditions internes et externes.

Mots-clés: Biogénèse des chloroplastes; TOC159; SUMOylation; *Arabidopsis thaliana*.

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Chloroplast: evolution, structure and functions

Theory of the Endosymbiosis

According to the Theory of Endosymbiosis, the chloroplast arose from a symbiogenetic event that occurred around 1.5 billion years ago, in which an ancestral eukaryotic cell endocytosed a cyanobacterium, an oxygenic photosynthetic bacterium. During evolution, the establishment of this intracellular symbiotic event led to the transfer of most of bacteria genes to the host cell genome (**Figure 1**). This has caused the development of the chloroplast as an organelle, and the conferment of photosynthesis. Today, sequence-based phylogenetic analyses confirm that all plastids descent from different eubacterial lineage, and, are considered monophyletic, including red, green, and glaucocystophyte algae. Plastids have evolved to become essential components for plant cellular functions. During plant growth and development, all plastid types can be considered as interrelated and interconvertible. They are derived from undifferentiated plastids called proplastids, which are present in the meristematic regions of the plant, and may differentiate into several forms, depending upon which function they perform in the cell. In fact, they are considered as a site of the manufacture and storage of important chemical compounds and can be classified in photosynthetic plastids, (chloroplast), and non-photosynthetic plastids (chromoplasts, amyloplasts, elaioplasts etc), by virtue of their storage components and internal structures.

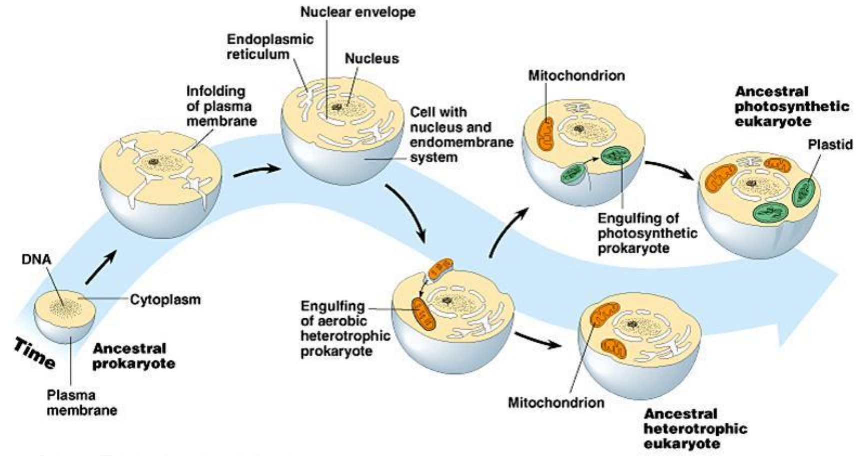

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Figure 1. Endosymbiosis in the history of plastid evolution. The first eukaryote may have originated from an ancestral prokaryote that had undergone membrane proliferation, compartmentalization of cellular function (into a nucleus, lysosomes, and an endoplasmic reticulum), and the establishment of endosymbiotic relationships with an aerobic prokaryote and, in some cases, a photosynthetic prokaryote to form mitochondria and chloroplasts, respectively (taken from Pearson Education, Inc).

Chloroplast biogenesis

Chloroplast biogenesis is a multistage process leading to fully differentiated and functionally mature plastids (Rudowska *et al.*, 2012) (**Figure 2**). Generally, the chloroplast develops from undeveloped proplastids, small organelles in leaf primordia that possess small vesicles but no photosynthetic complexes. Upon illumination, during natural photomorphogenesis, proplastids develop the thylakoid membrane network along with photosynthetic capacity (Rudowska *et al.*, 2011). During this differentiation thylakoids are formed and stacked in part into defined grana (Pogson *et al.*, 2015). They provide the platform of the light reactions of photosynthesis and their biosynthesis is a determining process of chloroplast biogenesis, which requires the coordinated synthesis of lipids together with proteins, pigments and cofactors (Kobayashi, 2016). The development from the precursors to mature photosynthetically active chloroplasts requires highly coordinated processes in different cell compartments. During the differentiation of the chloroplast, the following events take place: light perception and subsequent expression of nuclear and plastid pigment-binding proteins such as chloroplyll a/b-binding proteins into developing chloroplasts, insertion of these proteins into the thylakoid membranes, and protein assembly into functional complexes. When a seedling grows in the dark, its proplastids differentiate into etioplasts, subsequently the plant undergoes etiolation characterized by an elongated hypocotyl, an apical hook and closed cotyledons. Etioplasts have pro-lamellar bodies, a precursor structure of the thylakoid membranes, and represent primed organelles ready to undergo a rapid differentiation upon light exposure (Waters and Langdale, 2009). Indeed, the etioplast proteome shows drastic and rapid changes as early as two hours after illumination, reflecting a shift from a heterotrophic towards an autotrophic metabolism (Kleffmann *et al.*, 2007).

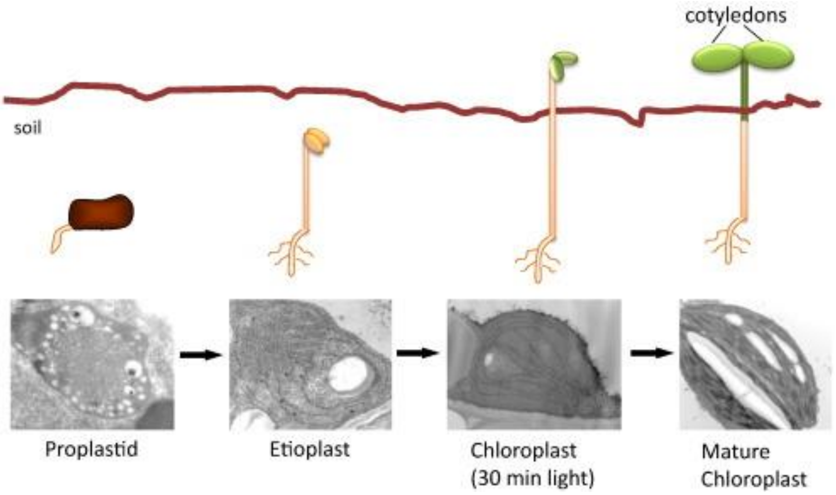

Figure 2. Schematic representation of de-etiolation stages in *Arabidopsis thaliana*. Chloroplast biogenesis and development in dicotyledonous seedlings alongside germination. (taken from Pogson *et al.,* 2015).

Structural organization of the chloroplast

The chloroplast is surrounded by two envelope membranes: the outer envelope membrane is highly permeable and allows free diffusion of small organic molecules between cytoplasm and the inter-membrane compartment, such as ions and metabolites, whereas the inner envelope membrane is less permeable and physically separates the internal part of the chloroplast from the cytoplasm; it also contains specific transporters to regulate the exchange of metabolites between the two compartments. Each of envelope membranes is a lipid bilayer composed of different percentage of phospholipids, galactolipids, and sulfolipids and are separated by an intermembrane-space called stroma. In this region, a system of interconnecting flattened membranes called thylakoids is the site of light absorption and ATP synthesis (F-ATPase), and it contains an aqueous solution called thylakoid lumen (**Figure 3**). In addition, plastid genome, ribosomes, primary starch granules and plastoglobules are located in the stroma. Thylakoids contain a large number of integral and peripheral membrane proteins, many associated with pigments including chlorophyll and carotenoids that are essential for photosynthetic activity. The ancient cyanobacterium contained thylakoids bearing phycobilisomes with blue and red antennal pigments on their cytosolic side. Red algae and glaucophytes retained the ancestral cyanobacterial phycobilisomes (and so are collectively termed biliphytes) and therefore they retained chloroplasts closely resembling the entire cyanobacterial ancestor while green plants (sub-kingdom Viridaeplantae), lost phycobilisomes and evolved chlorophyll a/b binding proteins (Cavalier-Smith *et al.*, 2000). Importantly, these membranes are functionally organized in grana, composed of tightly packed membranes containing mainly PSII complexes, and lamellae, single membrane spanning the stroma containing mainly PSI complexes. This structural difference is believed to prevent spill-over of excitation energy and uncontrolled electron flow during photosynthesis. The main aqueous compartment, the stroma, contains the enzymes performing the dark reactions of photosynthesis (also called the carbon assimilation process), multiple copies of the chloroplast genome and a protein synthesizing system of prokaryotic origin.

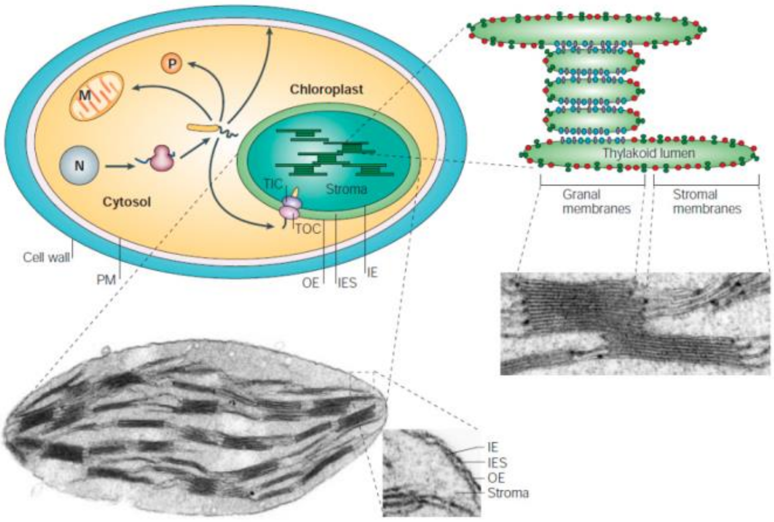

Figure 3. Diagram of a chloroplast inside a cell, showing thylakoid stacks. Shown here is a chloroplast inside a cell, with the outer membrane (OE) and inner membrane (IE) labeled. Other features of the cell include the nucleus (N), mitochondrion (M), and plasma membrane (PM). At right and below are microscopic images of thylakoid stacks called grana. Note the relationship between the granal and stromal membranes (taken from Soll and Schleiff, 2004).

Functions and metabolism of the chloroplast

Photosynthesis

In plants and green algae (i.e. photoautotrophic organisms) photosynthesis takes place in the chloroplast, which is surrounded by a double membrane and has its own genome. It is a process used by plants and other autotrophic organisms to convert light energy, normally from the sun, into chemical energy that can be used to fuel the organism’s activities. Photosynthesis consists of a series of reactions of oxido-reduction that use water molecules as primary source of electrons. The electrons are transferred through a series of carriers to an acceptor molecule that is then used to reduce carbon dioxide to produce carbohydrates and releasing as by-product molecular oxygen (O_2). The whole oxygenic photosynthesis is usually summarized by the formula:

$$6\ H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6\ O_2$$

It is important to mention that a complex series of reactions are involved in the process, which include two separate phases: the “light phase” or thylakoid reactions and the carbon fixation reactions (**Figure 4**).

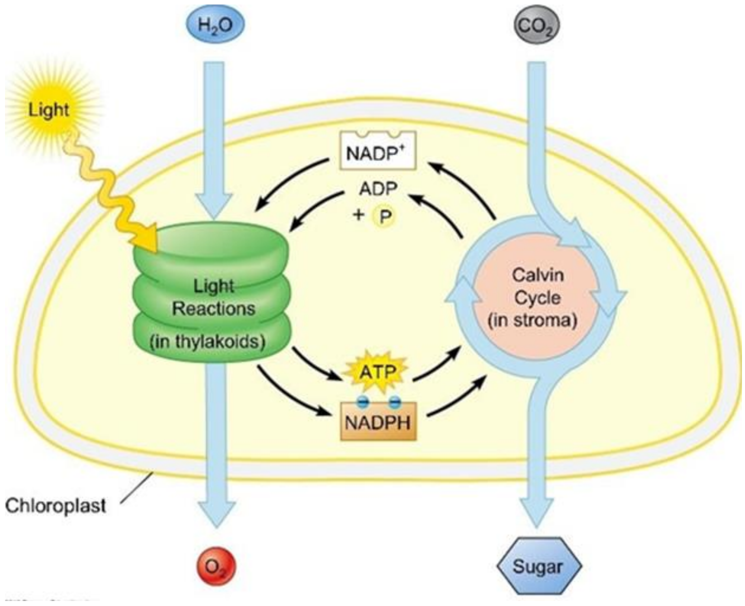

Figure 4. The intimate connection between light and dark reactions of photosynthesis. The light reactions take place at the internal membrane of the chloroplast, the thylakoids: the light harvesting complexes absorb and funnel light energy to the PSII and PSI reaction centers. These reaction centers contain specialized chlorophyll-protein complexes that catalyze the primary photochemical reactions. (taken from Pearson Education Inc. 2012).

The light-phase of photosynthesis

Photosynthesis is performed by two different sites of reaction that act in series: the photosystem II (PSII) and the photosystem I (PSI) that are connected by an electron transport chain in which an electron is transferred from a donor to an acceptor, according to a scale of redox potential as summarized in the "Z-scheme" (**Figure 5**). Photosystems (PSs) are multiprotein complexes which reside within thylakoid membrane. Each of PS is composed of different sets of specific proteins, such as light-harvesting complex (LHC) and chlorophyll protein complex (CP). For example, PSI is an integral membrane protein complex that uses light energy to mediate electron transfer from plastocyanin to ferredoxin. Two main subunits of PSI, PsaA and PsaB, are closely related proteins involved in the binding of P700, A0, A1 and Fx. PsaA and PsaB are both integral membrane proteins of 730 and 750 amino acids, that seems to contain 11 transmembrane segments. Instead, the core of PSII contains of pseudo-symmetric heterodimer of two homologous proteins D1 and D2, employed in the reaction centers. This reaction center is surrounded by light-harvesting complexes that enhance the absorption of light and transfer the energy to the reaction centers. Light-harvesting and reaction center complexes are membrane protein complexes that are made of several protein-subunits and contain numerous cofactors (Rutherford, 2003). The two PSs are excited by different light wavelengths: the PSII absorbtion peak is at 700nm while that of PSI is at 680nm, but in both cases the energy that comes from the sunlight can excite a chlorophyll electron increasing its potential energy. Briefly, PSII oxidizes water and reduces the plastoquinone pool; then an electronic cascade towards $NADP^+$ begins. The cytocrome b_6f complex catalyzes the transfer of electrons from plastoquinol to plastocyanin, releasing protons into the thylakoid lumen that contribute to create an electrochemical gradient used to synthesize ATP (Nelson and Yocum, 2006). PSI obtains an electron from plastocyanin and drives it to ferredoxin. Importantly, the release and accumulation of protons in the thylakoid lumen generates a proton motive force for synthesis of ATP (Allen and Pfannschmidt, 2000); both ATP and NADPH are energetic molecules used in the Calvin cycle to form sugars. Redox potential of ferredoxin can be alternatively used for nitrate assimilation and fatty acid desaturation (Ben-Shem, 2003). The catalytic mechanism of these four large membrane-protein complexes embedded in thylakoid membranes have been widely characterized due to the high-resolution structures made available in recent years (Allen and Pfannschmidt, 2000; Minagawa, 2004; Nelson, 2004; Nelson, 2006; Dekker, 2005; Allen, 2001). The overall equation for the light-dependent reactions under the conditions of non-cyclic electron flow in green plants is:

$$2\ H_2O + 2\ NADP+ + 3\ ADP + 3\ Pi + light \rightarrow 2\ NADPH + 2\ H+ + 3\ ATP + O_2$$

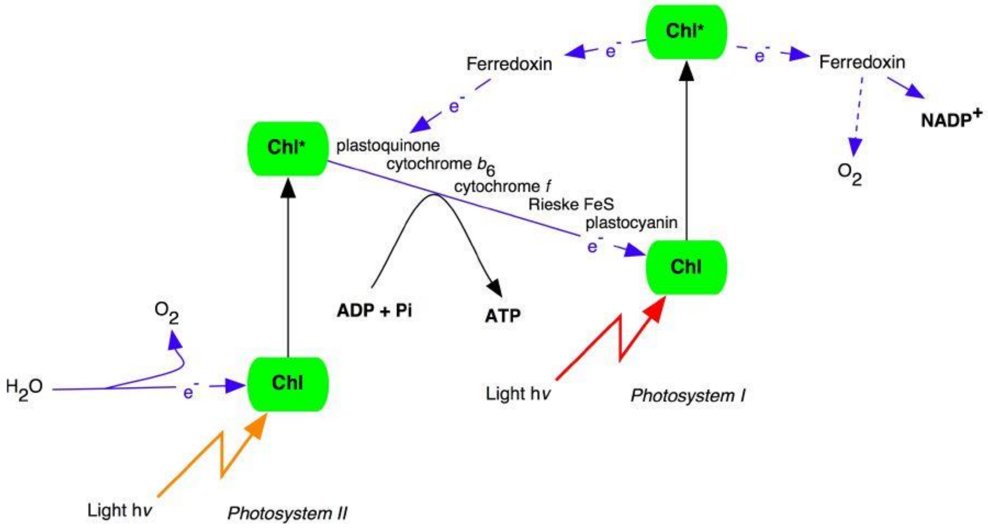

Figure 5. The Z-scheme shows the pathway of electron transfer from water to NADP+. The Z-scheme is an energy diagram for electron transfer in the "light reactions" of photosynthesis. The vertical energy scale shows each molecule's ability to transfer an electron to (to reduce) the next one from left to right. The ones at the top transfer electrons easily to those below them as it is a "downhill" reaction, energy, for electron transfer from those at the bottom to those above them it is an "uphill" reaction and requires input of outside energy: the light (photon). (taken from Allen, 2004).

The dark reactions of photosynthesis

The dark reactions or Calvin cycle reactions can be divided into three main stages: carbon fixation, reduction, and regeneration of the starting molecule. The site of the Calvin cycle reactions is the stroma of the chloroplast, where sugar is synthesized using ATP and NADPH produced in the light reactions. In plants, carbon atoms from CO_2 enter the interior of the leaf via pores called stomata to reach the stroma. Once there, the carbon atoms are fixed thank to an enzyme called RuBisCO (RuBP carboxylase-oxygenase) that catalyzes the attachment of CO_2 to ribulose bisphosphate (a five-carbon sugar). For each CO_2 that enters the cycle, two glyceraldehyde 3-phosphate (3-PGA) molecules are produced. Afterwards, ATP and NADPH are used to convert the 3-phosphoglycerin acid (3-PGA) molecules into molecules of a three-carbon sugar, glyceraldehyde-3-phosphate (G3P). ATP donates a phosphate group to the 3-PGA molecule, turning it into a molecule of 1,3-bisphosphoglycerate, that, afterwards, it is reduced by NADPH that donates two electrons turning the molecule into glyceraldehyde 3-phosphate (G3P). Some G3P molecules go to make sugar, while others must be recycled to generate the RuBP acceptors for the next cycle (**Figure 6**).

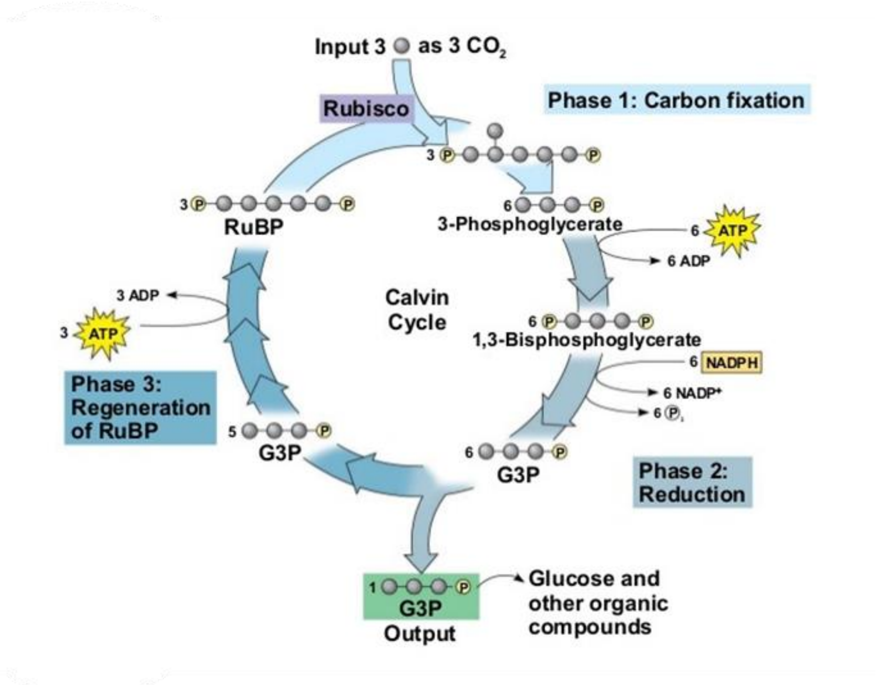

Figure 6. The Calvin cycle. In stage 1, the enzyme RuBisCO incorporates carbon dioxide into an organic molecule, 3-PGA. In stage 2, the organic molecule is reduced using electrons supplied by NADPH. In stage 3, RuBP, the molecule that starts the cycle, is regenerated so that the cycle can continue. Only one carbon dioxide molecule is incorporated at a time, so the cycle must be completed three times to produce a single three-carbon GA3P molecule, and six times to produce a six-carbon glucose molecule. (taken from Pearson Education Inc. 2014).

Other essential functions of the chloroplast

In theory, the union between a free-living cyanobacteria and a heterotroph host introduce many more metabolic capabilities beyond just photosynthesis to the host's repertoire. Autotrophs are able to synthesize whatever they need *de novo*. Conversely, heterotrophs are used to uptake a wide range of preformed macromolecules from their diet and they usually decompose these molecules to extract the building blocks necessary for their metabolism. Thus, although the main driving force for endosymbiosis between the heterotroph and the autotroph was photosynthesis, during their coevolution, some other metabolic capabilities of the autotroph ancestor became as essential as photosynthesis and new chimeric pathways were generated by reshuffling redundant functions. The essential character of the chloroplast beyond photosynthesis is well illustrated by those organisms that have further reduced the size of their plastid genome by losing the genes involved in photosynthesis (Cavalier-Smith, 2003). Parasitic plants and apicomplexan parasites such as the malaria parasites are notable examples. Many pharmacological and genetic studies carried out with these organisms have shown that their non-photosynthetic plastids (called apicoplasts) still provide essential functions. For instance, they include biosynthetic pathways for fatty acids, isoprenoids, iron sulfur cluster assembly, and a segment of the heme pathway in case of the malaria parasites (Ralph van Dooren *et al.,* 2004). Another indication for the essential function of the chloroplast beyond photosynthesis is the presence of essential open reading frames in its genome and in the genome of non-photosynthetic plastids such as those of *Epifagus virginiana*. The function of these genes is not directly linked to photosynthesis but at the same time is still not understood. Therefore, the chloroplast is not only an organelle to produce energy but seems to have maintained or acquired additional functions during evolution, which makes it indispensable for the cell even if photosynthesis is not required. These functions include amino acid synthesis, fatty acid synthesis, synthesis of purines and pyrimidines as well as sulfur and nitrogen metabolism.

Control of chloroplast biogenesis by gibberellic acid (GA)

Gibberellic acid (GA) is a phytohormone found in plants and fungi that belongs to a large family of tetracyclic diterpenoids. Seven GAs are thought to have biological activity (GA1, GA2, GA3, GA4, GA5, GA6 and GA7, respectively). In particular, GA4 has been identified as the main endogenous active gibberellin in germinating seeds (Ogawa *et al.,* 2003). Gibberellic acid is known to promote plant growth by encouraging the degradation of the DELLA proteins, a plant specific GRAS protein encoded by five genes (RGA, GAI, RGL1, RGL2 and RGL3, respectively). DELLAs are localized in the nucleus, which is in accordance with their role as regulators in gene expression (Dill *et al.,* 2001). However, recent findings have been shown that the gibberellin receptor GID1A can interact with DELLA proteins in the cytosol, before entering in the nucleus. The interaction is due to the binding between GID1A and gibberellin in the cytosol, whose initiates the gibberellin signaling (Livne and Weiss, 2014). All of them function as negative regulators of GA signaling and are proposed to bind to transcription factors and thereby modulate transcription events. GAI and RGA are considered negative regulators of the GA-signaling pathway for stem elongation, playing a predominant role in repressing vegetative growth. It has been shown that, in the *ga1-3/gai/rga* mutant vegetative growth is almost completely recovered, albeit there is no evidence that mutations in GAI and RGA improve germination or flower development of the *gal-3/gai/rga* mutant, suggesting that probably other DELLAs are involved in these processes (Dill, 2001). The expression of all DELLA genes is induced upon seed imbibition, in particular RGL1, RGL2 and RGL3 are mostly expressed in germinating seeds, while GAI and RGA are ubiquitously expressed in all tissues (Tyler *et al.,* 2004).

Recently, it has been shown that GA plays also an important role in regulating chloroplast biogenesis and photosynthesis (Alabadi *et al.,* 2008; Cheminant *et al.,* 2001). DELLA proteins regulate the levels of POR (protochlorophyllide oxidoreductase enzymes) and carotenoids during de-etiolation and also influence the grana stacking of the thylakoids (Jiang *et al.,* 2012). According to Cheminant *et al.,* GAs modulate the development of young chloroplasts during de-etiolation, and, at the same time, enhance the photosynthetic activity of mature chloroplasts (Cheminant *et al.*, 2011). GA is perceived by the GA receptor GID1, which is a soluble protein localized in both cytoplasm and nucleus (Ueguchi-Tanaka *et al.*, 2007; Sun, 2008). This pathway is repressed by DELLA proteins in their role as nuclear transcriptional regulators (Sun, 2010). The current model for GA signaling proposes that the hormone binds to its receptor GID1 promoting the association of GID1 with DELLA and triggering its ubiquitin-mediated proteasomal degradation. The binding of GA to GID1 enhances the interaction between GID1 receptor and DELLA proteins. A specific ubiquitin E3 ligase (SCF SLY1/GID2) is required for polyubiquitylation of DELLAs and their subsequent degradation by the 26S proteasome. (Dill *et al.*, 2004; Fu *et al.*, 2004). SLY1 is part of the SCF (SKP1, CULLIN1, F-box)-type E3 ubiquitin ligase complex that promotes polyubiquitylation of specific proteins. In addition to GA-dependent proteolysis, the activity of DELLA may also be modulated by post-translational modification, such as glycosylation and phosphorylation. In fact, it has been shown that SPYNDLY (SPY) transferase may activate DELLA by GlcNAc-modification. However, EARLIER FLOWERING 1 (EL1) encoding a casein kinase in rice may phosphorylate and activate DELLA, functioning as repressor of GA (Sun, 2010) (**Figure 7**).

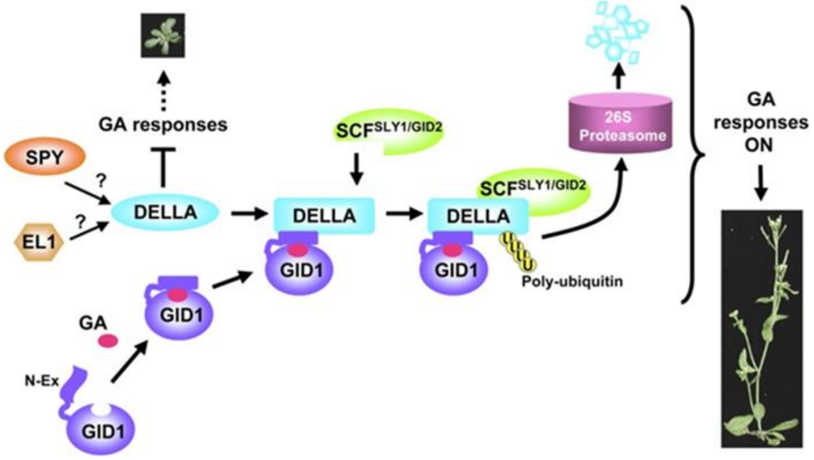

Figure 7. Model of GA signaling in plants. Bioactive GA binding induces a conformational switch in the N-Ex of GID1 for DELLA binding, which in turn promotes a conformational transition in the GRAS domain of the DELLA protein for SCFSLY1/GID2 recognition. DELLA protein will then be polyubiquitinated and degraded via the ubiquitin-proteasome pathway. SPY (an OGT) may activate DELLA by GlcNAc-modification, whereas EL1 (a casein kinase in rice) may phosphorylate and activate DELLA (taken from Sun, 2010).

Routing proteins into the chloroplast

Preprotein targeting to the chloroplast

In higher plants, the majority of plastid proteins are encoded by the plant nuclear genome. They are synthesized in cytosolic ribosomes as nuclear-encoded chloroplast proteins, also known as precursor proteins, that contain a cleavable N-terminal sequence called transit peptide (TP), necessary for targeting them to the chloroplast (Agne and Kessler, 2009), as well as for directing their translocation across the chloroplast envelope (Bruce, 2001). It is generally believed that the translocation across the chloroplast envelope happens via a post-translational mechanism, whereas preproteins are assisted by soluble cytosolic factors that maintain them in an unfolded conformation. The cytosolic protein 14-3-3 is a molecular chaperone that forms a dimer, where each monomer is able to bind one target precursor protein, and interacts specifically with chloroplast precursor proteins, recognizing their phosphorylated transit sequences. The formation of the precursor guidance-complex keeps the preprotein in a highly import-competent state and the dissociation of it requires ATP. Once a preprotein arrives at the chloroplast surface, its translocation is mediated by translocons located at the outer and inner envelope membranes of the chloroplast, named TOC and TIC, respectively (Bedard and Jarvis, 2005), and it is recognized by receptors components of the TOC, which specifically and selectively interact with the transit peptide. This mechanism was explained in three biochemically steps by using the *Pisum sativum* (pea) as chloroplast model system (Schnell and Blobel, 1993; Perry and Keegstra, 1994): the first step is the binding of the transit peptide at the chloroplast surface, which is a reversible process and doesn’t require energy (Perry and Keegstra, 1994; Kouranov and Schnell, 1997); the second step is the insertion across the outer membrane, where the target preprotein forms an early import intermediate (“early import intermediate stage”) with the import machinery. This step is irreversible and is promoted by hydrolysis of low concentration of GTP and ATP ($\leq$ 100 μM) (Schnell and Blobel, 1993). The requirement for ATP has been attributed to the activity of molecular chaperones necessary to bind and stabilize preproteins during their transport across the outer membrane (Chen and Schnell, 1999; Schnell *et al.,* 1994). A third and final step, known as “late import intermediate stage”, is the complete translocation across both envelope membranes into the chloroplast stroma and requires high concentrations of ATP (> 1 mM) in the stroma (Pain and Blobel, 1987; Theg *et al.,* 1989; Hsou-min Li *et al.,* 2020; Nakai, 2020). Molecular chaperones such as Hsp60, Hsp70 or Hsp100 (Kessler and Blobel, 1996; Nielsen *et al.,* 1997; Shi and Theg, 2010) may function as import motors or assist folding of imported proteins in the stroma. Stromal processing peptidase (SPP) is then responsible to remove the transit peptide through hydrolytic protease action. Structurally, transit peptides are highly divergent in length (from 20 to 80 amino acids), composition and organization; they are rich in Serine and Leucine

(Bhushan *et al.,* 2006). Transit peptides show multiple semi-conserved subdomains with complex relationships that seem to be involved at different stages of the targeting and translocation process (Lee *et al.,* 2006; Lee *et al.,* 2008; Rensink *et al.,* 1998). The lipids found within plastid membranes are emerging as players in the targeting, insertion and assembly process of proteins in plastid membranes (Dörmann and Benning, 2002). The outer chloroplast envelope is strongly negatively charged and enables formation of ionic interactions between preproteins and lipids on the chloroplast surface (Fulgosi and Soll, 2011). However, the import of precursor proteins into chloroplasts requires protease-sensitive components on the outer envelope membrane (Friedman and Keegstra, 1989) and the identification of these protease sensitive components became a question of great interest in the late 1980s and early 1990.s

The TOC-TIC machinery

The first attempts to identify components of the import machinery date back to the late 1980s, when several laboratories employed a variety of biochemical techniques to identify protein components of the chloroplast protein import apparatus using isolated pea chloroplast as model system (*Pisum sativum)* (Schnell *et al.,* 1994; Perry *et al.,* 1994). Schnell et al successfully used the “early” and “late import intermediates” to do so. They incubated the small subunit of Rubisco fused to ProteinA either in the presence of low (0.1mM ATP) or high concentrations (5mM ATP) of ATP to generate the “early intermediate” or “late intermediate”, respectively. They then isolated the pS-ProtA from the “early” and “late” intermediates using IgG column chromatography. The resulting protein complexes were separated by SDS-PAGE and their components identified by chemical Edman degradation. Three proteins that are now known as Toc159, Toc34 and Toc75 were associated with the “early intermediate”, Toc standing for translocon at the outer chloroplast membrane and the number indicating the molecular mass. The same three proteins were also associated with “late intermediate” but in addition a protein now known as Tic110 (Tic standing for translocon at the inner membrane of the chloroplast) and unknown protein at approx. 35 kDa were also present (**Figure 8**). More recently, the small flowering plant *Arabidopsis thaliana* became alternative model system for studying chloroplast protein import, thanks to its genomic sequence data and the fact that Arabidopsis is more amenable to molecular-genetic analyses than pea (Jarvis *et al.,* 1998).

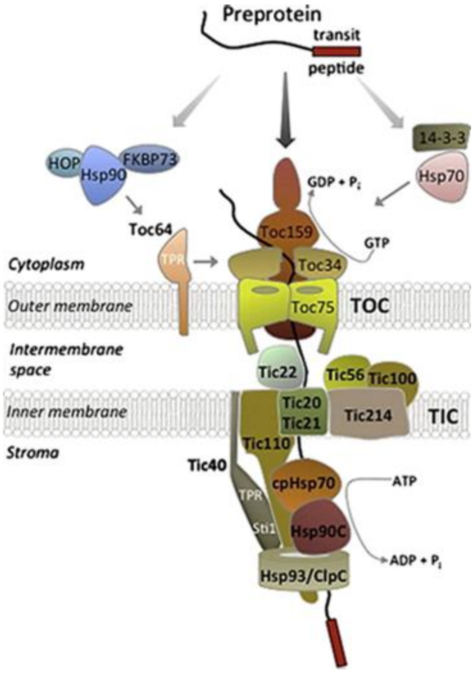

Figure 8. Schematic representation of the TOC and TIC complexes. The schematic illustration indicates the targeting of preproteins to the TOC complex and their transfer to the TIC complex and the stroma. Preproteins may use three different ways to reach the Toc75 channel, I) some non-phosphorylated preproteins use the Toc64/Hsp90 pathway; II) preproteins directly bind to the Toc159 receptor; III) preproteins phosphorylated at their transit peptide use the "guidance complex" containing Hsp70 and 14-3-3. After outer membrane translocation, preproteins transfer across the intermembrane space. Finally, the preproteins cross the inner membrane via the TIC complex followed by transit peptide cleavage by the stromal processing peptidase (SPP) (taken from Paila *et al.,* 2015).

The TOC core complex

Toc159, -34 and -75 are today considered the TOC core complex. The total mass of the TOC and TIC complexes has been estimated at between 500 kDa to 1 MDa in pea (Chen *et al.,* 2007; Scheiff *et al.,* 2007) and around 1 MDa in *Arabidopsis thaliana* (Kikuchi *et al.,* 2009). In addition to the TOC core components, other TOC proteins have been identified later as new components of the complex, such as Toc64 and Toc12, which have been assigned roles in the recognition of the preproteins and tethering of an intermembrane space Hsp70 to the exit site of the TOC complex, respectively (Sohrt *et al.,* 2000; Becker *et al.,* 2004). It has been also shown that the TOC complex interacts with cytosolic factors, such as heat shock proteins, implicated in guiding cytosolic preproteins to the chloroplast envelope.

Toc159: gateway to the chloroplast

Toc159 is a tripartite protein composed of an N-terminal acidic domain (A-domain), a central GTP binding domain (G-domain) and a C-terminal membrane anchoring domain of 52kDa (M-domain) (Chen *et al.,* 2000; Hiltbrunner *et al.,* 2001). Both the A- and G-domain are exposed to the cytosol whereas the M-domain is anchored in the outer membrane of the chloroplast envelope (Hirsch *et al.,* 1994). AtToc33 is mostly constituted of its G-domain and is anchored in the outer membrane by a short C-terminal part, which contains a stretch of hydrophobic amino acids (Chen and Schnell, 1997). It was initially identified as an 86 kDa fragment (Toc86 or Toc159GM) because of the high sensitivity of its A-domain to proteolysis (Bolter *et al.,* 1998; Kessler *et al.,* 1994; Waegemann and Soll, 1991). Toc159 has been proposed to be involved in the initial preprotein binding (Chen *et al.,* 2000). The initial docking and insertion of Toc159 receptor at the TOC complex requires its intrinsic GTPase activity and the interaction with the G-domain of Toc33. The association of the two G-domains stimulates membrane insertion of Toc159 and association with the translocon (Bauer *et al.,* 2002; Smith *et al.,* 2002). Additionally, the M-domain of Toc159 interacts with Toc75, which, along with Toc33, is a necessary component for the proper integration of Toc159 into the chloroplast outer envelope (Wallas *et al.,* 2003). The M-domain alone was showed to partially complement the preprotein import defect in the *ppi2* mutant (Lee *et al.,* 2003). In *Arabidopsis thaliana*, characterization of the *ppi2* mutant (plastid protein import 2), an atToc159 T-DNA insertion mutant, has shown that the differentiation of proplastids into chloroplasts is blocked, resulting in an albino phenotype (Bauer *et al.,* 2000), a condition where the plant cannot develop photo-autotrophically. Recently, it was discovered that the A-domain of Toc159 exists as a separate soluble phosphoprotein, but the physiological role of the separated A-domain remained elusive (Agne *et al.,* 2010). The function of the A-domain of Toc159 is still under investigation although it is non-essential for chloroplast biogenesis. Indeed, a T-DNA construct encoding only the G- and M-domains of Toc159 complemented the *ppi2* mutant (Lee *et al.,* 2003; Agne and Kessler, 2009).

The small GTPases: Toc33 and Toc34

Toc34 is the second membrane-anchored GTPase involved in the recognition of precursor proteins. Structurally, atToc33 is mainly constituted by the G-domain and contains a sequence of hydrophobic amino acids at its C-terminus (Chen and Schnell, 1997). Toc34 belongs to the class of TA (Tail Anchored proteins) and requires an AKR2A (Bae *et al.,* 2008) for membrane insertion into the outer membrane lipid bilayer of the chloroplast in its GTP-bound form (Qbadou *et al.,* 2003). Interestingly, in Arabidopsis, knock-out mutants for the two isoforms show distinct phenotypes: atToc33, the *ppi1* mutant, has a pale green phenotype disappearing after the first weeks of development, indicating that atToc33 is involved during the early stages of plant development, while, the *ppi3* mutant plant, knock-out for atToc34, has no visible phenotype apart from delayed root growth. Also, the *ppi1* mutant exhibits a defect in expression and import of photosynthetic related proteins, suggesting that atToc33 is mainly involved in import of photosynthetic proteins (Kubis *et al.*, 2003). These observations suggest that atToc34 has a more important role for plastid biogenesis in roots and that its function overlaps with that of atToc33 (Constan *et al.*, 2004). Additionally, the double mutant *ppi1*/*ppi3* is embryo lethal, indicating that the combined function of these two proteins is essential for protein import into plastids in different tissues during early development (Constan *et al.*, 2004).

The TOC GTPase receptors

Toc159 and Toc34 belong to the distinct family of septin GTPases (Weirich *et al.*, 2008) within the TRAFAC class (Sun *et al.*, 2002; Aronsson and Jarvis, 2011), and both are of eukaryotic origin (Reumann and Keegstra, 1999). Structurally, they share homology in the GTP-binding domain. In *Arabidopsis thaliana*, a family of four large Toc159 GTPases has been identified: at TOC159, atTOC132, atTOC120 and atTOC90 (Hiltbrunner *et al.*, 2001; Jarvis *et al.*, 1998). These homologues of Toc159 share structural similarities in the C-terminal anchoring domain (M-domain) and central GTPase domain (G-domain), differing at the N-terminal Acidic domain (A-domain) in length and sequence (Bauer *et al.*, 2000; Hilbrunner *et al.*, 2001) (**Figure 9**). The conserved G-domain of the Toc GTPases reveals classical motifs for guanine nucleotide binding and hydrolysis (Bourne *et al.*, 1991). Another GTPase is Toc34, a membrane-anchored protein smaller than Toc159. In pea, only one member has been found so far while two isoforms of Toc34 (atToc34 and atToc33) have been identified in Arabidopsis (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). Co-immunoprecipitation experiments demonstrated that atToc159 preferentially associates with atToc33, while atToc120, and/or atToc132 preferentially form a complex together with atToc34 (Ivanova *et al.*, 2004). Interestingly, the toc34 (*ppi3*) knock-out mutant has no visible defect, while the toc33 (*ppi1*) mutant displays a pale green phenotype with a chloroplast biogenesis defect as for toc159 mutant phenotype (*ppi2*), supporting the proposition that these latter two receptor isoforms function in the same complex and preprotein import pathway (Jarvis *et al.*, 1998; Bauer *et al.*, 2000; Kubis *et al.*, 2003, 2004; Constan *et al.*, 2004). During the last decade, many debates have arisen to understand which of the two acts as the primary receptor for the protein import (Chen *et al.*, 2000; Schleiff *et al.*, 2003). Recent evidence considers both the GTPases as mediating receptors involved in the recognition of the transit peptide (Kouranov and Schnell, 1997).

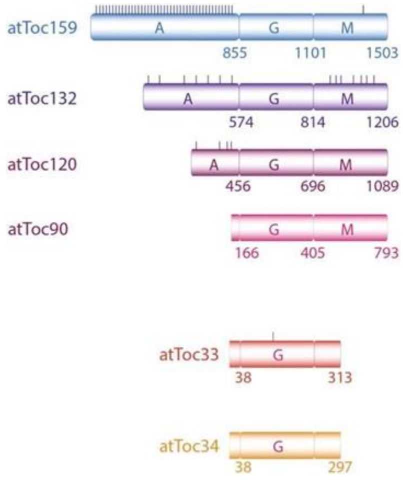

Figure 9. Schematic representation of TOC159 homologues. The translocon of the outer membrane complex includes two GTPase-receptors: TOC159 and TOC33 as well as additional homologues. TOC159 consists of a GTPase domain (G) flanked by a C-terminal membrane anchoring domain (M) and an acidic N-terminal region (A). In *Arabidopsis thaliana*, four TOC159 and two TOC34 isoforms have been identified. TOC159 homologues differ primarily in their A-domain sequences and lengths. Experimentally identified phospho-serine and -threonine residues (PhosphAT4.0) are schematically indicated by short vertical lines (taken from Demarsy *et al.*, 2014).

Toc75 protein-conducting channel

Toc75 is the protein between the TOC and the TIC translocons. It is of prokaryotic origin (Reumann *et al.*, 1999) and was identified as a key component of the transport machinery and assigned the role of protein conducting channel (Perry and Keegstra, 1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995; Hinnah *et al.*, 2002; Schleiff *et al.*, 2003). It is synthesized as a preprotein and contains chloroplast and organellar targeting information for its insertion into the chloroplast outer membrane (Tranel and Keegstra, 1996). The model plant A. *thaliana* has three genes encoding for homologues of pea Toc75 (psToc75): atTOC75-I, atTOC75-III, and atTOC75IV (Ivanova *et al.*, 2004). Structurally, Toc75 has a two-domain structure typical of the Omp85 family and it is formed by a N-terminal domain containing POTRA (POlypeptide TRansport Associated) (Sanchez-Pulido *et al.*, 2003), and a C-terminal β-barrel domain. The N-terminus of Toc75 contains a bi-partite targeting peptide. The N-terminal part of its transit peptide engages the TOC and TIC complexes and is cleaved by the stromal processing peptidase, while the C-terminal part consists of a glycine-riche sequence that spans the intermembrane space and is cleaved by an envelope-localized protease (Ma *et al.*, 1996). To date, two Toc75 coding genes have been identified to be present in the pea genome: Toc75, which codes for the protein-conducting channel and Toc75-V, was discovered based on its sequence similarity with the bacterial homologue. It has been proposed that Toc75-V might be a direct ortholog of the cyanobacterial ancestor, and Toc75 a paralog (Eckart *et al.*, 2002).

The TIC complex

TIC complex is the last stage of preprotein import into the stromal compartment. Composition and function of its components are still under debate. Tic110 was the first TIC component to be recognized (Kessler and Blobel, 1996; Lubeck *et al.*, 1996). Afterwards, other components of the TIC complex have bene identified: Tic62 (Kuchler *et al.*, 2002), Tic55 (Caliebe *et al.*, 1997), Tic40 (Stahl *et al.*, 1999), Tic32 (Hormann *et al.*, 2004), Tic22 (Kouranov and Schnell, 1997), Tic21 (Ma *et al.*, 1996), Tic20 (Chen *et al.*, 2002). The number of the different TIC proteins corresponds to their molecular mass in kDa. Tic110 is essential for plastid biogenesis in Arabidopsis, indeed T-DNA insertion lines are embryo-lethal (Inaba *et al.*, 2003), and appears to participate in the import of all transit peptide mediated import pathways of plastids as it associates in TOC-TIC complexes with any component of the Toc159 GTPase family (Kovacs-Bogdan *et al.*, 2010). Molecular studies and computational analysis have identified Tic110, Tic21 and Tic20 to be the putative components of the inner membrane preprotein conducting channel (Heins *et al.*, 2002; Reumann and Keegstra, 1999; Teng *et al.*, 2006); also, biochemical analysis have shown that Tic40 has a large hydrophilic domain projecting in the stroma that it might function as a co-chaperone, facilitating preprotein translocation across the inner membrane of the chloroplast through the interaction with ClpC (Bedard *et al.*, 2007; Chou *et al.*, 2003). Other TIC components such as Tic62, Tic55, and Tic32, have been proposed to act as redox regulators for import of preproteins (Caliebe *et al.*, 1997; Hormann *et al.*, 2004; Kuchler *et al.*, 2002).

Post-translational modifications at the chloroplast

Phosphorylation

Plants adjust their metabolism according to environmental stimuli by induced adaptation mechanisms, which ultimately lead to changes in intracellular protein levels and activities. In addition to modulating gene expression, which results in relatively long-term changes, rapid responses are required in order to maintain the functionality and fitness of plants upon sudden shift in ambient conditions. Therefore, plants have evolved several mechanisms called post-translational modifications (PTMs) that are able to modify stability, activity, interaction or subcellular localization to proteins by adding a polypeptide modifier (Prabakaran *et al.*, 2012) and thus providing mechanisms for the rapid adjustment of cellular metabolism (Mann and Jensen, 2003) (**Figure 10**). In general, for some proteins, post-translational modifications (PTMs) can take place prior to, or after holding. Recent studies have accumulated evidences about the crucial role of a multitude of modifications generated by proteolytic cleavage and covalent addition of modifying groups to amino acids, including phosphorylation, acetylation, methylation, glycosylation and ubiquitylation in the regulation of chloroplast proteins.

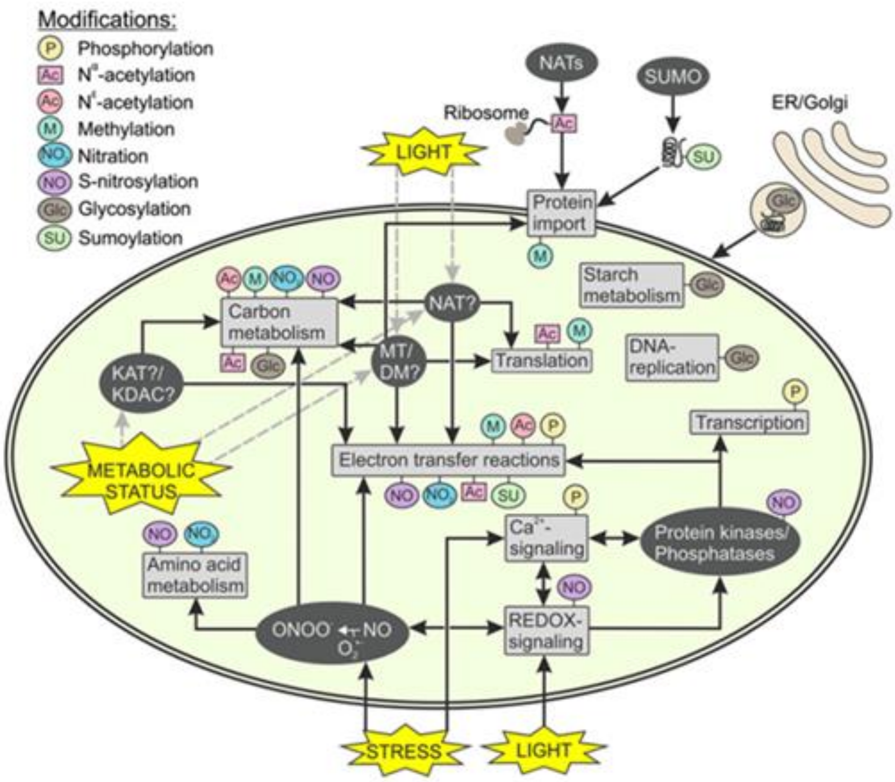

Figure 10. Overview of the possible PTMs of chloroplast proteins. Changes in internal and external conditions (yellow stars) require rapid regulation of many processes inside the chloroplast. The main processes regulated via PTMs are shown in gray boxes. Different types of modifications affecting these processes are marked with colored pins, and the enzymes/molecules executing the modifications are marked with dark gray ellipses. Enzymes yet uncharacterized are indicated with question marks. Black arrows mark known interactions, and hypothetical relations are marked with dashed gray arrows. KAT, Lys acetyltransferase; KDAC, Lys-deacetylase; NAT, Na-acetyltransferase; MT, methyltransferase; DM, demethylase; SUMO, SUMOylation machinery (taken from Lehtimäki *et al.*, 2015).

In chloroplasts, phosphorylation is the best-characterized post-translational modification, which regulates photosynthetic performance and other responses to environmental stimuli, thanks to the continue action of kinases and phosphatases enzymes. This regulatory mechanism affects the function of a great number of proteins and in plants, approximately 1,000 genes have been identified as potential kinases, which predominantly phosphorylate serine, threonine and tyrosine residues (Sugiyama *et al.*, 2008). It has been reported that phosphorylation regulates the activity of both TOC34 and TOC159 in pea (Fulgosi and Soll, 2002; Jelic *et al.*, 2002), while in *Arabidopsis*, TOC33 can be phosphorylated at serine-81 (Aronsson *et al.*, 2006). Up to date, studies on phosphoproteomics in *Arabidopsis thaliana* revealed a hyperphosphorylation at the A-domain of TOC159 protein, but no evidence of phosphorylation at the G- and M- domains. A recent finding on the phosphorylation of the A-domain of TOC159 has reported that a new integral membrane protein called KOC1 present in the cytosol is associated with the TOC complex, engaging for full import activity and de-etiolation process. In fact, it has been shown that in Arabidopsis koc1 mutant the import of photosynthetic preproteins diminishes when the A-domain of TOC159 is phosphorylated (Zufferey *et al.,* 2017). A negative effect of phosphorylation has been shown to influence the GTP-binding to TOC34 in pea and TOC33 in *Arabidopsis* (Sveshnikova *et al.*, 2000; Jelic *et al.*, 2003), causing a defect in the TOC complex integrity.

Ubiquitylation

Recent exciting discoveries from several studies indicate that ubiquitylation, which is one of the most important cellular processes for protein modification in eukaryotic organisms, is involved in the regulation of host defense signaling and may also play an important role in plant disease resistance. Thus, it is not surprising to find that ubiquitin plays critical roles in diverse aspects of plant biology, from growth and development to responses to biotic and abiotic stimuli. Approximately 6% of *Arabidopsis* protein coding genes are suspected to have functions related to ubiquitin modification and several putative Ubiquitin ligases have been identified as defense regulators (Devoto *et al.,* 2003). Moreover, Ubiquitin can itself be a substrate for further ubiquitylation and both the extent of ubiquitylation and the lysine residue, chosen for ubiquitin chain formation, influence the fate of the modified protein. So far, the best-characterized function of ubiquitylation that distinguishes it by other post-translational modifications is the degradation of target proteins through the 26S proteasome. In fact, in the ubiquitin-proteasome system (UPS), the degradation of proteins involves an intricate enzymatic cascade that activates, transfers and binds ubiquitin to cellular proteins. This modification is an ATP-dependent process, carried out by three classes of enzymes: an “ubiquitin activating enzyme” (E1) that forms a thio-ester bond with ubiquitin, a highly conserved 76-amino acid protein. This reaction allows subsequent binding of ubiquitin to an “ubiquitin conjugating enzyme” (E2), followed by the formation of an isopeptide bond between the carboxy-terminus of ubiquitin and a lysine residue on the substrate protein. The latter reaction requires an “ubiquitin ligase” (E3). Then, the ubiquitinated target protein is usually received by the 26S proteasome for degradation. Substrate specificity for ubiquitylation is determined by the E3 ligases (a protein that recruits the E2 ubiquitin-conjugating enzyme) (**Figure 11**). Structurally, the proteasome is a cylindrical complex of proteins with a main function of degrading unneeded or damaged proteins by proteolysis. It is formed by two subcomplexes called 19S (regulatory particle) and 20S (core particle), which are responsible for the recognition and degradation of ubiquitinated proteins. In the last decade, the major progress on the study of the ubiquitin E3 ligases has been accomplished, unravelling the function of several PUB E3 ligases that appear to be widely connected to stress-related aspects. One of them is CHIP, a PUB E3 ligase characterized to have three additional tetratricopeptide repeats (TPRs), making transgenic plants to be hypersensitive towards high and low temperature regimens (Yan *et al.*, 2013). In *Arabidopsis thaliana*, AtCHIP ubiquitylates FtsH1, a subunit of the chloroplast FtsH protease complex localized in the cytosol, affecting proteolytic processes in the organelle (Shen *et al.*, 2007a; Shen *et al.*, 2007b) and mono-ubiquitylates PP2a, a protein phosphates 2a involved in modulating ABA response (Luo *et al.*, 2006). In recent findings, it has been shown that CHIP may alter the number of proteases available in the stroma and in the thylakoid membranes and, therefore, affects the protein quality control as well as the protein homeostasis in the chloroplast (Shen *et al.*, 2007).

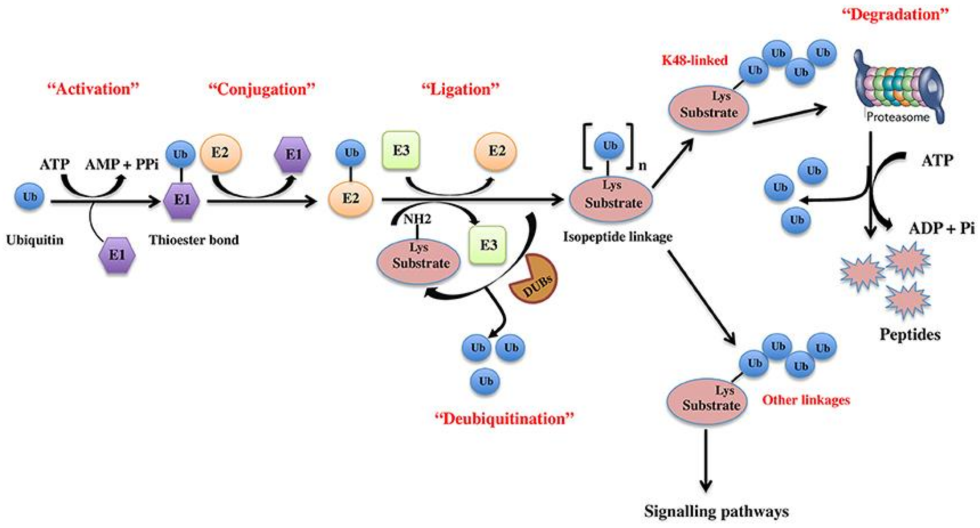

Figure 11. Ubiquitylation mechanism. This process requires the sequential actions of three enzymes, an activating enzyme (E1) that forms an ATP-dependent labile thioester linkage with the carboxyl-terminal group of ubiquitin through its cysteine thiol group, thereby activating the C-terminus of ubiquitin for nucleophilic attack; a conjugating enzyme (E2) that transiently carries the activated ubiquitin molecule as a thiol ester; and an ubiquitin ligase (E3) that finally transfers the activated ubiquitin from the E2 to the ε-amino group of acceptor lysine residue of the substrate (taken from Gupta *et al.*, 2018).

SUMOylation

The ability of plants to cope with environmental challenges requires stress responses designed to mitigate the damage inflicted. The stress–induced modification of intracellular proteins by small ubiquitin-like modifier (SUMO) has recently emerged as an additional line of defense in eukaryotes. In fact, the Small Ubiquitin-related MOdifier, (SUMO), a ubiquitin-like protein (UbL) modifies covalently a large number of proteins with important roles in many cellular processes, including gene expression, chromatin structure, signal transduction and maintenance of the genome. Compared to post-translational modifications such as phosphorylation or acetylation, ubiquitylation and SUMOylation are structurally complex. In addition, they share similarities between the machinery that attaches SUMO to substrate proteins and the enzymes that participate in ubiquitylation.

SUMOylation is a post-translational modification involved in various cellular processes, such a nuclear-cytosolic transport, transcriptional regulation, and protein stability (Hay, 2005). It is a reversible and dynamic process, which requires a specific set of enzymes (**Figure 12**). The SUMO conjugation pathway is mechanistically analogous to the ubiquitin conjugation pathway and the enzymes involved in the SUMO pathway are similar to those involved in the ubiquitin pathway, although they have no role in the ubiquitylation or any other ubiquitin-like proteins and are specific for SUMOylation. SUMO forms are formed as inactive precursor proteins. The pathway begins with a SUMO-activating enzyme (SAE), which carries out an ATP-dependent activation of the SUMO carboxyl terminus, transferring the activated SUMO to a SUMO-conjugating enzyme (SCE) called Ubc9. Afterwards, SUMO is transferred to a lysine residue present in the protein substrate with the assistance of one of the several SUMO-protein ligases (E3). Many of the lysine residues, where SUMO becomes attached, are in the short consensus sequence ψKXE/D where ψ is a large hydrophobic amino acid, generally isoleucine, leucine or valine; K is the lysine residue that is modified; X is any residue; E is a glutamic acid and D is an aspartic acid (Rodriguez *et al.*, 2001). The cleavage of SUMO from its target proteins, is called deSUMOylation or deconjugation, and is catalyzed by SUMO-specific proteases (also known as ubiquitin-like protein processing enzyme - ULPs) that specifically cleave at the carboxyl terminus of SUMO. This step leads to obtain mature SUMO exposing carboxyl terminus glycine residues that generates active form of the protein (Li and Hochstrasser, 2000; Schwienhorst *et al.*, 2000). In addition, the cleavage takes place after a conserved glycine-glycine motif required for the SUMO-substrate linkage formation. This linkage is an isopeptide bond between the carboxyl terminus of SUMO and an ε-amino group of a lysine residue in the target protein. Plants, fungi as well as animals have the same glycine-glycine motif at the cleavage site, with the only difference of the carboxyl termini of SUMO4, SUMO6 and SUMO7, which deviates at the penultimate position. In fact, SUMO4 and SUMO6 have a serine-glycine motif, while SUMO7 an alanine-glycine motif instead

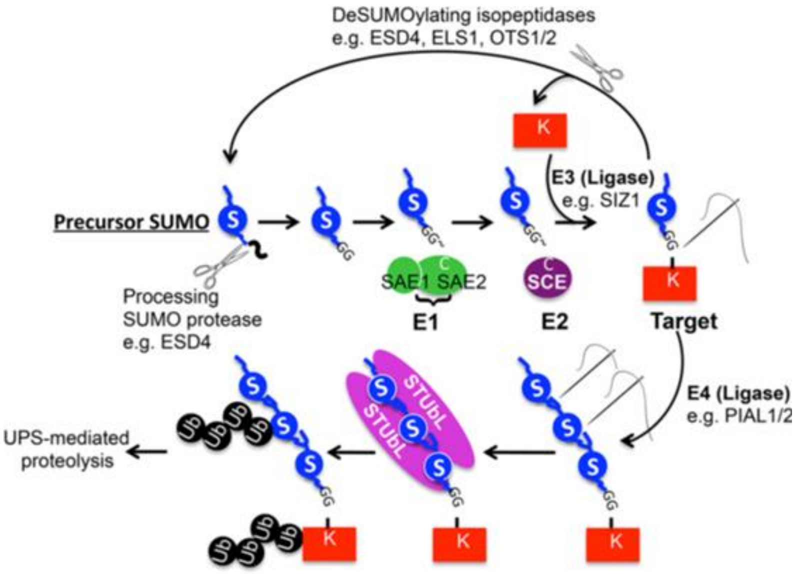

Figure 12. SUMOylation mechanism. SUMO is produced as a precursor protein with a C-terminal extension. SUMO proteases cleave off the C-terminal tail to expose the reactive carboxyl group of the C-terminal Gly. The SAE (or El) with its two subunits (SAE1 and SAE2) forms a thioester bond with this Gly residue to prepare for its transfer to the SCE (or E2). In addition to the thioester bond, the SCE binds SUMO non-covalently as well and eventually transfers SUMO to a target protein, usually with the aid of a third enzyme (E3), the SUMO ligase such as SIZ1. Targets, now covalently modified by SUMO through an isopeptide bond, perform specific functions, which are subsequently terminated by either removing SUMO from the target protein (deconjugation) or by regulated proteolysis. SUMO proteases with isopeptidase activity specifically and precisely hydrolyze the isopeptide bond, releasing free SUMO and target protein. Alternatively, a polySUMO chain forms through the activity of SUMO ligases (E4) such as PIAL1 and PIAL2, and this chain recruits STUbLs, which ubiquitinate both SUMO and the target protein and target them for degradation by the 26S proteasome (taken from Elrouby, 2015).

SUMO-activating enzyme (SAE)

The first enzyme involved in the SUMOylation pathway is a SUMO-activating enzyme (SAE), also called E1. It is a heterodimer of 40 kDa and 70 kDa proteins (Dohmen *et al.*, 1995; Desterro *et al.*, 1999), composed of a small subunit named SAE1 and a large subunit named SAE2 (Kurepa, 2003; Saracco, 2007). In turn, two genes encode SAE1, SAE1a (At4g24940) and SAE1b (At5g50580) respectively and are contained in segments duplicated between the chromosome 4 and 5 and are expressed with 81% amino acid sequence identity. The goal of expressing two different isoforms could provide a selectivity for the activation of specific SUMO isoforms (Kurepa, 2003). While the larger subunit SAE2 is expressed only by one single copy gene in the *Arabidopsis* genome, and forms a heterodimer with either isoform of SAE1 (Kurepa, 2003). The SUMO-activating enzyme catalyzes a four-part reaction. The first one is ATP-dependent and occurs at the adenylation domain of SAE2. It enables the bond formation between the glycine residue at the C-terminus of SUMO and ATP. After that, in the second reaction, the adenylate is conversed to a thioester, binding SUMO to a conserved cysteine domain. The third and fourth final reactions, the activated SUMO is transferred to a cysteine residue in the SUMO-conjugating enzyme (SCE) (Kurepa, 2003; Novatchkova, 2004).

SUMO-conjugating enzyme (SCE)

Once activated by SAE, SUMO can be transferred to the active site of the second enzyme called SUMO-conjugating enzyme, (SCE, also known as E2) (Saracco, 2007) to form a SUMO-E2 thioester intermediate (Johnson and Blobel, 1997; Desterro *et al.,* 1997). As the SUMO-activating enzyme, SCE is predominantly a nuclear protein (Seufert et al., 1995). In *Arabidopsis thaliana*, two loci encode this enzyme, resulting in two proteins SCE1a and SCE1b. However, the latter misses 53 residues at the N-terminus. Furthermore, no transcribed SCE1b could be detected in an experiment using anti-SCE1b antibodies, suggesting it is only a pseudogene (Kurepa, 2003). In contrast, SCE1a is highly expressed in multiple tissues (Kurepa, 2003). SCE1a contains an Ub-conjugating enzyme catalytic domain including a cysteine residue that is positionally conserved. This cysteine is the site for forming of a thiol-ester bond with SUMO (Kurepa, 2003). The SCE serves to catalyze the formation of an isopeptide bond between the target protein and SUMO, in conjunction with a third involved enzyme, an E3 ligase (Novatchkova, 2004) (Saracco, 2007). While in ubiquitylation pathway, there are many different types of conjugating enzymes but only a single ubiquitin protein in SUMOylation pathway, only one conjugating enzyme exists for eight different SUMO proteins (Bachmair, 2001). SUMO-conjugating enzyme is more conserved when compared with the other enzymes involved in the SUMOylation pathway (Kurepa, 2003). Embryo lethality has been observed in *sce* single mutants (Saracco, 2007). The presence of only one SCE in SUMOylation contrasts with the ubiquitin pathway where the multiple ubiquitin-conjugating enzymes participate in ubiquitylation of distinct sets of substrates (Bachmair *et al.,* 2001). In baker’s yeast *Saccharomyces cerevisiae,* the enzyme is called Ubc9 because of its similarity to ubiquitin-conjugating enzymes, while it is called Hus5 in the fission yeast *Schizosaccharomyces pombe*.

SUMO ligases

SUMO ligases are enzymes that bind, directly or indirectly, to a specific protein substrate, promoting the transfer of SUMO from a thioester intermediate to the amino group of a lysine residue in the protein substrate. The attachment to the target protein requires the presence of a consensus sequence in the SUMO-modified proteins, ΨKXE/D, where Ψ is a hydrophobic aliphatic residue, K is the lysine residue that is modified, X is any residue, E is a glutamic acid and D is an aspartic acid (Rodriguez *et al.*, 2001). A general characteristic of the SUMO E3 ligases is their conserved SP-RING domain, which is important for the interactions between the SCE enzyme and the protein substrates (Sachdev *et al.*, 2001). This particular domain contains one zinc ion and possesses a set of residues that stabilize the domain structure by a hydrogen bonding and van der Waals contacts (Bennett and Scheres, 2010). In animals and fungi, three types of SUMO ligases have been identified and named SIZ/PIAS, RanBP2, and Pc2, all of which interact with SCE enzyme, enhancing SUMOylation in vivo and in vitro. SIZ2/NFI1 in *Saccharomyces cerevisiae* and PIAS in animals share structural similarities with the SIZ group present in plants, possessing a RING-like domain for binding to the SCE-SUMO complex (Johnson and Gupta 2001; Kahyo *et al.*, 2001). While, RanBP2 (Ran-Binding Protein 2; Pichler *et al.*, 2002), does not share any relation to SIZ type SUMO ligases and it is located at the cytoplasmic filaments of the nuclear pore complex, interacting with SUMOylated RanGAP and GTPase proteins.RanGAP was itself one of the SUMO targets identified (Saitoh *et al.*, 1998). So far, its finding is restricted to animals and it was first recorded during studies on the nuclear core complex in mammalian cells. Pc2 protein belongs to the Polycomb Group (PcG) and was reported to be structurally unrelated to SIZ/PIAS or RanGAP (Kagey *et al.*, 2003). PcG proteins are able to form large multimeric complexes, which are microscopically detectable as distinct foci present within the cell and named PcG bodies.

In plants, two SUMO E3 ligases have been identified in the *Arabidopsis* genome, namely SAP and Miz 1 (also called SIZ1) and Methyl Methansulfonate-Sensitivity protein 21 (also called MMS21 or High Ploidy 2 (HPY2). Previous studies have reported that SIZ1 functions as a mediator of several environmental stimuli. For example, it can modulate the expression of different genes involved in phosphate starvation and low temperature (Miura *et al.*, 2005; 2007) as well as regulate heat shock proteins under high temperature (Yoo *et al.*, 2006). It has also been found to have a function in the response to drought stress (Catala *et al.*, 2007) as well as to modify the activity of abscisic acid (ABA) insensitive 5 (ABI5), a transcription factors involved in ABA signaling (Miura *et al.*, 2009). In addition, it is involved in the homeostasis of salicylic acid (SA); in fact, the accumulation of SA in siz1 mutant disturbs both the innate immunity response and flowering time (Jin *et al.*, 2008). Recent studies have also reported that SIZ1 participates in copper accumulation and/or the response to copper as well as in the regulation of nitrogen assimilation (Chen *et al.*, 2011; Park *et al.*, 2011). From a structural point of view, SIZ1 E3 ligases are characterized by four motifs: a N-terminal SAP (for scaffold attachment factor A/Bacinus/PIAS) motif, a PINIT (for Pro-Ile-Asn-Ile-Thr) motif, an SP-RING zinc finger domain, and a SUMO-interacting domain called SXS. In addition, SIZ1 contains all the protypical domains of SIZ/PIAS proteins and a PHD motif (for plant homeodomain) (Mi Sun *et al.,* 2010).

MMS21, instead, is an important regulator of cell proliferation and cytokinin signaling in root development (Huang *et al.,* 2009); in fact, its mutant *mms21-1* exhibits a short-root phenotype and different responses to exogenous cytokinins (Huang *et al.,* 2009). It has also been found to be involved in DNA damage response (Zhang *et al.,* 2013). Recent studies have shown that in Arabidopsis, MMS21 is associated with the E2Fa/DPa complex in cell cycle regulation (Liu *et al.,* 2016). Yeast-two Hybrid screening have shown that MMS21 interacts with the Chromatin Remodeler BRAHMA (BRM) protein and this interaction enhances the attachment of SUMO3 isoform on BRM protein (Zhang *et al.,* 2013). Another example of the MMS21 activity has been reported in *Saccharomyces cerevisiae* where MMS21 protein might be required to promote nuclear function (Kim *et al.,* 2016).

deSUMOylation pathway and SUMO-specific proteases

SUMO-specific proteases function to catalyze the cleavage of SUMO from its target protein and play at least two functions in the SUMOylation mechanism. The first is to remove SUMO from the target protein, making the modification reversible, and the second is to provide a source of free SUMO, which will be used for future conjugation to other proteins. Only two classes of deSUMOylating enzymes have been identified and characterized so far. They are called ULP and ULP-like proteases and function removing precisely SUMO from the target proteins. SUMO-specific proteases contain about a 200 amino acid domain (ULP domain), with cleaving activity (Mossessova and Lima, 2000). Nuclear magnetic resonance spectroscopy studies have shown no sequence similarities between the enzymes that cleave SUMO with those that cleave ubiquitin (Dohmen *et al.,* 2004). In baker's yeast, two deSUMOylating enzymes have been found, ULP1 and ULP2, respectively; they function to catalyze the carboxyl terminus of SUMO and to remove SUMO from the isopeptide-linked conjugates (Li and Hochstrasser, 2000). In mammalian genome, seven genes have been identified to encode proteins with ULP domain. These proteins are called SENPs (sentrin proteases), and, with the exception of SENP8, which cleaves at the carboxyl terminus of the small ubiquitin-like modifier NEDD8 (also known as RUB 1) (Mendoza *et al.,* 2003), all the remaining members of the SENP group are specific for SUMO. Homologs of SENPs in *Arabidopsis thaliana* are called AtULPs (Kurepa *et al.,* 2003).

In *A. thaliana*, data base searches have identified 67 genes with similarity to the SUMO protease domain. However, due to the difficulty of evaluating the enzyme specificity, it results unlikely that all of them are specific for SUMO. Although a vast number of deSUMOylating enzymes have been reported to being present in human and yeast (Gong *et al.*, 2000; Li and Hochstrasser, 2000; Johnson, 2004), they remain largely understudied. To understand better the role of SUMO proteases in plants, new investigations under stress conditions have been made, contributing towards their understanding. Particularly in *Arabidopsis*, seven SUMO-specific proteases are currently identified: EARLY IN SHORT DAYS 4 (ESD4), ULP1a/ESD4 LIKE SUMO PROTEASE (ELS1), ULP1b, ULP1c,/OVERLY TOLERANT TO SALT 2 (OTS2), ULP1d/OTS1, ULP2a, and ULP2b (Kurepa *et al.*, 2003; Colby *et al.*, 2006; Miura *et al.*, 2007a; Miura and Hasegawa, 2010; Hermkes *et al.*, 2011). Among them, three bona fide SUMO proteases have been characterized using physiological, biochemical and genetic approaches such as ESD4, OTS1, and OTS2 (Reeves *et al.*, 2002; Murtas *et al.*, 2003; Conti *et al.*, 2008, 2014). Several studies have also been performed in crop plants such as rice, wheat, and maize, using computational proteomic techniques to identify alignments of the conversed ULP catalytic domain. However, no SUMO-specific proteases have been characterized in crop plants so far. In particular, both ELS1 and ESD4 are widely expressed and show similarities in their sequences (Hermkes *et al.*, 2011). According to Murtas et al, ESL1 is ubiquitously expressed and localized in the cytoplasmic compartment, while ESD4 is localized to the nuclear periphery (Murtas *et al.*, 2003). In addition, loss-of-function els1 mutant has a mild phenotypic consequence, while esd4 mutant is severely dwarfed (Murtas *et al.*, 2003). Recent studies on both their functions have shown that ESD4 may be involved in SUMO recycling from the substrates, while ELS1 may play a role in SUMO maturation (Hermkes *et al.*, 2001).

SUMO-interacting motif (SIM)

It has been shown by Kerscher *et al.* that some proteins can interact together only when one of them is SUMOylated (Kerscher, 2007). The binding of SUMO to a target protein (or its interaction) is mediated by short amino acid consensus sequences called SUMO-interacting motifs (SIMs). In contrast with SUMOylation, which requires a covalent bond formed by an E3 SUMO ligase, the SUMO interaction does not occur through a covalent link and no enzymes are required. This mechanism has been demonstrated for RanGAP1 and RanBP2, where the interaction between both proteins can occur only if SUMO is attached to RanGAP1 (Joseph *et al.*, 2004). In TOC159 the predicted covalent SUMOylation site is present in its M-domain and a SUMO-interacting motif (SIM) is present in its G-domain. The amino acid sequence forming the core of the SIM motif has a conserved sequence, but the neighboring amino acids can vary (Nelis, 2015). Structurally, the SIM is embedded in a groove between the ß-strand and the α-helix in the structure of SUMO and can bind in parallel and antiparallel orientation in the groove (Kerscher, 2007). The core is flanked by negatively charged amino acids and serine and threonine amino acids can also be found adjacent to the core. These amino acids are easily phosphorylated and can therefore create a negative charge in the SIM core (Song *et al.* 2004). Previous studies have shown that the SIM core is hydrophobic and is composed by 3-4 aliphatic residues with the following sequence Val/Ile – X – Val/Ile – Val/Ile (Hecker *et al.*, 2005). Valine and Isoleucine are very similar; therefore, they can be interchanged without affecting the SUMO binding, allowing some variation in the SIM core. This fact has been confirmed by Song *et al.*, by testing the interaction between SIM and different synthetic peptides (Song *et al.*, 2004). The interaction occurs between the hydrophobic amino acids in the SIM and the aromatic amino acids present in SUMO protein. The change of a single amino acid in the core of the SUMO-interacting motif severely affects the interaction with binding partners.

Chloroplast quality control – balancing energy production and stress

The way that cells balance stress production with chloroplast turnover is inherently heavily dependent on the nucleo-cytosolic compartment (Rochaix and Ramundo, 2018). In chloroplast biogenesis, the complex signaling network, that comprises both anterograde and retrograde signaling chains, coordinates the two cellular compartments by sending reciprocal messages from one organelle to another in which nucleus-encoded factors modulate chloroplast gene expression (i.e. transcription, RNA processing, translation, and assembly of photosynthetic complexes). The flux of protein import into chloroplasts depends on several elements such as developmental programs, stress conditions, plant responses. (Lopez-Juez, 2007; Yu *et al.*, 2012). Consequently, the capacity of the protein import system to adapt must be rapid and efficient in order to facilitate the changes in gene expression that impact the plastid proteome. A set of chloroplast quality control mechanisms have evolved to monitor protein import through the TOC-TIC machinery. They inhibit the accumulation of photosynthetic proteins in the cytoplasm as well as the elimination of misfolded and mistargeted imported proteins in the stroma. However, it has been shown that several *toc* and *tic* mutants that reduce the efficiency of import tend to accumulate cytosolic preproteins (Agne *et al.*, 2009; Lee *et al.*, 2003; Ivanova *et al.*, 2004; Jarvis *et al.*, 1998). A significant transcriptional upregulation of cytosolic chaperones and the cytosolic ubiquitin-proteasome system (UPS) takes place in a response to defects induced by the absence of atToc159 in the *ppi2* mutant (Bischof *et al.*, 2011; Lee *et al.*, 2009), and inhibition of the 26S proteasome in these plants, resulting in the appearance of chloroplast preproteins in the cytosol. However, studies conducted in both algae and plants indicate that post-transcriptional and post-translational processes are also involved in the regulation of the turnover of stressed damaged chloroplasts. For example, stress conditions can induce photo-oxidative damage or inhibit plastid protein synthesis (Ramundo *et al.*, 2013; Hua and Viestra, 2016). In fact, plants evolved post-translational mechanisms engaging turnover of chloroplast proteins to maintain plastid homeostasis. One of these well-known mechanisms is the ubiquitin proteasome system (UPS) by which ubiquitylated proteins are degraded by the multi-subunit protease 26S proteasome. An example is the heat shock protein Hsc70-4, a cytosolic Hsp70 isoform, and the cytosolic E3 CHIP ubiquitin ligase that in genetic studies on Arabidopsis have been reported to generate defective chloroplasts when overexpressed (Lee *et al.,* 2009). Subsequently, further studies have revealed that the combination of CHIP and Hsp70-4 mediates the fate of imported chloroplast proteins by recognizing the transit sequence when misfolded, promoting their ubiquitylation and, subsequently, degradation by the 26S proteasome (Lee *et al.,* 2009). Other examples of proteasome substrates are insoluble protein aggregates or even entire organelles. PUB4 is an E3 ligase protein-quality controller, which was identified as a novel component of CLV3/CLE acting downstream of the cell-signaling pathway in root meristem size as a regulator of cell proliferation and of the timing of asymmetric cell division, important for the final root architecture (Kinoshita *et al.*, 2014). Other studies show PUB4 responsible for removing damaged plastids through an autophagic mechanism. In this scenario, reactive oxygen species (called ROS) are generated by excessive activity of the chloroplast electron transfer chain. These defective chloroplasts confer oxidative-damage to plant cells. In particular, it has been noticed that when chloroplasts accumulate $^1O_2$ (singlet oxygen) both the photosynthetic capacity and the ultrastructure of the organelle are damaged (Woodson *et al.,* 2015). Consequently, plants evolved a mechanism to remove damaged plastids through PUB4 as quality control mediator (Hua and Viestra, 2016). This allows to hypothesize that PUB4-mediated quality control removes damaged plastids through an autophagic mechanism (**Figure 13**).

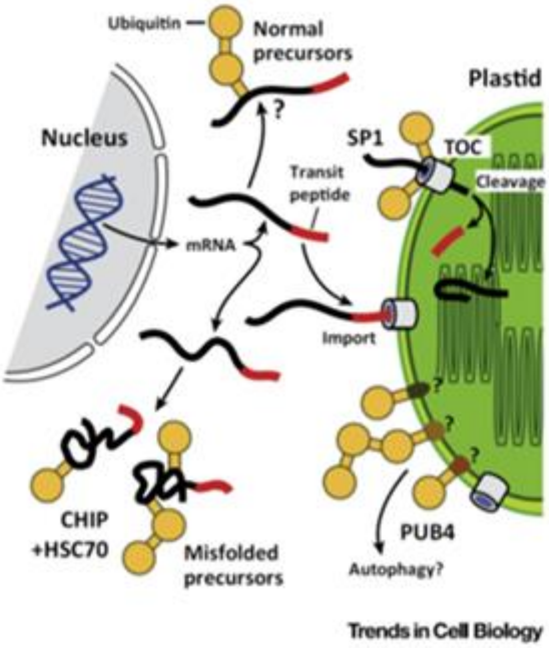

Figure 13. Ubiquitylation mediated regulation and quality control of chloroplasts. Many plastid proteins are expressed from nuclear genes and imported into the chloroplast via transit peptide signals. Ubiquitin (orange color) controls this process and eliminates damaged chloroplasts by: (i) removing mis-folded precursors by CHIP-mediated ubiquitylation; (ii) removing normal precursors before import through E3s such as DIF, (iii) controlling plastid protein import by SP1-mediated ubiquitylation and subsequent 26S proteasome-directed degradation of components of the TOC import complex, and (iv) eliminating damaged chloroplasts by ubiquitylation of unknown plastid outer membrane proteins by PUB4, which is possibly followed by autophagic breakdown in the vacuole (taken from Hua and Viestra, 2016).

General thesis outline and aims

Chloroplast biogenesis requires the import of a large variety of precursor proteins from the cytoplasm. These photosynthetic proteins are normally expressed and accumulated in wild type plants, resulting in an efficient protein import system performed by the import receptor TOC159, able to maintain the basal level of protein import into chloroplasts (Bauer *et al.,* 2000). They are imported post-translationally into the chloroplast through the TOC-TIC protein complex (Translocon of the Outer and Inner membranes of the Chloroplast, respectively). Additional factors direct the preproteins from the cytosol to the TOC complex: the so-called guidance complex binds to TOC receptors in a GTP-dependent manner to deliver precursor proteins. Also, plant hormones, considered as developmental messengers with antagonist and synergetic effects, are viewed as important elements during chloroplast biogenesis. Therefore, this present research focuses on the mechanisms that control chloroplast biogenesis at the level of chloroplast protein import.

The first part of this project is to investigate the interaction between TOC159 and the DELLA protein RLG2, a transcription factor that functions as negative regulator of gibberellic acid (GA) signaling in seed germination. Gibberellic acid is a phytohormone known to promote seed germination, plant growth, and other GA-dependent pathways, by promoting the degradation of the inhibitory DELLA proteins. Here, in the first chapter, I aim to contribute to the analysis of the TOC159/DELLA RGL2 interaction and how it might control the onset of chloroplast biogenesis during germination and the import of photosynthetic preproteins.

The second part of my thesis is devoted to the role of covalent TOC159 SUMOylation, as post-translational modification, and to the non-covalent SUMO-binding. TOC159 is known to be post-translationally modified (ubiquitylated and phosphorylated). Furthermore, the ubiquitin proteasome system is known to mediate the regulation and quality control of chloroplasts during plant development. However, recent studies have shown that SUMOylation may contribute to this regulation. For this reason, I will analyze the relation between TOC159 and the SUMOylation system. More specifically, the aim was to identify and verify the existence of SUMO interacting and SUMOylation sites in TOC159. I would then use mutagenesis to test the effects of SUMO- interaction and -ylation on abundance and stability of TOC159 under various conditions and initially under low gibberellic acid (GA). The results may provide a new element to the molecular framework of posttranslational modifications regulating TOC159, and concomitantly chloroplast biogenesis, during early plant development.

Preface to Chapter I:

This chapter consists of a published manuscript (Shanmugabalaji *et al.*, 2018. Current Biology 28, 1 – 8. Chloroplast Biogenesis Controlled by DELLA- TOC159 Interaction in Early Plant Development. doi.org/10.1016/j.cub.2018.06.006) that shows how gibberellic acid (GA) controls chloroplast biogenesis during seed germination. Under low GA conditions, when germination and greening are inhibited, TOC159 binds to the DELLA protein RGL2.

This direct interaction with the DELLA RGL2 promotes TOC159 degradation by the Ubiquitin-Proteasome System (UPS). Importantly, we show that the UPS-dependent degradation of TOC159 does not depend on SP1, a RING-type ubiquitin E3 ligase, known to target components of the TOC complex under stress conditions.

This work is the result of a collaboration with the Seed Molecular Genetics group at the University of Geneva. I have contributed the following experiments to this publication and carried them out under the guidance of Dr. Shanmugabalaji Venkatasalam:

Figure 1A: I performed a time course to analyze TOC159, TOC33, TOC75, and RGL2 protein accumulation during early developmental stages.

Figure S1A, B, C: I checked the impact of ABA and PAC on wild-type, *rgl2* and *3Δdella* mutant seedlings.

Figure 3E: I generated pTOC159:GFP-TOC159GM/*ppi2* transgenic plants to check the role of ubiquitylation under low GA conditions using CoIP.

Figure 3F, G: I analyzed the role of SP1 on accumulation of the TOC complex under low GA conditions.

Chapter I

Chloroplast Biogenesis Controlled by DELLA-TOC159 Interaction in Early Plant Development

Summary

Chloroplast biogenesis, visible as greening, is the key to photoautotrophic growth in plants. At the organelle level, it requires the development of non- photosynthetic, color-less proplastids to photosynthetically active, green chloroplasts at early stages of plant development, i.e., in germinating seeds. This depends on the import of thousands of different preproteins into the developing organelle by the chloroplast protein import machinery [1]. The preprotein import receptor TOC159 is essential in the process, its mutation blocking chloroplast biogenesis and resulting in albino plants [2]. The molecular mechanisms controlling the onset of chloroplast biogenesis during germination are largely unknown. Germination depends on the plant hormone gibberellic acid (GA) and is repressed by DELLA when GA concentrations are low [3, 4]. Here, we show that DELLA negatively regulates TOC159 protein abundance under low GA. The direct DELLA-TOC159 interaction promotes TOC159 degradation by the ubiquitin/proteasome system (UPS). Moreover, the accumulation of photosynthesis-associated proteins destined for the chloroplast is downregulated post- transcriptionally. Analysis of a model import substrate indicates that it is targeted for removal by the UPS prior to import. Thus, under low GA, the UPS represses chloroplast biogenesis by a dual mechanism comprising the DELLA-dependent destruction of the import receptor TOC159, as well as that of its protein cargo. In conclusion, our data provide a molecular framework for the GA hormonal control of proplastid to chloroplast transition during early plant development.

Experimental model and subject details

Plant materials and growth conditions

Wild-type and mutant plants were in the Columbia-0 (Col-0), Wassilewskija (Ws), and Landsberg *erecta* (L*er*) ecotype. The *sp1-3* and *rgl2-13* mutants used in this study were in the Col-0 ecotype and previously described [24, 36]. The *sly1-2 rgl2-13* double mutant was generated by crossing homozygous *rgl2-13* and *sly1-2* plants followed by genotyping. For genotyping the *sly1-2* allele, PCR was per- formed using dcaps primers followed by *Dra*I digestion. The *della* multiple mutant (N16298) are in the L*er* background and were described previously [21]. The different combinations of *della* mutants used (rgl2-SK54 rga-28 gai-t6) and the pentuple *della* mutant are in the Col-0 background as previously described [37]. The *ppi2* mutant, NTAP/WT, and NTAP-TOC159GM/*ppi2* lines used in this study were in the Ws ecotype and previously described [38]. Plants were grown either on soil under long-day conditions (16 h light (120 mmol m^{-2} s^{-1}), 8 h dark, 21°C, 70% relative humidity) or on Murashige and Skoog (MS) medium under short-day conditions (8 h light (120 mmol m^{-2} s^{-1}), 16 h dark, 21°C, 70% relative humidity).

Seedling treatment

Seeds were surface-sterilized and placed on MS medium containing the desired concentrations of paclobutrazol (PAC) (5 mM, 2 mM, 1 mM), abscisic acid (ABA) (5mM), or both. Plates were then placed under standard growth conditions for 66 hours, unless indicated otherwise. For *in vivo* co-immunoprecipitation (CoIP) experiments and proteasome inhibitor experiments, the seedlings were further treated with MG132 (100 mM) in liquid MS medium by vacuum infiltration for 20 min. The MG132 vacuum-infiltrated seedlings were immersed in liquid MS medium for the next 12 hours. The corresponding volumes of solvents for PAC, ABA, and MG132 (ethanol, DMSO) were used in control experiments.

Plant transformation and transgenic lines

The coding sequence for TOC159GM was amplified from the pET21d-TOC159GM construct [38] with TOC159GM –F(GATE)/ TOC159GM –R(GATE) primers and ligated into the pENTR221 vector using BP clonase (Invitrogen) and inserted into the pB7WGF2 binary vector (http://www.psb.ugent.be/gateway/) using the LR clonase (Invitrogen), resulting in a vector coding for the GFP- TOC159GM fusion under the control of the 35S promoter. The TOC159 promoter was PCR amplified from genomic DNA using pTOC159-F(*Spe*I)/pTOC159-R (*Hind*III) primers, digested with

*Spe*I/*Hind*III, and used to replace the cauliflower mosaic virus 35S promoter in the GFP-TOC159GM construct. The binary construct pTOC159:GFP-TOC159GM was introduced into *Agrobacterium tumefaciens* C58 and stably transformed into heterozygous *ppi2* plants using the floral dip method [40]. Transformants were selected on phosphinotricine-containing medium and complemented homozygous lines were selected and named pTOC159:GFP-TOC159GM/*ppi2*.

METHOD DETAILS

Yeast two-hybrid assays

The DELLA protein N-terminal part results in auto-activation in the yeast two-hybrid assay [20]. To avoid auto-activation, the sequence encoding a RGL2 fragment between amino acids 113 and 547 was amplified by PCR using primers RGL2-F(*Nde*I)/ RGL2-R(*Nco*I), digested with *Nde*I/*Nco*I, and ligated into the pGBKT7 (Clontech) vector to generate BD-DRGL2. The cDNA library obtained from 36-hour-old 5 mM PAC-, 3 mM ABA-treated *Arabidopsis* seedlings fused to the GAL4-AD in pGADT7 (Clontech) vector transformed into yeast strain Y187, and BD-DRGL2 transformed into yeast strain Y2H GOLD, was used as the bait vector for yeast two-hybrid screening. The independent transformants were selected on SD –Leu –Trp –Ade –His plates with 5 mM 3-aminotriazol (3-AT). The cDNA fragments corresponding to the TOC159 GTPase domain (TOC159G), TOC159 membrane domain (TOC159M), and TOC33 GTPase domain (TOC33G) were ligated into the pGADT7 (GAL4-AD) and pGBKT7 (BD fusion) vectors. The full-length cDNA sequences of RGL1, RGL2, RGL3, RGA, and GAI in pET28a were digested with *Not*I and further treated with Klenow enzyme to fill in the cohesive end and digested with *Nde*I. The blunt-ended and *Nde*I-digested fragments were ligated into the SmaI/*Nde*I sites of the pGADT7 vector. Yeast cells carrying the bait vector were then co-transformed with the prey plasmids containing the full-length DELLA fragments and/or the various TOC159 and TOC33 coding sequences. The empty bait vector (BD) was used as a negative control. BD-T, AD-p53, and AD-Lam were used as the positive and negative controls.

Bimolecular fluorescence complementation (BiFC) assay

Full-length RGL2 was PCR amplified from pET28a-RGL2 [10] using primers RGL2–F(GATE)/RGL2–R1(GATE), ligated into the pENTR221 vector by BP clonase (Invitrogen), and inserted into the binary vectors pYFN43 and pYFC43, containing the N- and C-terminal halves of GFP, respectively. TOC159GM from pENTR221 (described above) was inserted by LR-reaction (Invitrogen) into the binary pYFN43 and pYFC43 vectors [39]. The C–F(GATE)/C–R(GATE) primers were used to remove the *ccdB* cassette from the pENTR221 vector using BP clonase (Invitrogen) and inserted into the binary vectors pYFN43 and pYFC43 without any coding sequence. The vectors encoding the YFN and YFC fusions with or without TOC159GM/RGL2 constructs were transformed into *A. tumefaciens* C58Ci strain. All different combinations of constructs were co-infiltrated into leaves of 2–3-week-old *Nicotiana benthamiana* and the p19 protein of tomato bushy stunt virus [41] was used to suppress gene silencing in *N. benthamiana* leaves. Infiltrated leaves were analyzed after 2 days using a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

Transient expression assays

pB7WGF2 binary vector coding for the GFP-TOC159GM fusion under the control of the 35S promoter (described above). The coding sequence of RGL2 was PCR amplified from pET28a-RGL2 using primers RGL2 –F(GATE)/RGL2 –R(GATE), ligated into the pENTR221 vector using BP clonase (Invitrogen), and inserted into the pB7WGR2 binary vector (http://www.psb.ugent.be/ gateway/) using LR clonase (Invitrogen) to recombine RFP-RGL2 fusion under the control of the 35S promoter. Both constructs were transformed into *A. tumefaciens* strain C58Ci. Single constructs (GFP-TOC159GM, RFP-RGL2) and the combination of both constructs were co-infiltrated into leaves of 2–3-week-old *N. benthamiana*; the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in *N. benthamiana* leaves. Infiltrated leaves were analyzed after 2 days under a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

Confocal laser scanning microscopy

Seeds of the *Arabidopsis thaliana* chloroplast protein import reporter line 35S:RbcS(TP)-GFP/WT were surface-sterilized and plated on MS medium containing 1 mM PAC under short-day conditions for 5 days. The fluorescence was directly observed under a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

RNA extraction and gene expression analysis

Total RNA was extracted from seeds using the phenol:chloroform method as described [42]. Total RNAs were treated with RQ1 RNase-Free DNase (Promega) and reverse-transcribed using Improm II reverse transcriptase (Promega) and oligo(dT)15 primer (Promega) according to the manufacturer’s recommendations. Quantitative RT-PCR was performed by using QuantStudio Real Time PCR (ThermoFisher Scientific) and Power SYBR Green PCR master mix (Applied Biosystems). Relative transcript level was calculated by using the comparative DCt method and normalized to the *PP2A* (*At1g69960*) gene transcript levels.

Protein extraction and immunoblotting

Samples of identical fresh weight from each plant were frozen in liquid nitrogen and proteins were extracted using AP extraction buffer (100 mM Tris pH 8, 2% b-mercoptoethanol, 4% SDS, 20% glycerol) followed by acetone precipitation. SDS-PAGE and immunoblotting were performed as described [35, 42]. After protein transfer, the nitrocellulose membranes were stained with amido black. To probe the blots, primary antibodies recognizing TOC159 [2], TOC75 [30], TOC33 [31], TOC132 [24], TOC120 [32], FBN1A [33], MDH [34] and RGL2 [10] were used. To detect photosynthesis-associated proteins, antibodies recognizing RBCS, RBCL, PSBA, PSBO, and LHCB2 were purchased from Agrisera, Sweden. Additional antibodies recognizing affinity tags or non-photosynthetic housekeeping proteins were IgG (Cell Signaling Technology), anti-Ubiquitin (Cell Signaling Technology), anti-MYC (Cell Signaling Technology), anti-GFP (Sigma), anti-actin (Sigma), and anti-UGPase (Agrisera). Secondary antibodies were anti-rabbit IgG conjugated with horseradish peroxidase (Millipore), or anti-mouse IgG conjugated with horseradish peroxidase (Sigma). Chemiluminescence was detected using ECL Plus Western Blotting Detection Reagents (Pierce) and developed using a GE Amersham Imager 600. Band intensities were quantified using ImageQuant TL (GE Healthcare) software.

In planta CoIP from transient expression system in *N. benthamiana*

Full-length RGL2 was PCR amplified from pET28a-RGL2 using the primers RGL2–F(GATE)/RGL2–R2(GATE), inserted into the pENTR221 vector by BP clonase (Invitrogen), and further recombined into the pEarleyGate 203 binary vector [43] with LR clonase to obtain a RGL2-MYC fusion construct. The GFP-TOC159GM (described above) and RGL2-MYC fusion constructs were trans- formed into *A. tumefaciens* strain C58Ci and co-infiltrated into 2–3-week-old *N. benthamiana*; the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in *N. benthamiana* leaves. Total proteins were extracted in lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.2% v/v protease inhibitors (Sigma P9599), 20 mM MG132, and 10% glycerol. Anti-GFP antibodies conjugated to microbeads (mMACS GFP-tagged beads; Miltenyi Biotec) were used to isolate the immunoprotein complexes. The eluates were resolved by SDS-PAGE, and GFP-TOC159GM and RGL2-MYC were detected using anti-GFP and anti-MYC antibodies, respectively.

In vivo CoIP

36-hour-old NTAP/WT and NTAP-TOC159GM/*ppi2*, PAC (5 mM)-treated transgenic seedlings were transferred to MG132-containing medium for 12 hours. Total proteins were extracted in ‘‘lysis buffer (as above)’’and IgG-Sepharose resin was used to isolate protein complexes. Eluates were analyzed by western blotting using anti-RGL2, IgG, anti-TOC75, anti-LHCB2 and anti UGPase antibodies for detection of RGL2, NTAP-TOC159GM, TOC75, LHCB2, and UGPase protein. 36-hour-old pTOC159:GFP-TOC159GM/*ppi2*, PAC (5 mM) treated transgenic seedlings were transferred to MG132-containing medium for 12 hour. One-week-old pTOC159:GFP-TOC159GM/*ppi2* transgenic seedlings transferred to MG132-containing medium for 12 hours were used as a control. Total proteins were extracted in ''lysis buffer (as above)'' and anti-GFP antibody conjugated to microbeads (mMACS GFP-tagged beads; Miltenyi Biotec) was used to isolate protein complexes. Ubiquitinated TOC159GM protein complexes were detected by western blotting using anti-GFP, anti-Ubiquitin, and anti-UGPase antibodies for detection of GFP-TOC159GM, Ubiquitinated protein complexes, and UGPase protein. 35S:RbcS(TP)-GFP/WT seeds were grown in the presence or absence of PAC (1 mM) for 5 days and tested for ubiquitination as described above. Total proteins were extracted in ''lysis buffer (as above),'' and anti-GFP antibodies conjugated to micro beads (mMACS GFP-tagged beads; Miltenyi Biotec) were used to isolate immunoprotein complexes. Eluates were analyzed by west- ern blotting and probed with anti-GFP and anti-Ubiquitin antibodies for detection of RbcS(TP)-GFP, GFP, and Ubiquitinated protein complexes. 72-hour-old estradiol inducible pRGL2:HA-RGL2, PAC (10 mM)-treated transgenic seedlings with or without estradiol (50 mm). Total proteins were extracted in lysis buffer containing 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1% Triton X-100, 1mM DTT, 0.1% protease inhibitors (Sigma P9599), 5 mM MG132, and 10% glycerol, and HA-beads (Anti-HA affinity matrix; Roche) were used to isolate protein complexes. Eluates were analyzed by western blotting and probed with anti-HA, anti-RGL2, anti-TOC159, and anti-UGPase antibodies for detection of HA-RGL2, RGL2, TOC159, and UGPase protein.

Expression and purification of recombinant proteins

RGL2-His tag was overexpressed in *Escherichia coli* strain BL21(DE3) transformed with expression vector pET28a-RGL2-HIS. Expression was induced using 0.4mM isopropyl-b-D-thiogalactopyranoside at 25°C for 12 h. Bacterial pellets were lysed by sonication in 50 mM Tris-HCl pH 8, 150 mM NaCl, and 5 mM imidazol followed by centrifugation for 30 min at 14,000 rpm (Sorvall, SS-34). The supernatant was filtered through a 0.45mM nitrocellulose filter and RGL2-His$_6$ protein was purified from the supernatant fraction by nickel-nitrilotriacetic acid agarose affinity chromatography. Eluates were dialyzed against phosphate buffered saline (PBS). 10% glycerol was added to the purified RGL2-His$_6$ protein and stored at —80°C.

Chloroplast TOC159 targeting and protein import assay

Isolation of pea chloroplasts and *in vitro* protein import assays were performed as described [44, 45]. Isolated chloroplasts were incubated with *in vitro*-synthesized [^{35}S] methionine-labeled TOC159 (TNT_T7 Quick-coupled Transcription/Translation System; Promega) in the presence or absence of recombinant RGL2 protein, using bovine serum albumin as a negative control for recombinant RGL2 protein. As a negative control of TOC159 targeting, *in vitro* translated [^{35}S]-methionine labeled preprotein of the small subunit of Rubisco (p-RBCS) was incubated with isolated chloroplasts in the presence or absence of recombinant RGL2 protein, using BSA as a control. Selected samples were treated with the thermolysin protease to remove non-imported [^{35}S]-methionine labeled proteins. 25 mg of re-isolated chloroplasts were separated by SDS-PAGE. Gels were stained with Coomassie Blue, dried, and analyzed using a Phosphorimager (Molecular Imager FX; Bio-Rad).

Quantification and statistical analysis

For protein quantification, immunoblot band intensities were quantified using ImageQuant TL (GE Healthcare) software and the data are shown as mean ± SEM. Sample size, n, for each experiment is given in the figure legends. For transcript quantification, the data are shown as mean ± SEM. Sample size, n, for each experiment is given in the figure legends. Statistical analysis was carried out by Student's t test, with p values higher than 0.05 being considered non-significant (n.s.) while p values lower than 0.05 being considered significant for the analyzed data and indicated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. For [^{35}S]-methionine-labeled protein quantification, radioactive bands were quantified using ImageQuant TL (GE Healthcare) software and the data are shown as mean of two independent biological samples having given apparently similar results.

Results and discussion

Chloroplasts are the photosynthetic organelles of plants and members of the larger plastid family. Chloroplast biogenesis as the transition from proplastids (undifferentiated plastids in plant embryos) to chloroplasts in very young plants has rarely been investigated so far and promises new insight into the controlling mechanisms. The assembly of the photosynthetic apparatus during chloroplast biogenesis depends on the import of thousands of different preproteins by the chloroplast protein import machinery. The core of the preprotein import machinery at the outer chloroplast membrane consists of the TOC75 protein-conducting channel and the import receptors TOC33 and TOC159 [5–7]. In seeds, DELLA factors cooperate to stall seedling establishment by promoting abscisic acid (ABA) synthesis and accumulation upon seed imbibition. *Arabidopsis* DELLAs are encoded by five genes: *RGL1*; *RGL2*; *RGL3*; *RGA*; and *GAI* [3]. Gibberellic acid (GA)-dependent downregulation of DELLA abundance is central in chlorophyll and carotenoid biosynthesis during the differentiation of functional chloroplasts and also regulates chloroplast division and grana stacking in mesophyll cells [8, 9].

TOC159 and RGL2 protein levels are inversely regulated during seed germination

RGL2 plays a predominant role in repressing seedling establishment because *RLG2* mRNA expression is positively regulated by ABA, thus introducing a positive feedback loop sustaining high RGL2 accumulation [10–12]. On Murashige and Skoog (MS) medium, favorable to germination, RGL2 protein was detected until 24 hr after imbibition, after which increasing GA concentrations lead to its downregulation, consistent with previous results [13]. TOC159 protein levels began to increase strongly after RGL2 disappeared 36–48 hr after imbibition (Figure 1A). Under unfavorable germination conditions, such as canopy light or high temperatures, GA synthesis is repressed, which promotes DELLA accumulation. To mimic those conditions, seeds were placed on MS medium containing paclobutrazol (PAC), an inhibitor of GA synthesis. As expected, PAC-treated seeds accumulated high RGL2 protein levels, which remained high while TOC159 levels were reduced, consistent with the absence of greening (Figure 1B). We also compared the effects of PAC on wild-type (WT) to those on the *della* single mutant *rgl2* and the triple mutant *rgl2/gai/rga*, in which seedling germination and chloroplast biogenesis proceeds despite reduced concentrations of GA (Figure S1A). In PAC-treated WT seeds, TOC159 levels were strongly diminished when compared to untreated seeds, and in *rgl2* and *rgl2/gai/rga* mutants, no PAC effects could be observed (Figures S1B and S1C). Like TOC159, TOC33 was diminished in WT in the presence of PAC (Figures 1C and 1D). Interestingly, PAC treatment had no effect in WT on the levels of TOC132 and TOC120, two homologs of TOC159 that are not specifically required for chloroplast biogenesis (Figures 1C and 1D) [14].

TOC159 protein level is downregulated posttranscriptionally under low GA

We next explored how DELLA factors could repress TOC159 accumulation when GA synthesis is inhibited. Under low-GA conditions, RGL2, together with other DELLA factors, promotes endogenous ABA accumulation [10, 11, 15]. Thus, in PAC- treated WT seeds, the diminished TOC159 protein levels (Figures 1B, 1C, and 1F) could potentially be attributed to endogenous ABA repressing *TOC159* mRNA accumulation or negatively regulating TOC159 accumulation posttranscriptionally. We tested these hypotheses in turn. PAC-treated WT and *rgl2* seeds had similar *TOC159* mRNA expression, showing that low TOC159 accumulation in PAC-treated WT seeds relative to *rgl2* mutant seeds cannot be attributed to low *TOC159* mRNA levels (Figure 1E) [16]. Mutant seeds lacking one or several DELLA factors do not accumulate endogenous ABA upon imbibition in presence of PAC. In order to evaluate whether endogenous ABA negatively regulates TOC159 accumulation posttranscriptionally, we added ABA together with PAC in the germination plates in order to compensate for low endogenous ABA levels in mutant seeds lacking DELLA factors. Thus, we incubated PAC-treated *rgl2*, *rgl2/gai/rga*, and *rgl1/rgl2/rgl3/rga/gai* (*della*-null mutant) seeds with ABA, which blocked their germination and greening as for WT seeds (Figure S1A). Strikingly, TOC159 levels remained low in PAC- plus ABA-treated WT seeds relative to *rgl2*, *rgl2/gai/rga*, and *rgl1/ rgl2/rgl3/rga/gai* (Figures 1F and S1B–S1D). We also analyzed TOC159 protein levels in the *sly1-2* mutant, which accumulates high RGL2 protein levels upon imbibition [10]. The *sly1* mutant cannot germinate or green unless the tegument is removed [17]. *sly1-2* seeds had visibly lower TOC159 levels compared to WT and *rgl2-13*. The *sly1-2* phenotype was rescued in the *sly1-2/rgl2-13* double mutant, which had near- WT levels of TOC159 (Figure 1G). Collectively, these results therefore are not consistent with the notion that high endogenous ABA levels triggered by DELLA factors under low-GA conditions are responsible for repressing TOC159 accumulation in seeds. Rather, they strongly suggest that DELLA factors could directly repress TOC159 accumulation posttranscriptionally in a manner unrelated to their role in stimulating endogenous ABA levels.

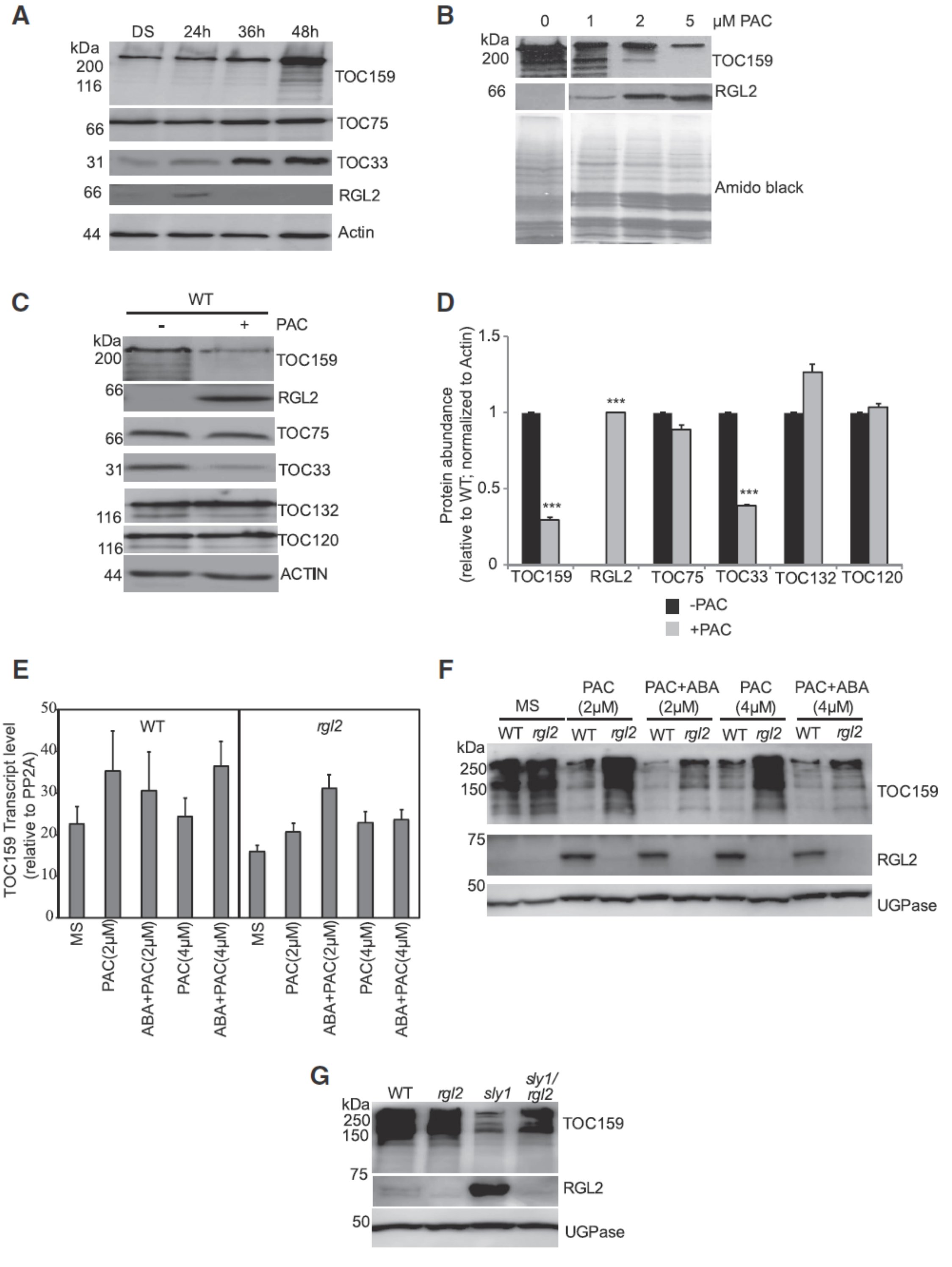

Figure 1. RGL2 Negatively Regulates TOC159 Protein under Low GA. (A) Western blot analysis of WT dry seeds (DSs) and seedlings germinated for 24, 36, and 48 hr, using antibodies to TOC159, TOC75, TOC33, and RGL2. Actin was used as a loading control. **(B)** Total protein extracts from 66-

hr-old WT seedlings grown in the presence or absence of PAC (1, 2, and 5 mM) were analyzed by immuno- blotting using antibodies to TOC159 and RGL2. Amido Black staining of the membrane shows equal loading. **(C and D)** Total protein extracts of 66-hr-old WT seedlings grown in the presence or absence of PAC (5 mM) were analyzed by immunoblotting using antibodies to TOC159, RGL2, TOC132, TOC120, TOC75, and TOC33 (C), and specific bands were quantified and normalized with respect to actin. Data are relative to WT (D). Error bars indicate ± SEM (n = 4). Student's t test; ***p < 0.005. **(E)** TOC159 transcript levels measured from WT and rgl2 seedlings grown in the absence or presence of PAC (2 or 4 mM), ABA (5 mM), and PAC+ABA. Data represent means ± SD of TOC159 mRNA level relative to PP2A (n = 4). **(F)** Western blot analysis of WT and rgl2 seedlings grown in the absence or presence of PAC (2 or 4 mM), ABA (5 mM), and PAC+ABA, using anti- bodies to TOC159, RGL2, and UGPase (loading control). **(G)** Total protein extracts of 24-hr-old WT, rgl2, sly1, and sly1/rgl2 seedlings, analyzed by immunoblotting using antibodies to TOC159, RGL2, and UGPase. See also Figure S1.

TOC159 and DELLA proteins interact directly

Up to here, our understanding of the role of RGL2 and other DELLAs in proplastid to chloroplast transformation during seed germination is very limited. To explore it, we carried out a yeast two-hybrid screen using RGL2 as the bait, and TOC159 itself was identified as an interaction partner of DELLAs. The TOC159 protein is composed of three domains: the N-terminal acidic domain (A-domain), central GTPase domain (G-domain), and C-terminal membrane domain (M-domain) (Figure S2) [2]. All DELLA factors physically interacted with the TOC159 G-domain in the two-hybrid assay (Figure 2A). The G-domain of the receptor TOC33 failed to interact with DELLA, indicating specificity of the DELLA–TOC159 G-domain interaction (Figures 2A and S2). Co-immunoprecipitation of GFP-TOC159GM and MYC-RGL2 from extracts of co-infiltrated *Nicotiana benthamiana* leaves confirmed the interaction *in planta* (Figure 2B). In vegetative plants, GFP-TOC159GM localizes at the outer envelope of the chloroplast membrane, whereas RFP-RGL2 is localized in the nucleus. How- ever, when RFP-RGL2 and GFP-TOC159GM were expressed together, fluorescence co-localized in the nucleus (Figure 2C). Fluorescence also appeared in the nucleus by bimolecular fluorescence complementation (BiFC) upon co-expression of YFC- TOC159GM and YFN-RGL2 in *N. benthamiana* leaves (Figure 2D). NTAP-TOC159GM isolated from transgenic seedlings grown in the presence of PAC was associated with RGL2 as well as TOC75 (Figure 3A). In a reverse experiment, TOC159 associated with RGL2-HA purified from estradiol-inducible *RGL2-HA* seedlings grown in the presence of PAC (Figure 3B). This corroborates the notion that RGL2 interacts with TOC159 *in vivo*. Moreover, recombinant RGL2 inhibited the *in vitro* outer membrane insertion of synthetic TOC159 in isolated chloroplasts, suggesting that it also functions to sequester TOC159 (Figure S3). Consistent with this notion, DELLAs have been shown to sequester interaction partners other than TFs in the nucleus, such as the prefoldin complex [18].

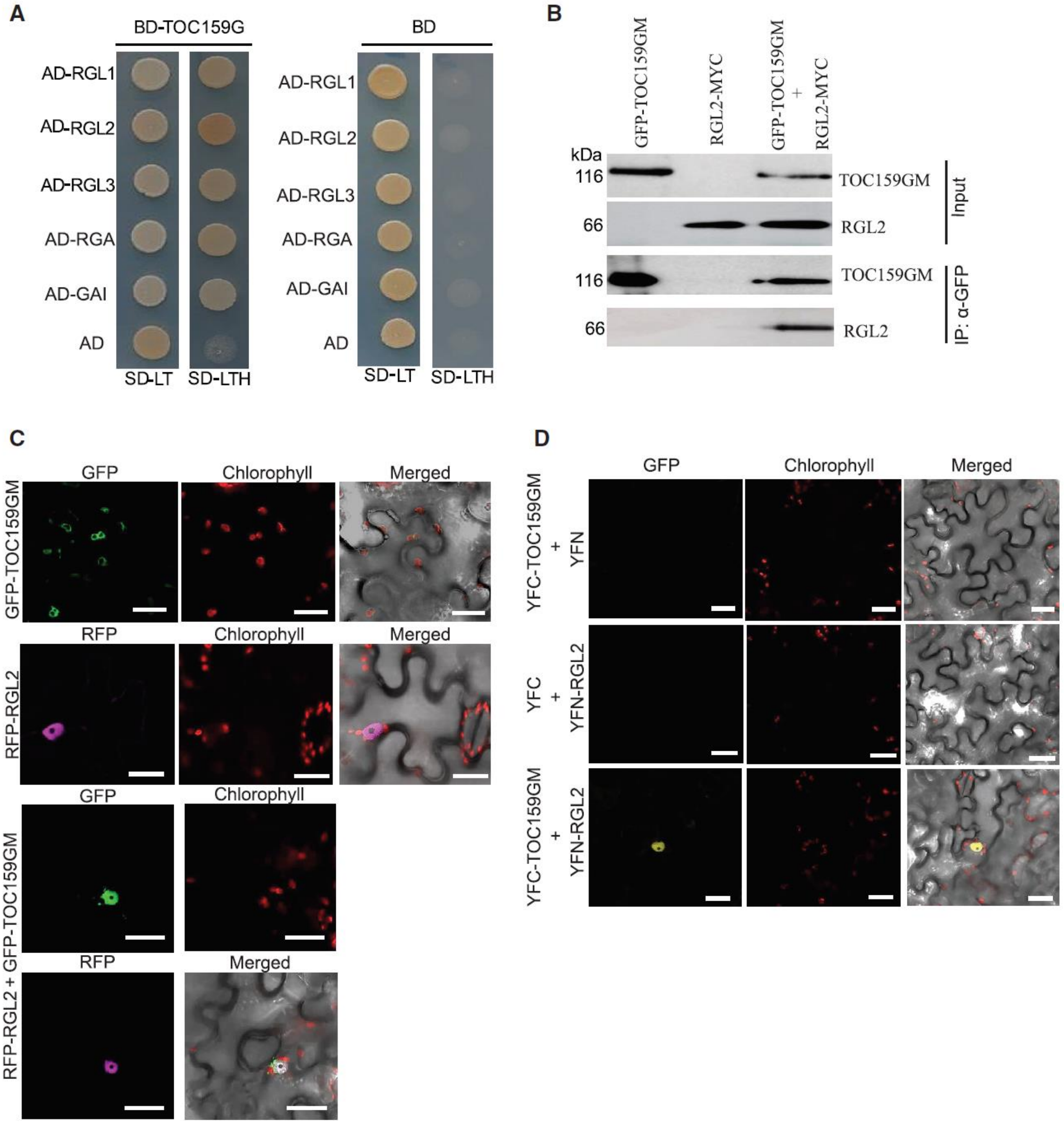

Figure 2. DELLA Specifically Interacts with TOC159 G-Domain. (A) Yeast two-hybrid interaction between the TOC159 G-domain and DELLA proteins on –Leu, –Trp and –Leu, –Trp, –His medium. AD, activation domain; BD, binding domain; empty vector was used as a control. **(B)** Co-immunoprecipitation from *N. benthamiana* leaves expressing GFP-TOC159GM, RGL2-MYC, or both. Total protein extracts (input) were incubated with anti- GFP beads, and the recovered proteins (IP) were analyzed by western blotting using anti-GFP and MYC antibodies to detect TOC159GM and RGL2, respectively. **(C)** Confocal microscopy images showing localization of GFP-TOC159GM and RFP-RGL2 in transiently transformed *N. benthamiana* leaves and co-localization confocal microscopy

images of GFP-TOC159GM and RFP-RGL2 in co-transformed *N. benthamiana* leaves. The scale bars represent 20 mm. **(D)** BiFC confocal microscopy images showing TOC159GM and RGL2 interaction in *N. benthamiana* leaves co-expressing TOC159GM and RGL2 fused to the C- (YFC) and N-terminal (YFN) portions of YFP, respectively. No fluorescence was observed with the corresponding empty vectors (upper panel). The scale bars represent 20 mm. See also Figure S2.

DELLA promotes TOC159 degradation by the ubiquitin/proteasome system

Besides their role in interfering with transcription factor (TF) activity [19–22], it has been demonstrated that DELLA mediates phytochrome interacting factor (PIF) degradation by the ubiquitin/proteasome system (UPS) [23]. TOC159 is a known target of ubiquitination and proteasome degradation [24, 25]. There- fore, DELLAs may promote TOC159 degradation via the UPS to repress chloroplast biogenesis under low-GA conditions. To evaluate this hypothesis, we first addressed whether TOC159 regulation by the UPS could be detected. PAC-treated WT seedlings incubated with MG132 (a proteasome inhibitor) had increased TOC159 levels, implicating degradation by the UPS (Figures 3C and 3D). Ubiquitination of TOC159 was confirmed after immunoprecipitating GFP-TOC159GM and anti-ubiquitin western blotting in the presence of PAC (Figure 3E).

Ubiquitin-proteasome-mediated degradation of TOC159 is SP1 independent under low GA

Chloroplast outer membrane ubiquitin E3 ligase SP1 has been implicated in the remodeling of the TOC complex by the UPS during plastid developmental transitions as well as stress, and TOC159, -75, and -33 are known targets of SP1 [24, 26]. To address the role, if any, of SP1 in DELLA-mediated TOC159 degradation, we analyzed PAC-treated *sp1* mutant seedlings. In the presence of PAC, TOC33, -75, and -159 were decreased in WT, but not in *rgl2* or *rgl2/rga/gai*. Surprisingly, only TOC33 and -75, but not TOC159, were increased in the presence of PAC in *sp1* when compared with WT (Figures 3F and 3G). This provides evidence for a role of SP1 in the PAC-induced degradation of TOC33 and TOC75 but suggests that a currently unknown ubiquitin ligase is specifically involved in DELLA-dependent degradation of TOC159 by the UPS.

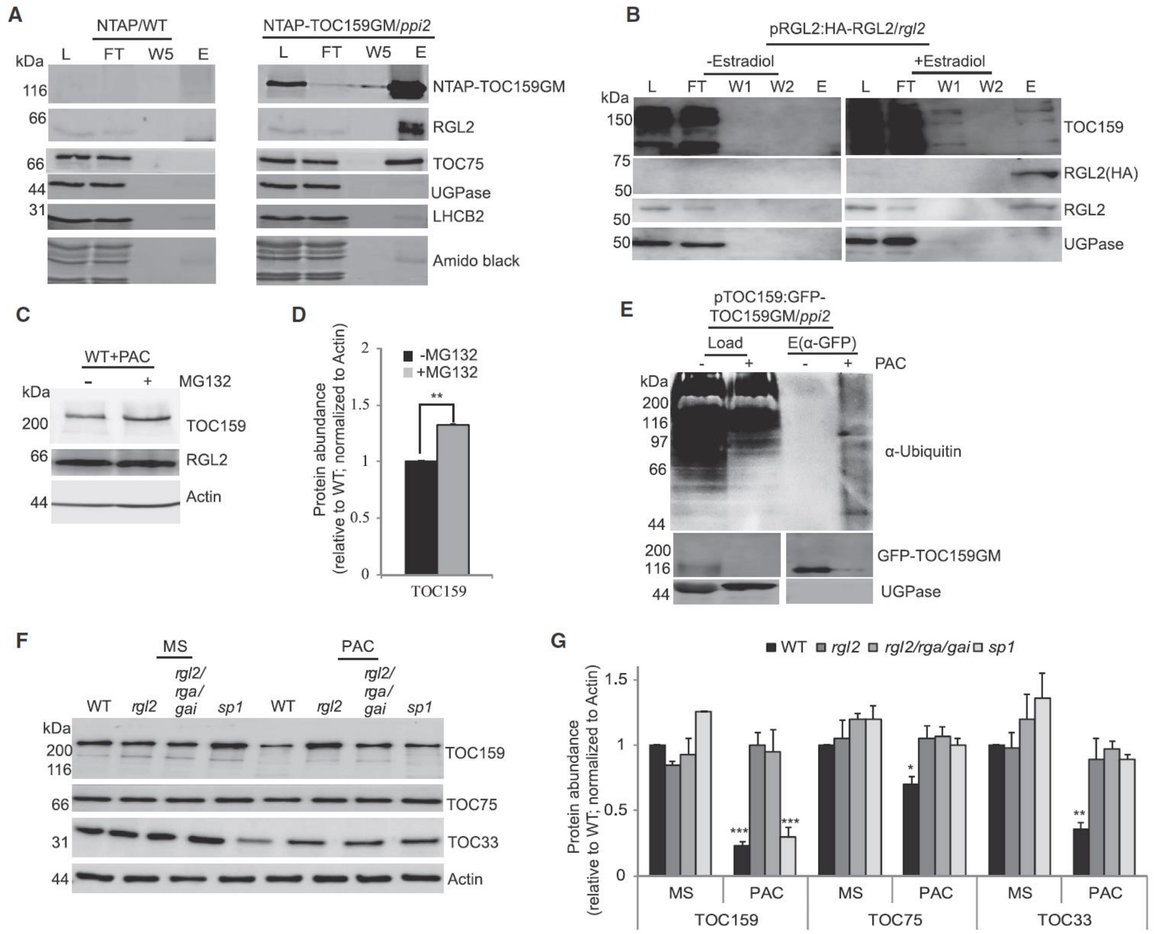

Figure 3. TOC159 Interacts with RGL2 and Is Degraded via the UPS under Low GA. (A and B) Immunoprecipitated NTAP-TOC159GM protein complex was analyzed by western blotting using immunoglobulin G (IgG) (to detect NTAP-TOC159GM), antibodies to RGL2, TOC75, UGPase, and LHCB2; NTAP/WT seedlings were used as a negative control (E, eluate; FT, flow-through; L, load; W5, last wash; A), and immunoprecipitated RGL2-HA protein complex was analyzed by western blotting using antibodies to HA (to detect HA-RGL2), RGL2, TOC159, and UGPase (B). **(C and D)** Total protein extracts of 3-day-old WT seedlings grown on PAC treated with or without MG132, analyzed by immunoblotting using antibodies to TOC159, RGL2, and actin as a loading control (C), and specific bands were quantified and normalized with respect to actin. Data are relative to WT (D). Error bars indicate ± SEM (n = 4). Student's t test; $^{**}p < 0.01$. **(E)** Immunoprecipitated GFP-TOC159GM protein complex was analyzed by western blotting using antibodies to GFP (to detect GFP-TOC159GM), ubiquitin, and UGPase. **(F and G)** Total protein extracts of 66-hr-old seedlings grown on MS medium in the presence or absence of PAC were analyzed by immunoblotting using antibodies to TOC159, TOC75, and TOC33 proteins. Actin was used as a loading control (F), and specific bands

were quantified and normalized to WT grown without PAC (G). Error bars indicate ± SEM (n = 3). Student's t test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. See also Figure S3.

Levels of photosynthesis-associated preproteins are regulated at the posttranslational level under low GA

We provide evidence that DELLA interacts with TOC159 under low-GA conditions, which leads to degradation of TOC159 by UPS and diminishes the levels of the chloroplast protein import receptor. This in itself would be expected to impede chloroplast biogenesis. However, it also raises the question of the fate of preproteins destined for the chloroplast during early seed germination. Very high concentrations of ABA or PAC (20 mM) repress the expression of photosynthesis-associated genes in the embryo [27]. However, the expression of most photosynthesis- associated genes did not change significantly in WT in the presence of moderate PAC concentrations (5 mM) when compared with *rgl2* in early seed germination [16]. We then investigated the accumulation of photosynthesis-associated as well as several non-photosynthetic proteins (FBN1A, FBN4, and MDH) in the presence or absence of low PAC concentrations (1 mM). In the absence of PAC, WT seedlings accumulated higher levels of photosynthesis-associated proteins, but not of the non- photosynthetic proteins (Figures 4A, 4B, S4A, and S4B). More- over, *rgl2* accumulated higher levels of photosynthesis-associated proteins than WT at the early stages of seed germination (Figures S4C and S4D). These results suggest regulation of pre-protein abundance at the posttranscriptional level.

An un-imported model preprotein is degraded by the UPS under low GA

It has been shown that un-imported chloroplast-destined preproteins accumulating in the cytosol are targeted for the UPS by the C terminus of Hsc70-interacting protein (CHIP) ubiquitin E3-ligase [28]. To address un-imported preprotein fate under low-GA conditions, we used RbcS(TP)-GFP (the transit peptide of the small subunit of rubisco preprotein fused to GFP) expressed in WT as a model. Seedlings treated with low PAC concentrations were import deficient as judged by the absence of imported GFP protein (Figure 4C). Furthermore, higher molecular mass, un-imported RbcS(TP)-GFP preprotein was detected by western blotting (Fig-ure 4C) together with weak GFP fluorescence at the chloroplast periphery (Figure 4D). In the absence of PAC, imported RbcS(TP)-GFP was detected by western blotting (Figure 4C) and resulted in strong GFP fluorescence inside chloroplasts (Figure 4D). These results show that low GA inhibits import of the model chloroplast preprotein and negatively regulates its concentration. Immunoprecipitation of un-imported RbcS(TP)-GFP under low GA followed by western blotting revealed higher molecular mass bands. Anti-ubiquitin western blotting indicated that these are due to ubiquitination of RbcS(TP)-GFP (Figures 4E and 4F).

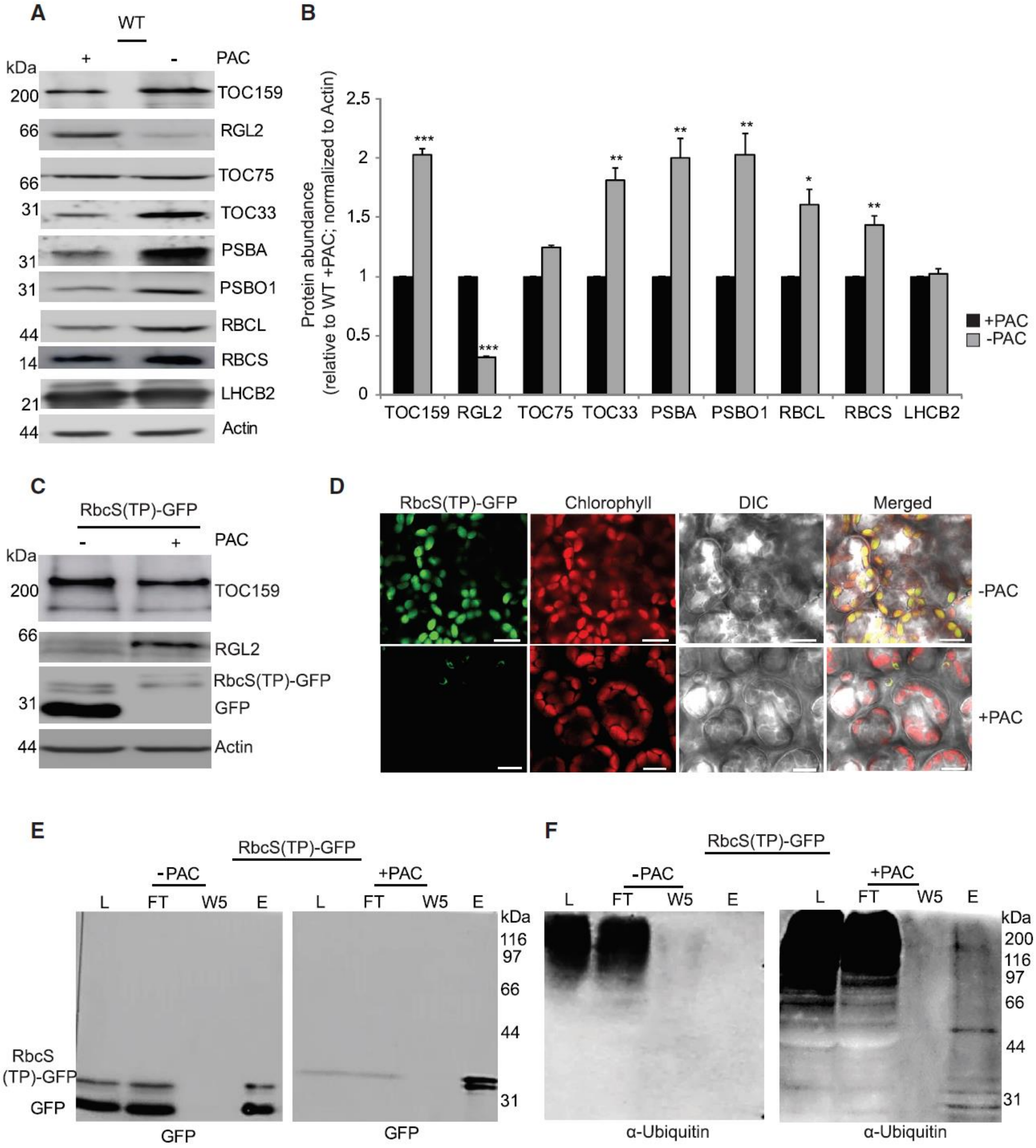

Figure 4. Low GA Concentration Reduces Accumulation of Photosynthesis-Associated Proteins and Promotes UPS-Mediated Degradation of the Un-imported Model Preprotein RbcS(TP)-GFP.

(A and B) Total protein extracts of 48-hr-old WT seedlings grown in the presence or absence of PAC (1 mM), analyzed by immunoblotting using antibodies to TOC159, RGL2, TOC75, TOC33, and several photosynthesis-associated proteins (A). Specific bands were quantified and normalized with respect to actin. Data are relative to WT + PAC (B). Error bars indicate ± SEM (n = 4). Student's t test; *p < 0.05; **p < 0.01; ***p < 0.005. **(C)** Total protein extracts of 5-day-old RbcS(TP)-GFP-expressing seedlings grown in the presence or absence of PAC (1 mM), analyzed by immunoblotting using antibodies to TOC159, RGL2, GFP, and actin (loading control). **(D)** Confocal microscopy images of cotyledons of 5-day-old RbcS(TP)-GFP-expressing seedlings grown in the presence or absence of PAC (1 mM). The scale bars represent 20 mm. **(E and F)** Immunoprecipitation of RbcS(TP)-GFP from 5-day-old seedlings treated with and without PAC. Total protein extracts (L) were incubated with anti-GFP beads, and immunoprecipitated proteins were analyzed by western blotting using antibodies to GFP (to detect RbcS(TP)-GFP products; E) and ubiquitin (F). Different but identically loaded blots were used for western analysis (F). See also Figure S4

Conclusions

Our studies provide a molecular framework for the GA hormonal control of chloroplast biogenesis during seed germination. Environmental stress decreases the concentrations of GA during seed imbibition, and when GA levels are low the DELLA (RGL2) accumulates [10–12]. Under such conditions, seed germination as well as chloroplast biogenesis are attenuated. Under low GA, a posttranslational mechanism contributes to the delay of chloroplast biogenesis by limiting the chloroplast protein import pathway at the level of the TOC complex. Direct interaction with DELLA promotes the degradation of the TOC159 import receptor by the UPS prior to its assembly in the TOC complex. In the yeast two-hybrid screen, TOC159 interacted with all five DELLAs. This suggests that DELLA-promoted TOC159 degradation as a means to control protein import into the chloroplast occurs at additional stages of development or under different types of stress in vegetative plants. As the import receptor TOC159 is essential for chloroplast biogenesis (the *ppi2* loss-of-function mutant has an albino phenotype), it is a very suitable target for interference with chloroplast biogenesis [2]. The chloroplast outer membrane ubiquitin E3-ligase SP1 [24] does not appear to be required for the DELLA-dependent degradation, presumably because the affected TOC159 never integrates into the outer membrane. Moreover, co-localization of TOC159 and RGL2 in the nucleus suggests that a cytoplasmic or nuclear E3-ligase is implicated. In contrast to TOC159, the reduced levels of TOC33 under low GA could be attributed to SP1 activity. SP1-independent degradation of TOC159 hints at the activity of an un- known E3-ligase. In the *ppi2* mutant lacking TOC159, many nu- clear genes encoding photosynthesis-associated proteins are strongly downregulated [2]. Under the low-GA/PAC conditions used in this study, TOC159 was diminished but the photosynthesis-associated genes were still expressed as in the absence of PAC. Still, the corresponding proteins were accumulating at reduced levels. Using RbcS(TP)-GFP as an *in vivo* model protein for chloroplast import, we provided evidence that ubiquitination of a preprotein in the cytosol and degradation by the UPS occurs when import is blocked. In view of the low-GA-induced import defect, this mechanism may be responsible for the removal of preproteins from the cytosol. Potentially, the cytosolic ubiquitin E3-ligase CHIP is involved in this scenario, as it is known to target un-imported preproteins [28]. When conditions become favorable for seed germination, GA accumulates to higher levels. The GA-Gibberellin insensitive dwarf1 (GID) complex binds to DELLA, leading to its ubiquitination by the F-box protein SLY1 and the SCF E3 ligase followed by degradation via the UPS [29], which in turn stabilizes TOC159. The comparatively low levels of TOC159 in the *sly1* mutant that accumulates RGL2 to high levels lend support to this scenario. Upon destruction of DELLA, TOC159 is immediately available for assembly into the TOC complex, which allows full import of photosynthesis-associated proteins into the chloroplast. Such a mechanism can assure a rapid switch from heterotrophic to photoautotrophic growth (visible as seedling greening) as soon as conditions allow. This in turn may improve the seedling's chances for survival in a challenging and stressful environment.

Supplemental Information

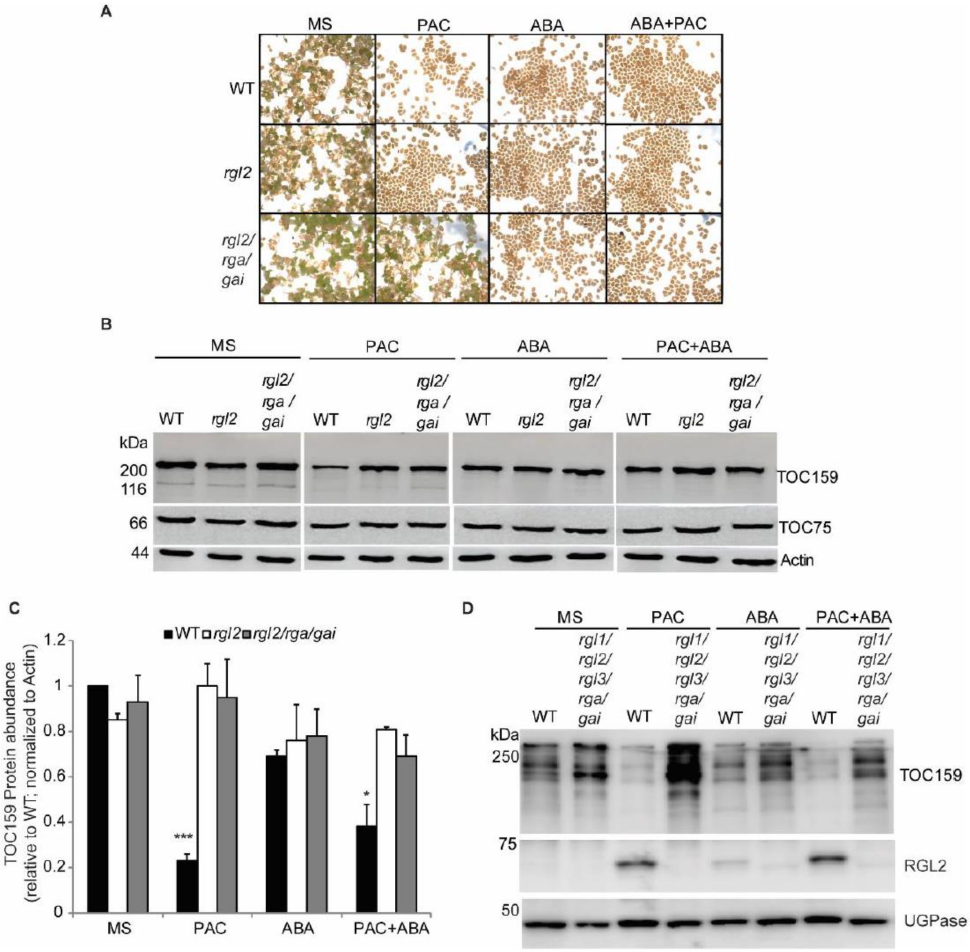

Figure S1. TOC159 protein level is not regulated by ABA, related to Figure 1. (A-C) WT, *rgl2,* and *rgl2/rga/gai* seedlings grown on MS medium with or without PAC (5 µM), ABA (5 µM), and ABA+PAC, documented at 66 hours **(A)** and total protein extracts were analyzed by immunoblotting using antibodies to TOC159, TOC75, actin **(B)** and quantification of specific bands were normalized with respect to actin. Data are relative to WT **(C)** Error bars indicate ±SEM (n=3). (Student's t-test; *p<0.05; ***p<0.005). For quantification, the same data were used in Fig. 3F for TOC159 protein quantification. **(D)** Western blot analysis of WT and *rgl1/rgl2/rgl3/rga/gai* (*della* null mutant) seedlings grown in the absence or presence of PAC (5 µM), ABA (5µM), and PAC+ABA, using antibodies to TOC159, RGL2, and UGPase (loading control).

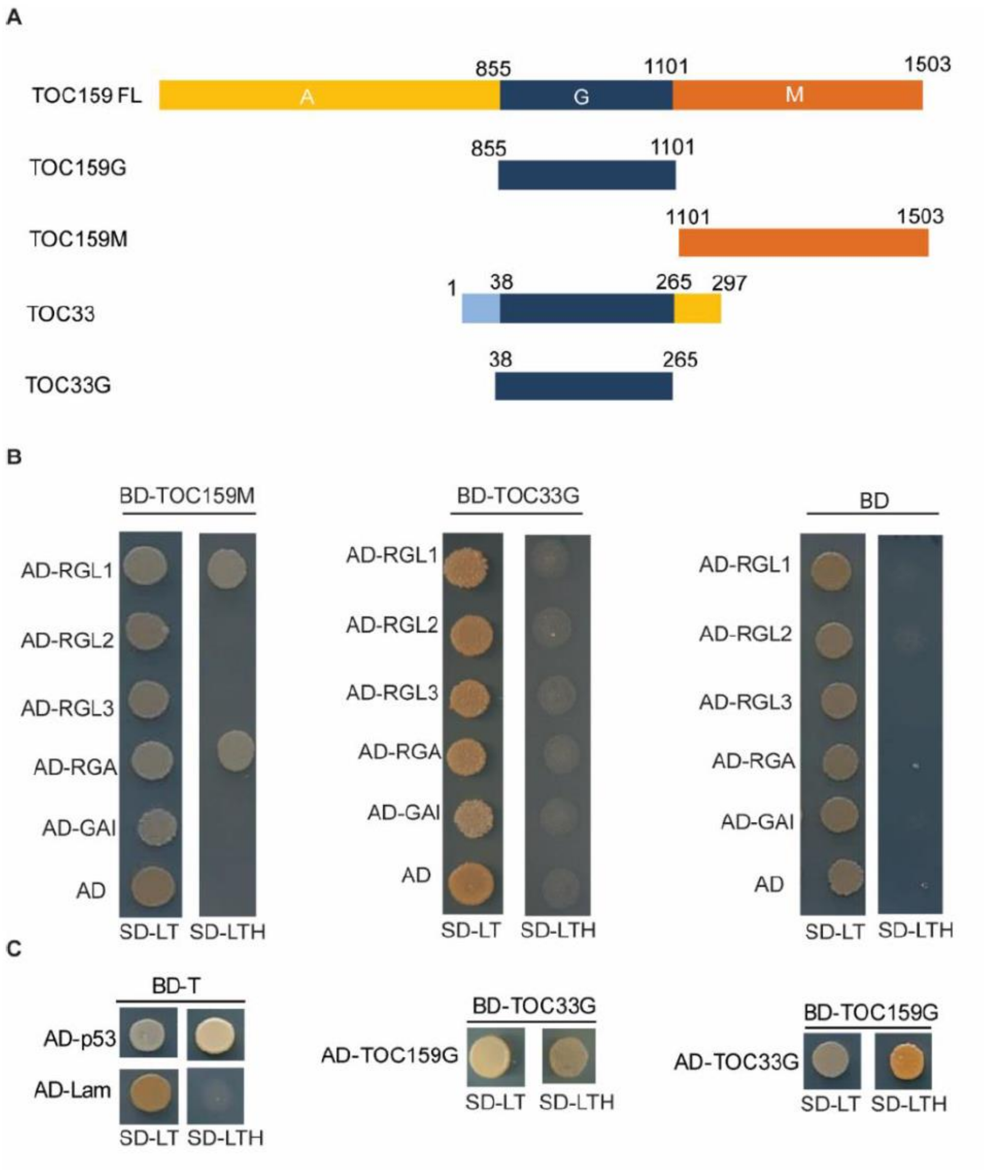

Figure S2. DELLAs interact with the G-domain of TOC159 but not with that of TOC33, related to Figure 2. (A) Schematic representation of full-length TOC159 composed of N-terminal acidic domain (A-domain), GTPase domain (G-domain; TOC159G), and C-terminal membrane domain (M-domain; TOC159M), and full-length TOC33 with GTPase domain (TOC33G). **(B)** Yeast two-hybrid analysis of BD-TOC159M and BD-TOC33G interactions with AD- RGL1, AD-RGL2, AD-RGL3, AD-RGA, and AD-GAI vector on –Leu, –Trp (SD-LT) and – Leu, –Trp, –His (SD-LTH) media. Empty bait vector was used as a negative control. (C) Yeast two-hybrid analyses of (left panel) BD-T interaction with AD-p53 and AD-Lam vector on SD-LT and SD-LTH media, as a control for functional yeast two-hybrid assays; and of interactions of (center panel) BD-TOC33G with AD-TOC159G and (right panel) vice versa on SD-LT and SD-LTH media, as a positive control for the BD-TOC33G interaction.

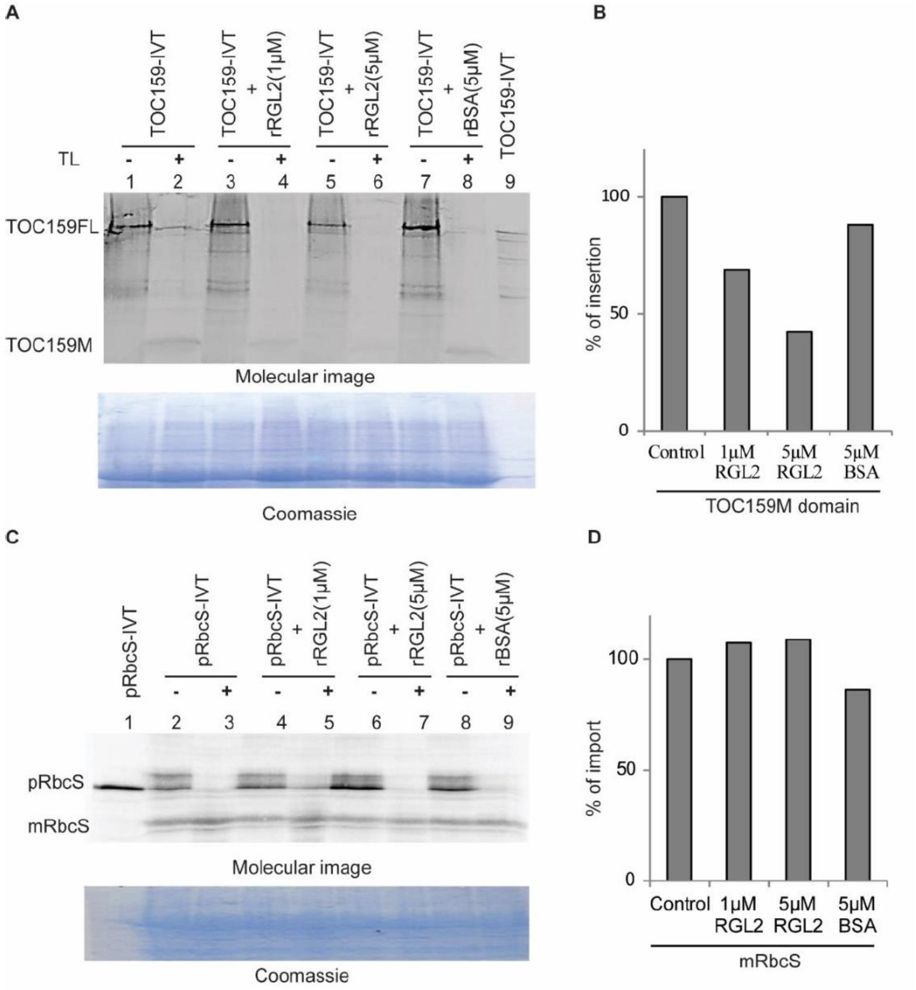

Figure S3. Recombinant RGL2 (rRGL2) binds to TOC159 and interferes with its insertion into the chloroplast membrane, related to Figure 3. (A-B) In vitro-synthesized, [35S]methionine-labeled TOC159 (TOC159FL) was incubated with isolated pea chloroplasts in the presence or absence of recombinant RGL2 or BSA. Chloroplasts were reisolated and incubated with of thermolysin (TL) Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control. Quantitative analysis of data from replicate experiments using ImageQuant TL software (GE Healthcare). The TL-resistant fragment (TOC159M) of in vitro-synthesized [35S]-methionine labeled TOC159 incubated with chloroplasts in the absence of rRGL2 or BSA (lane 2 in A) was set to 100% (B). **(C-D)** In vitro-translated, [35S]-methionine labeled pRbcS was incubated with isolated pea chloroplasts in the presence of recombinant RGL2 or BSA. Chloroplasts were reisolated and incubated with TL. Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control. Quantitative analysis of data from replicate experiments using ImageQuant TL software (GE Healthcare). Imported, TL-resistant [35S]-methionine labeled mRbcS in the absence of rRGL2 OR BSA (lane 3 in C) was set to 100% (D).

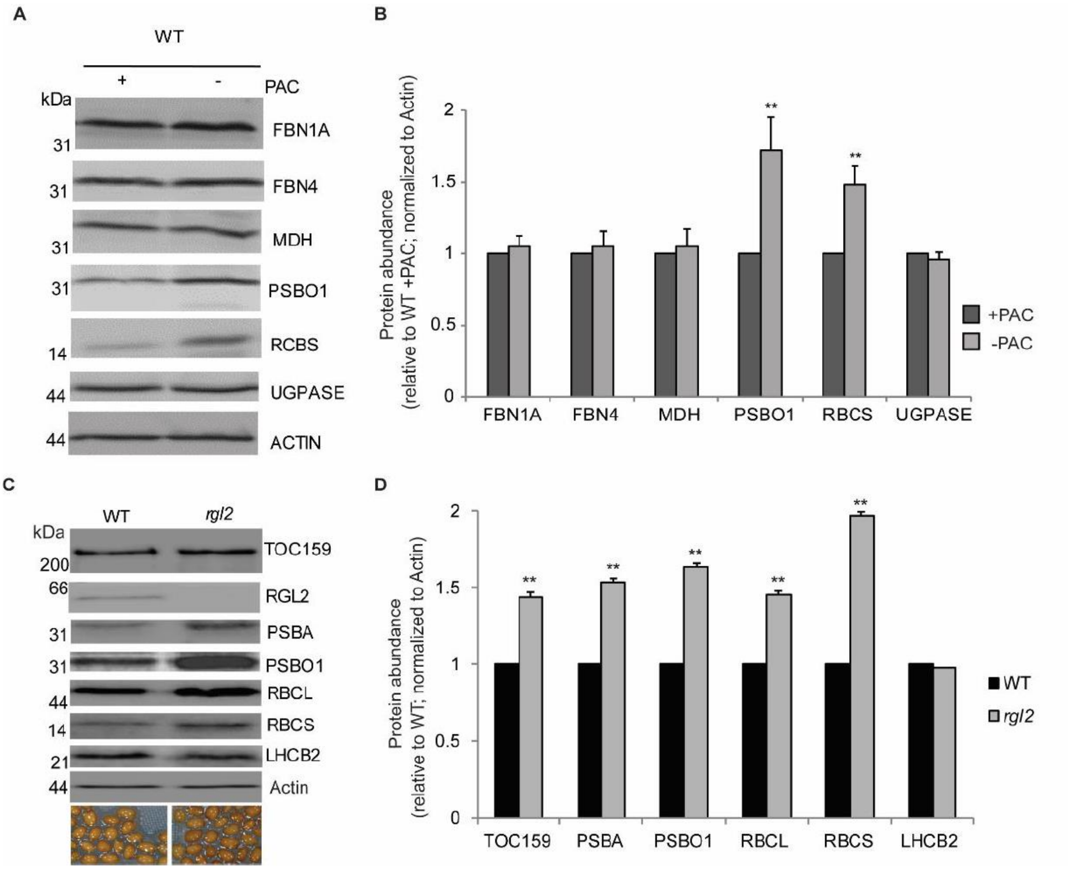

Figure S4. Low GA does not reduce accumulation of non-photosynthetic proteins and precocious accumulation of photosynthesis-associated proteins in rgl2, related to Figure 4. (A and B) Total protein extracts of 48-hour-old WT seedlings grown in the presence or absence of PAC (1 µM), analyzed by immunoblotting using antibodies to chloroplast non-photosynthetic proteins fibrillin 1A (FBN1A), fibrillin 4 (FBN4), malate dehydrogenase (MDH), cytosolic UGPase and photosynthesis-associated proteins PSBO1, RBCS (A) Specific bands were quantified and normalized with respect to actin. Data are relative to WT+PAC (B). Error bars indicate ±SEM (n=4). (Student's t-test; *p<0.05; **p<0.01; ***p<0.005). **(C-D)** Total protein extracts of 28-hour-old WT and rgl2 seedlings were analyzed by immunoblotting using antibodies to TOC159, RGL2, and several photosynthesis-associated proteins and specific bands were quantified and normalized with respect to actin. Data are relative to WT. Error bars indicate ±SEM (n=3). (Student's t-test; **p<0.01).

Primer name	**Sequence (5′→ 3′)**
RGL2-F(*Nde*I)	GCTTCTCATATGCTTGACACGACC
RGL2-R(*Nco*I)	GCAAGCTTCGGTACTCAGGCGAG
TOC159G-F(*Eco*RI)	CGTTCCGAATTCATGTCCAATTCTAATGTCACAATGGC
TOC159G-R(*Bam*HI)	GGAG AGGCGGGATTCCTTATCGGAAACCAAATAC
TOC159GM-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCCAATTCTAATGTCACAATGGC
TOC159GM-R(GATE)	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGTACATGTCGTACTTGTCGTTCGTCGC
pTOC159-F(*Spe*I)	CACTGAAAGCTTAAACCTGATTTCAC
pTOC159-R (*Hind*III)	CGACCACCACTAGTATCCTTCCGCTTTGC
RGL2-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGAGAGGATACGGAGAAACATGG
RGL2-R1(GATE)	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GGC GAG TTT CCA CGC CGA GG
RGL2-R2(GATE)	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATA GGC GAG TTT CCA CGC CGA GG
C-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAG TCCAAATGTTCTTCTCAAACG
C-R(GATE)	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATGTTTTTATTTTGTCACAC
TOC159G-F(*Eco*RI)	CGT TCCGAATTCATGTCCAATTCTAATGTCACAATGGC
TOC159G-R(*Bam*HI)	GGAGAGGCGGGAATTCTTATCGGAAACCAAATAC
TOC159 INT	GAATAGGGTTTTAATCGGAAG
TOC159 A3R	TGCCACATCAACATGCACTG
LB5	GATGCAATCGATATCAGCCAATTTTAGAC
PP2A RT F	GGACCGGAGCCAACTAGGA
PP2A RT R	GCTATCCGAACTTCTGCCTCATT
TOC159 RT F	CACAGTCTTGCTCTAGCTAG
TOC159 RT R	CTCCTCTGACCACATATGCC
rga-28F	ACCACGGGACTTCTTCTTCAT
rga-28R	TGGACTAAACGAACACCGTTC
rgl1-Sk62F	TGAAGAGAGAGCACAACCACC
rgl1-Sk62R	AGCTCGAAGACCGAGTTAACC
rgl3-3F	GTTACATACACACATGTTCCTCGTTCTCGA
rgl3-3R	ATTGCCTCTTCTAGTAATGCCTTCAATGAC
gai-t6F	AGCATCAAGATCAGCTAAAG
gai-t6R	CTAGATCCGACATTGAAGGA
gai-t6	TCGGTACGGGATTTTCGCAT
rgl2-13F	ATGGCTGAAGTAGCACAGAAGCTTGAACA
rgl2-13R	GGCAATCAGCTGTTGCCCGTCTCACTGGTG
sly1-2F	ACTCTCTTTACCTCTGGCCTCTTT
sly1-2R	ACTTACCAATCAACAAACTCC

Table S1. Primers used in this study. Related to STAR Methods.

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Preface to Chapter II:

Chloroplasts fulfill fundamental metabolic tasks, requiring enzymatic, structural and regulatory activities of a specific set of proteins. The proper function of these photosynthetic organelles requires a combination of specific protein involved in the biogenesis processes and the protein quality control mechanisms.

In Chapter I, I contributed to the analysis of the role of gibberellic acid (GA) in chloroplast biogenesis. The results showed that under low GA conditions TOC159 is ubiquitylated and targeted for degradation by the UPS upon DELLA binding.

Chapter II follows up on Chapter 1 by analyzing the post-translational modification of TOC159 by SUMO-(small ubiquitin-like modifier)-ylation and non-covalent SUMO binding. I address its involvement in the regulation of chloroplast biogenesis during early plant development. I carried out all of the experiments in this chapter under the guidance of Dr. Shanmugabalaji Venkatasalam.

Chapter II

SUMOylation of TOC159 regulates chloroplast biogenesis in early plant development

Summary

Chloroplast biogenesis describes the transition of non-photosynthetic proplastids to photosynthetically active chloroplasts in the cells of germinating seeds. Chloroplast biogenesis requires the import of thousands of nuclear-encoded preproteins and depends on the essential import receptor TOC159, mutation of which results in non-photosynthetic albino plants. The molecular mechanisms controlling the onset of chloroplast biogenesis are mostly unknown. We previously showed that ubiquitin-proteasome system (UPS)-dependent regulation of TOC159 levels contributes to the regulation of chloroplast biogenesis during early plant development. In addition, SUMOylation appears to fine-tune TOC159 levels during early plant development. The Small Ubiquitin-related Modifier protein (SUMO) may be covalently conjugated to (SUMOylation) or interact with a SUMO-interacting motif (SIM) of a target protein. We identified a SUMO-interacting motif (SIM) in the TOC159 GTPase (G-) domain that interacts with the SUMO3 isoform. Moreover, we report that the TOC159 membrane (M-) domain is SUMOylated by SUMO3 and that a single K to R substitution (K1370R) in the M-domain suppresses SUMOylation. Expression of the TOC159K1370R mutant in the *toc159* mutant (*ppi2*) complemented its albino phenotype. Compared to wild type TOC159, TOC159K1370R was destabilized under UPS-inducing stress conditions. However, TOC159K1370R recovered to same protein level as wild type TOC159 in the presence of a proteasome inhibitor. Thus, SUMOylation partially stabilizes TOC159 against UPS-dependent degradation under stress conditions. Our data contribute to the evolving knowledge of a molecular framework that tightly control proteostasis of the TOC159 import receptor during proplastid to chloroplast transition.

Introduction

Plants, being sessile organisms, must adapt to a large variety of biotic and abiotic stresses in order to survive. Therefore, they need quick and reversible responses to change physiological metabolisms, which ultimately lead to modifications in intracellular protein turnover and activities. In the last two decades, thanks to the genome largely solved and innovative molecular biology tools, scientists have centered their attention on how the genetic code affects all aspects of life to the perpetual changing of the environment. Several studies have accumulated evidence from plants indicating that they have evolved a set of mechanisms known as post-translational modifications (PTMs), to help to regulate multiple developmental programs in response to the environment. These are the fastest among all the plant responses and their modifications are generated by proteolytic cleavage and covalent addition of modifying groups to amino acids (Augustine and Viestra, 2018). In the chloroplast, the best characterized PTM is phosphorylation, regulating photosynthetic performance according to environmental stimuli through the action of kinase and phosphatase enzymes (Sugiyama *et al.,* 2008). Another important cellular process for protein modification is ubiquitylation, which amongst other processes is involved in the host defense signaling and in plant disease resistance. This well-studied PTM targets proteins for degradation by the 26S proteasome (Devoto *et al.,* 2003). Recently, studies focused on SUMOylation, a PTM related to ubiquitylation. Small Ubiquitin-like Modifier (SUMO) is a 15-kDa protein that covalently modifies a large number of proteins involved in many cellular processes, including gene expression, signal transduction, and genome maintenance (Gareau and Lima, 2010). In addition, it has been shown that SUMOylation regulates protein localization and activity (Gill, 2004). This reversible and dynamic mechanism starts with the attachment of SUMO to the target protein by a conjugation pathway, mechanistically analogous to the ubiquitylation system. SUMO is produced as a precursor protein with a C-terminal extension that needs to be processed by SUMO proteases (ULP, ubiquitin-like protein protease) to expose the reactive carboxyl group of a glycine residue. The process is activated by the E1 SUMO-activating enzyme (SAE) that requires ATP for the formation of the thioester bond between the cysteine and the glycine residues present in the SAE1 and SAE2 subunities, respectively. Then, the E2 SUMO-conjugating enzyme (SCE) binds SUMO non-covalently and transfers it to the lysine residue in the target protein, forming an isopeptide bond (Augustine and Viestra, 2018). This lysine is located in a consensus sequence ψKXE/D. This step requires the presence of a third class of enzymes called E3 SUMO ligases, such as HIGH PLOIDY 2 (HPY2/MMS21) and SAP/MIZ1 (SIZ1), to enhance the process. Target proteins, now covalently modified by SUMO, perform specific functions, which may subsequently be reversed by SUMO proteases that hydrolyze the isopeptide bond between SUMO and the target protein (Yates *et al.,* 2016). SUMOylation plays a crucial role in plant development and stress responses (Park *et al.,* 2011). In fact, genetic analysis of SUMOylation in *Arabidopsis thaliana* has identified several single and double embryo lethal mutants (Saracco *et al.,* 2007; Takashi *et al.,* 2012). Studies using loss-of-function mutants of SUMO E3 ligases have reported that SIZ1 acts as a mediator of several environmental stimuli. For example, it can modulate the expression of different genes involved in phosphate starvation and low temperature (Miura *et al.,* 2007) as well as regulate the heat shock proteins under high temperature (Yoo *et al.,* 2006). SUMOylation also has a function in the response to drought stress (Catala *et al.,* 2007) and is involved in the modification of a transcription factor involved in ABA signaling (ABI5) (Miura *et al.,* 2009). While the other SUMO E3 ligase, MMS21, is an important regulator of cell proliferation and cytokinin signaling in root development (Huang *et al.,* 2009). The mms21-1 mutant exhibits a short-root phenotype and altered responses to exogenous cytokinines (Huang *et al.,* 2009).

Chloroplasts are unique organelles that carry out photosynthesis. Although chloroplasts contain their own genome, the majority of chloroplast proteins are encoded by the nuclear genome and imported. They are synthesized as preproteins by cytosolic ribosomes. These preproteins are imported post-translationally into the organelle, depending on the presence of a cleavable N-terminal targeting sequence (transit peptide). The transit peptide directs preproteins into the chloroplast and across its envelope and is proteolytically removed after import. Translocation of preproteins across the envelope is achieved by the joint action of protein complexes called Toc (Translocon at the Outer membrane of the Chloroplast) and Tic (Translocon at the Inner membrane of the Chloroplast) respectively. The action of Toc and Tic requires the hydrolysis of ATP and GTP at different levels indicating an energetic requirement of the import process. The core of the Toc complex is heterotrimeric consisting of two related GTP-dependent preprotein receptor GTPases, Toc159 and Toc34, which interact with a β-barrel membrane protein, Toc75, that forms a protein-conducting channel, and is regulated by specific interactions with nuclear encoded preproteins (Hinnah *et al.,* 1997; 2002). Toc159 and Toc34 at the chloroplast surface recognize the transit peptides of newly synthesized preproteins. They initiate translocation across the outer membrane by transferring preproteins to Toc75 through a series of intermolecular events controlled by their intrinsic GTPase activities (Kessler and Schnell, 2002; Li *et al.,* 2007; Chang *et al.,* 2012; Lee *et al.,* 2013). Toc159 appears to be a major point of entry for highly abundant photosynthesis-associated preproteins arriving at the translocon complex. It is therefore regarded as the major chloroplast protein import receptor. Toc159 has a three-domain structure: a highly acidic N-terminal domain (A-domain), a central GTP-binding domain (G-domain) and a C-terminal membrane anchor domain (M-domain) (Kubis *et al.,* 2003). Chloroplast biogenesis, the transition of non-photosynthetic proplastids to photosynthetically active chloroplasts, depends on the essential import receptor Toc159 and its mutation results in non-photosynthetic albino plants (Jarvis and Lopez-Juez, 2013; Bauer *et al.,* 2000). Chloroplast biogenesis is dependent on the plant hormone gibberellic acid (GA). DELLA proteins act as negative regulators of GA signaling, known to inhibit seed germination and other GA-dependent pathways (Shanmugabalaji *et al.,* 2018). Under stress conditions, the DELLA RGL2 promotes the ubiquitylation and degradation of Toc159 by Ubiquitin Proteasome System (UPS), thereby delaying the onset of chloroplast biogenesis. An earlier study relied on a Yeast-two-Hybrid screen to identify putative Arabidopsis SUMO substrates. The E2 enzyme (also known as SCE – SUMO conjugating enzyme) was used as bait. TOC159 was one of the high probability candidates containing a predicted SAS (SUMO Attachment Site – ψKXE/D) (Elrouby and Coupland, 2010). Therefore, we are interesting on the role of Small Ubiquitin Like Modifier (SUMO) mediated posttranslational modification of TOC159 at the early developmental stage. In this study we investigate the role of SUMOylation and SUMO-binding of TOC159 and its role in chloroplast biogenesis. Covalent SUMOylation of TOC159 occurs at the M-domain. The M-domain is SUMOylated by SUMO3 and a K1370R substitution in TOC159GM domain abolishes it. Expression of TOC159GMK1370R point mutant in *toc159* mutant (*ppi2*) complements the albino phenotype but led to further depletion of TOC159 under low gibberellic acid conditions. The TOC159 G-domain contains a SUMO-interacting motif (SIM) that interacts with the SUMO3 isoform in the yeast-two-hybrid assay. The data provides a molecular framework for SUMO-binding and SUMOylation of TOC159 showing that SUMOylation positively influences protein stability with regard to the UPS The mechanism may allow fine-tuning of TOC159-dependent chloroplast biogenesis during early plant development.

Materials & Methods

Plant materials and growth conditions

The *A. thaliana* Columbia- Wassilewskija (Ws) ecotype was used as wild types. The *ppi2* mutant and pTOC159-GFP-TOC159GM:*ppi2* line used in this study were in the Wassilewskija (Ws) ecotype previously described (Bauer *et al.,* 2000) (Shanmugabalaji *et al.*, 2018). Seedlings were grown on Murashige-Skoog (MS) medium with long-day conditions (16-h light, 8-h dark, 120 µmol x m-2 x s-1, 21 °C). Plants were grown either on soil under short-day conditions (8 hours dark, 16 hours light, 120 µmol x m-2 x s-1, 21 °C) for vegetative growth, and under long-day conditions (16-h light, 8-h dark, 120 µmol x m-2 x s-1, 21 °C) for flower development and seed production.

Seedling treatment

Surface-sterilized seeds were placed on MS medium supplemented with 2 µM or 5 µM paclobutrazol (PAC). The plates were incubated under short day conditions for 3 days. Proteasome inhibitor experiments were performed as described earlier (Shanmugabalaji *et al.*, 2018).

Plant transformation and transgenic lines

The K1370R point mutation was introduced into the binary construct pTOC159-GFP-TOC159GM using a site directed mutagenesis kit (Agilent-QuikChangeII) with the primers TOC159S3F and TOC159S3R and resulted in pTOC159-GFP-TOC159GM-K/R. The pTOC159-GFP-TOC159GM-K/R construct was introduced into *Agrobacterium tumefaciens* (C58 strain) and stably transformed into heterozygous *ppi2* plants using the floral dip method. Transformed plants were selected on phosphinotricine-containing medium and lines homozygous for the transgene as well as the *ppi2* mutation were isolated and named pTOC159:GFP-TOC159GM-K/R:*ppi2* (referred to as GFP-TOC159GM-K/R:*ppi2* plants).

Yeast two-hybrid assays

The pGBKT7-TOC159G (BD fusion) and pGBKT7-TOC159M (BD fusion) vector were introduced in to the yeast strain Y2H GOLD as previously described (Shanmugabalaji *et al.*, 2018). The full-length cDNA sequences of SUMO1, SUMO2, and SUMO3 were amplified using primers (S1F, S1R, S2F, S2R, S3F, S3R), digested with NdeI/ EcoRI and ligated into the corresponding sites of the pGADT7 vector. The empty bait vector (BD) was used as a negative control. Co-transformants were selected on SD –Leu –Trp and SD –Leu –Trp –His plates.

Confocal laser scanning microscopy

One week old seedlings of the GFP-TOC159GM:*ppi2* and GFP-TOC159GM-K/R:*ppi2* lines were directly observed under a Leica TCS SL confocal microscope. Fluorescence images were captured and analyzed using LCS lite software (Leica).

Protein extraction and immunoblotting

Identical amounts of samples were collected and proteins extracted using AP extraction buffer (100 mM Tris pH 8, 2% b-mercaptoethanol, 4% SDS, 20% glycerol) followed by acetone precipitation (Piskurewicz and Lopez-Molina 2011). The SDS-PAGE and immunoblotting were performed according to standard protocols. To probe the blots, primary antibodies recognizing TOC159 (Bauer *et al.,* 2000), TOC75 (Hiltbrunner *et al.,* 2001), TOC33 (Rahim *et al.,* 2009), GFP (Takara), SUMO3 (Agrisera), UGPase (Agrisera), actin (sigma) and RGL2 (Piskurewicz, 2008) were used. As markers for photosynthesis-associated proteins, antibodies recognizing PSBA and PSBO1 were purchased from Agrisera. Secondary antibodies were anti-rabbit IgG conjugated with horseradish peroxidase (Millipore), or goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma). Chemiluminescence was detected using ECL Plus Western Blotting Detection Reagents (Pierce) and developed using a GE Amersham Imager 600. Band intensities were quantified using ImageQuant TL (GE Healthcare) software.

In planta CoIP from transient expression system in *N. benthamiana*

Full-length SUMO3 was PCR amplified from cDNA using the primers SUMO3–F(GATE) and SUMO3–R2(GATE) and inserted into the pENTR221 vector by BP clonase (Invitrogen). It was recombined into the pEarleyGate 203 binary vector [43] with LR clonase to obtain a 35S-SUMO3-MYC fusion construct. The binary vector 35S-GFP-TOC159GM used in this study has been described previously (Shanmugabalaji *et al.,* 2018). The point mutation was introduced in the binary construct 35S-GFP-TOC159GM by using a site directed mutagenesis kit (Agilent-QuikChangeII) with the primers TOC159S3F and TOC159S3R, as results we obtained 35S-GFP-TOC159GM-K/R. The 35S-SUMO3-MYC, 35S-GFP-TOC159GM and 35S-GFP-TOC159GM-K/R were introduced into *Agrobacterium tumefaciens* (C58 strain). And co-infiltrated into 23 weeks old *N. benthamiana*. Immunoprecipitation to isolate the protein complexes from total protein extracts using GFP-tagged microbeads (Miltenyi Biotec) has been described previously (Shanmugabalaji *et al.*, 2018). Anti-GFP antibody were used to detect GFP-TOC159, GFP-TOC159GM-(K/R). Anti-SUMO3, and anti-MYC antibodies, respectively, were used to detect the SUMOylated TOC159GM.

Quantification and statistical analysis

For protein quantification on immunoblots, ImageQuant TL (GE Healthcare) software was used to measure band intensities and the data are shown as mean ± SEM. Statistical analysis was carried using the Student's t test, with p values higher than 0.05 being considered non-significant (n.s.) while p values lower than 0.05 being considered significant for the analyzed data and indicated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Results

SUMO3 interacts with the TOC159 G domain and is SUMOylated at the TOC159 M domain

An earlier study using an *in vitro* SUMOylation assay in E. *coli* showed that out of three SUMO isoforms (SUMO1, SUMO2, and SUMO3), only SUMO3 SUMOylated TOC159 (Elrouby and Coupland, 2010). TOC159 has a N-terminal A- (acidic-, a central G- (GTP-binding domain) and a C-terminal M- (membrane) domain. The A-domain is known to be non-essential for Toc159 function and exquisitely sensitive to protease activity. It was excluded and only constructs encoding the TOC159 G- and M-domains (TOC159GM) were used in this study. In addition to a covalent SUMOylation site TOC159 may also have SUMO-interaction motifs (SIM). To investigate the possibility of SUMO3 interaction with TOC159GM, we used the GPS-SUMO prediction algorithm to search for SUMO Interaction Motifs (SIM) in TOC159GM. Based on the search results there is a predicted SIM (VKVLP) in the G-domain that is conserved in other plant species. (**Figure 1A** and **B**). To analyze the physical interaction between TOC159GM and SUMO3, we performed yeast two hybrid assays using TOC159G and TOC159M separately as baits. The TOC159G domain interacted exclusively with SUMO3 but not SUMO1 and SUMO2. None of the SUMO isoforms interacted with TOC159M. (**Figure 1C**).

Next, we examined SUMOylation of TOC159 using the GPS-SUMO algorithm to search for covalent SUMOylation sites in TOC159GM. A high scoring SUMOylation site with a strongly conserved motif (TGVKLED) and containing a potentially SUMOylatable lysine (K1370) was identified within the M-domain. (**Figure1-table Supplement 1**) (**Figure 1D**). The Arabidopsis K1370 is well conserved in other plants species (**Figure 1E**).

To investigate the SUMOylation of TOC159GM, we infiltrated *Nicotiana benthamiana* with 35S-GFP-TOC159GM or GFP-TOC159GM-K/R each together with or without 35S-SUMO3-MYC. To analyze the infiltration experiments identical amounts of total extracts were subjected to immunoprecipitation using anti-GFP-beads followed by Western blotting. An anti-GFP antibody was used to indicate total expression of GFP-TOC159GM and GFP-TOC159-K/R and resulted in bands of similar intensities in all four experiments. Anti-MYC and anti-SUMO3 were used to analyze conjugation of SUMO3-MYC to GFP-TOC159GM and GFP-TOC159GM-K/R. The Western blotting using anti-MYC and anti-SUMO3 antibodies resulted in strong signals for GFP-TOC159GM (Fig 1F, lane 3) but only very weak ones for GFP-TOC159GM-K/R (Fig 1F, lane 4) when co-expressed with SUMO3-MYC. No signals were detected when the GFP-TOC159GM constructs were expressed in the absence of SUMO3-MYC (Fig 1F, lanes 1 and 2). Note that GFP-TOC159GM co-infiltration with SUMO3 resulted in a much higher molecular mass band when analyzed with anti-MYC or -SUMO3 than the main, strong GFP-

TOC159GM band detected with anti-GFP. It therefore appears that upon co-expression with SUMO3-MYC only a small fraction of GFP-TOC159GM was present in the SUMOylated form and that it had a much higher molecular mass than GFP-TOC159GM alone (**Figure 1F**).

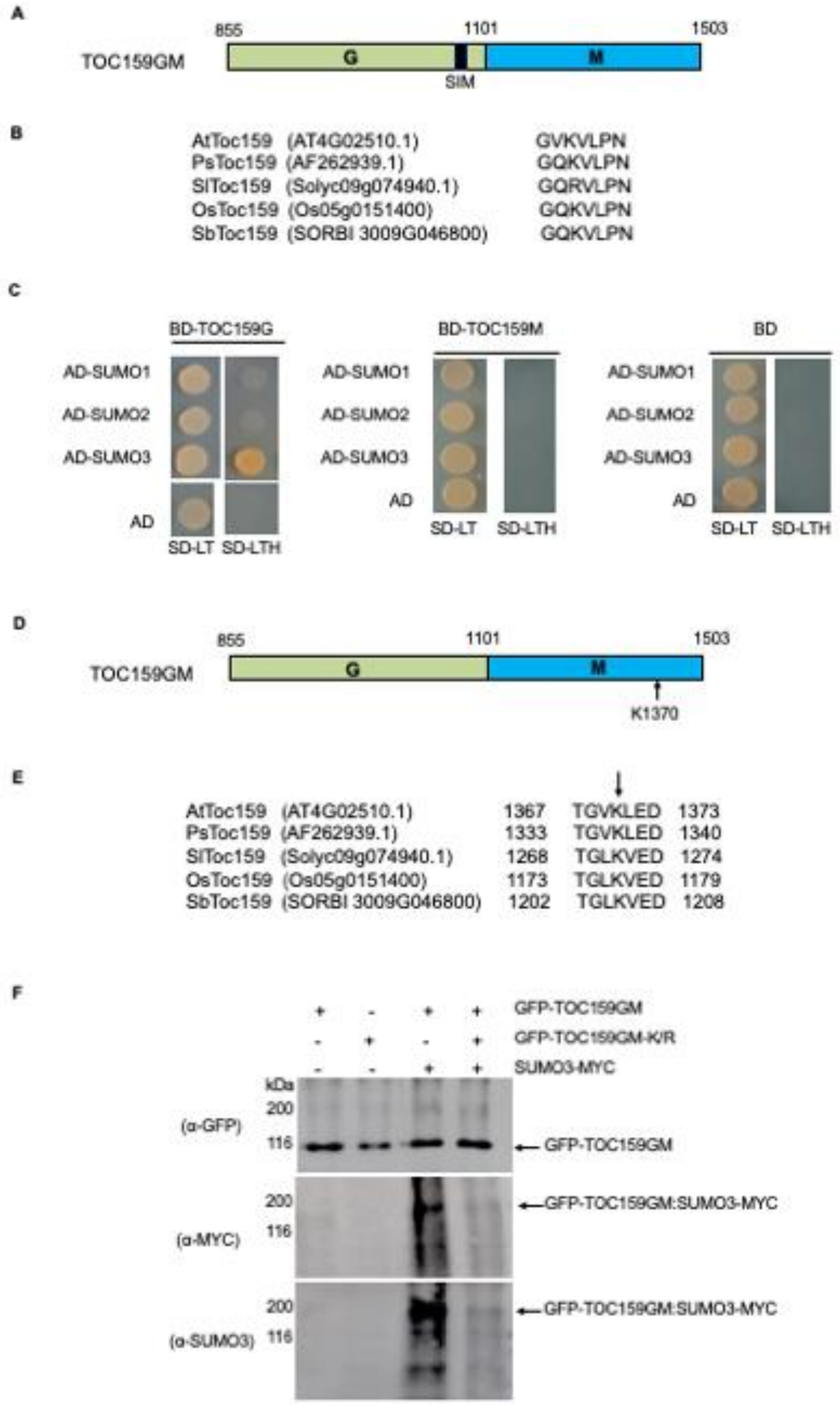


Figure 1. SUMO interaction and SUMOylation of TOC159GM. (A) Schematic representation of TOC159GM indicating the predicted SUMO Interaction Motif (SIM) in the G-domain. **(B)** Alignment of putative conserved SIM motifs in the G-domain of the following species: *Arabidopsis thaliana* (At), *Pisum sativum* (Ps), *Solanum lycopersicum* (Sl), *Oryza sativa* (Os), and *Sorghum bicolor* (Sb). **(C)** Yeast two-hybrid interaction assay of TOC159 G and M domain with SUMO proteins on –Leu, –Trp and –Leu, –Trp, –His medium. AD, activation domain; BD, binding domain; empty vector was used as a control. **(D)** Schematic representation of TOC159GM with indication of the predicted SUMOylation site K1370 (Lysine) at the M domain. **(E)** Alignment of the conserved predicted K1370 SUMOylation sites in the M-domain of a variety of species (as in Figure 1B). **(F)** Transient expression of GFP-TOC159GM, GFP-TOC159GM-K/R (SUMO mutant, K1370 replaced with R) with and without SUMO3-MYC in *Nicotiana benthamiana* leaves. Total protein extracts were subjected to immunoprecipitation with anti-GFP beads. The immunoprecipitated proteins from the expression of GFP-TOC159GM (lane 1) and GFP-TOC159GM-K/R (lane 2) alone as well as the co-expression with SUMO3 (lane 3 and 4) were analyzed by western blotting using anti-GFP, anti-MYC and anti-SUMO3 antibodies.

Non-SUMOylatable TOC159GM-K/R mutant complements the ppi2 mutation

Previous work has demonstrated that the A-domain of TOC159 is apparently dispensable but that the M-domain of TOC159 is essential for protein import into the chloroplast (Lee *et al.*, 2003). Earlier studies demonstrated that TOC159GM alone without the A-domain could complement the albino *ppi2* phenotype (Agne *et al.*, 2009). To characterize the effect of the K1370R mutation on the M-domain *in vivo*, we engineered transgenic lines expressing GFP-TOC159GM as well as GFP-TOC159GM-K/R under the TOC159 promoter in the *ppi2* background. Two independent transgenic lines of pTOC159-GFP-TOC159GM:*ppi2* and pTOC159-GFP-TOC159GM-K/R:*ppi2* (called GFP-TOC159GM:*ppi2 and* GFP-TOC159GM-K/R:*ppi2* plants hereafter) were isolated and the genotypes were confirmed by PCR using specific primer pairs (**Figure 2A, Figure 2-figure Supplement 1A**). Homozygous GFP-TOC159GM:*ppi2* and GFP-TOC159GM-K/R:*ppi2* transgenic lines gave green plants with almost identical chlorophyll accumulation and very close to that of wild type (**Figure 2C**). This indicated complementation of the visible *ppi2* phenotype. Western blotting analysis using GFP antibodies specifically detected GFP-TOC159GM and GFP-TOC159GM-K/R proteins. The blots revealed that TOC159GM and GFP-TOC159GM-K/R proteins accumulated to very similar levels in the respective transgenic lines. The blots were also probed with antibodies against TOC75 and TOC33, revealing no significant differences between the two transgenic lines (**Figure 2B**) (**Figure 2-figure supplement 1B and C**). To localize the GFP-TOC159GM and GFP-TOC159GM-K/R *in vivo* 7-day-old seedlings were analyzed by confocal fluorescence microscopy. The images show that both GFP-TOC159GM and GFP-TOC159GM-K/R give clear fluorescent signals at the chloroplast periphery that are consistent with outer envelope membrane localization (**Figure 2D**).

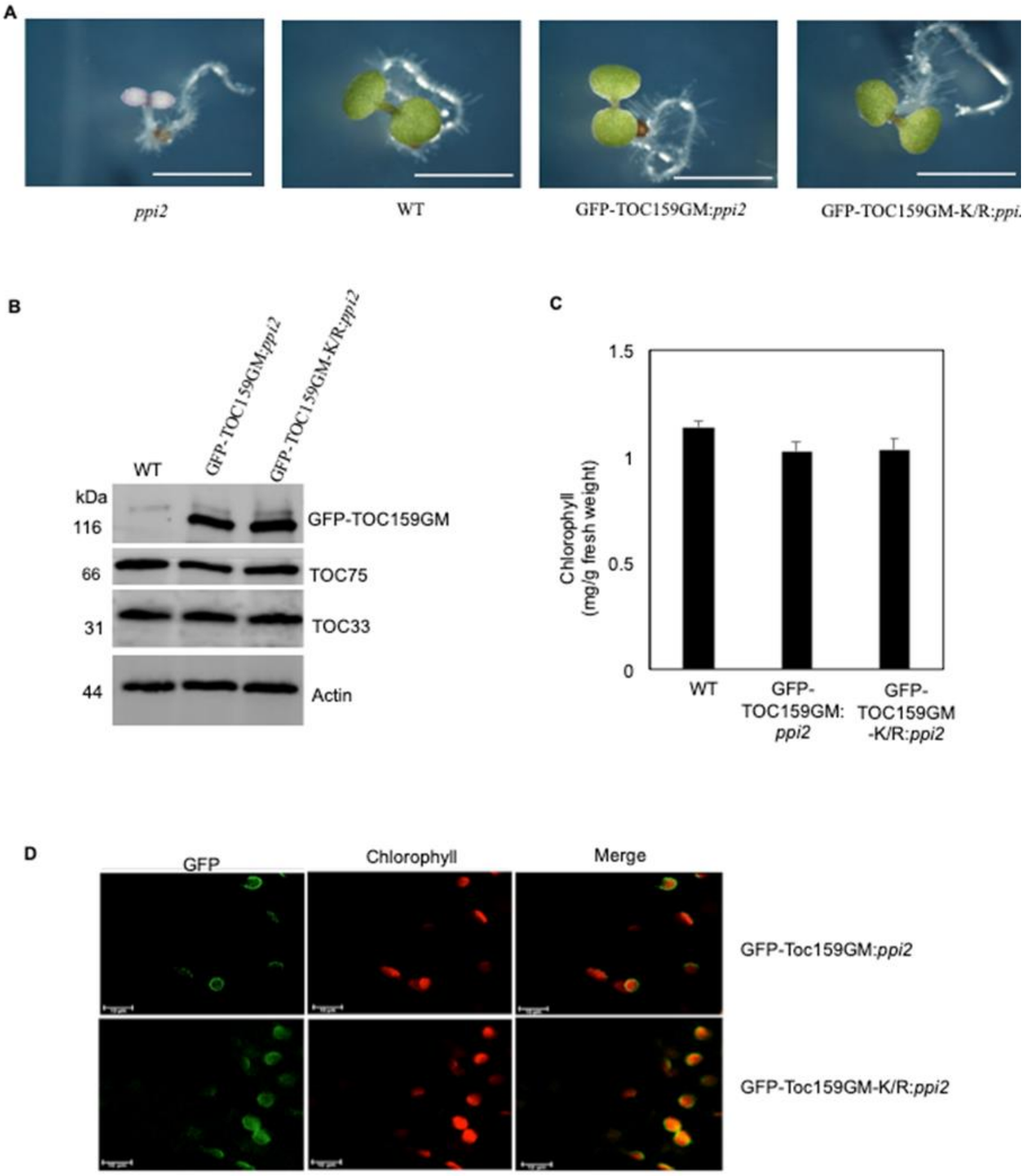


Figure 2. Complementation of *ppi2* (*toc159* mutant) by non-SUMOylatable Toc159GM-K/R. (A) 7 days old seedling phenotypes of *ppi2*, WT (Ws), GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2*. **(B)** Western blot analysis of total protein extracts from 7 days old seedlings of WT (Ws), GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2*. The blot was probed with anti-GFP, -TOC75 and TOC33 antibodies. Actin was used as a loading control. **(C)** Chlorophyll levels of wild type, GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2* from 7 days old seedlings. **(D)** Confocal microscopy

analysis of 7 days old GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2* seedlings. Green fluorescence (GFP, left-hand panel), red fluorescence (Chlorophyll, middle pane) and the overlay of the two (right-hand panel) are shown.

TOC159GM-K/R accumulation is diminished when compared to TOC159GM compared under low gibberellic acid conditions

Addition of paclobutrazol (PAC) to the MS growth medium can be used to inhibit GA biosynthesis and results in low GA conditions. Under low GA early during germination the DELLA RGL2 destabilizes TOC159 via the ubiquitin-proteasome system. To explore a possible role of SUMOylation of K1370 on protein stability GFP-TOC159GM:*ppi2* and GFP-TOC159GM-K/R:*ppi2* were allowed to germinate in the presence or absence of PAC. As reported earlier, the GFP-TOC159GM protein level was severely reduced in GFP-TOC159GM:*ppi2* seeds under low GA. In comparison, the level of GFP-TOC159-K/R in GFP-TOC159GM-K/R:*ppi2* seeds was even more diminished. Moreover, TOC75 and TOC33 levels were also lower in mutant TOC159GM-K/R:*ppi2* than in GFP-TOC159GM:*ppi2* under low GA whereas their levels were the same in the untreated lines (**Figure 3A and B**) (**Figure 3 - figure supplement 1 and 2**). PAC-treated seeds, low in GA, accumulated high levels of RGL2 protein. We also compared RGL2 protein levels between TOC159GM:*ppi2* and TOC159GM-K/R:*ppi2* in the presence of PAC. The results revealed that there was no difference in RGL2 accumulation between the two lines (**Figure 3C**).

SUMOylation partially stabilizes the TOC159 under low gibberellic acid conditions

To determine whether the diminished stability of GFP-TOC159-K/R under low GA conditions is also due to UPS-mediated degradation, GFP-TOC159GM-K/R:*ppi2* and GFP-TOC159GM:*ppi2* seeds were germinated under low GA and subjected to treatment with or without the proteasome inhibitor MG132. Western blot analysis demonstrated that both GFP-TOC159-K/R and GFP-TOC159 were rescued by MG132 and accumulated to the same level. (**Figure 3E and F**) (**Figure 3 - figure supplement 3**). The results suggest that SUMOylation partially protects GFP-TOC159GM against UPS-mediated degradation under low GA when compared to GFP-TOC159GM-K/R.

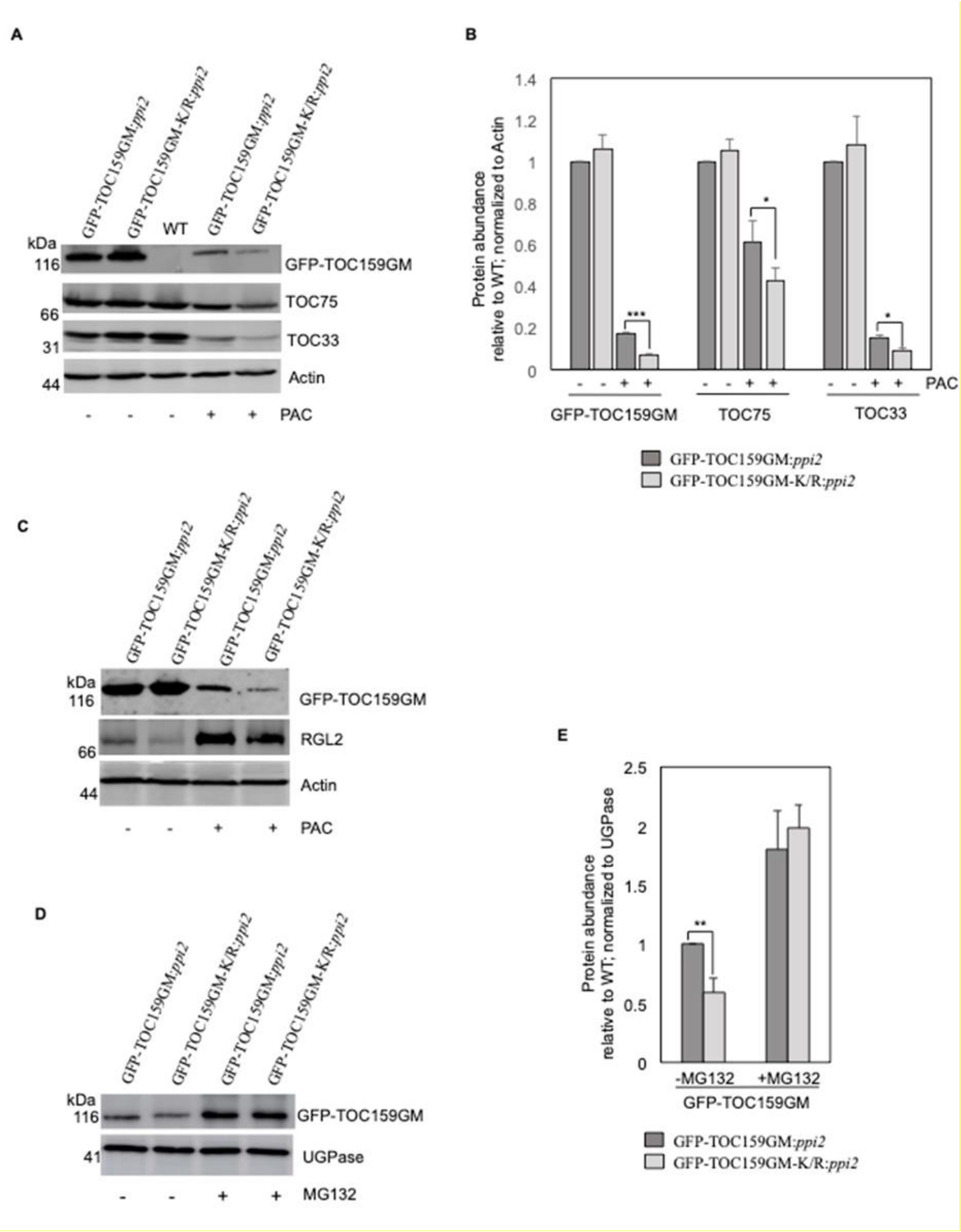


Figure 3. SUMOylation partially protects TOC159 from UPS-mediated degradation. (A) Immunoblotting of total protein extracts from three days old seedling of GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2* grown in the presence or absence of PAC (5 µM). WT (Ws) was used as the control for antibody specificity. The blot was probed with anti-GFP, -TOC75, and -TOC33 antibodies. Anti-actin was used for a loading control. **(B)** Specific bands corresponding to GFP, TOC75, TOC33 and actin were quantified (A). The quantified bands were normalized to GFP-Toc159GM in GFP-TOC159GM:*ppi2* plants grown in the absence of PAC. Error bars indicate ± SEM (n = 3). Student's t test; ∗$p < 0.05$; ∗∗∗$p < 0.005$. **(C)** Immunoblotting of total protein extracts from three days old GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2* seedlings grown in the presence or absence of PAC (5 µM). The blot was probed with anti-GFP and -RGL2 antibodies. Anti-actin was used for a

loading control. **(D)** Total protein extracts of three day old GFP-Toc159GM:*ppi2* or GFP-Toc159GM-K/R:*ppi2* grown seedling grown on PAC and subsequently treated with or without MG132 were analyzed by immunoblotting using anti-GFP antibodies and anti-UGPase for a loading control. **(E)** The specific bands corresponding to GFP and UGPase were quantified (C). The quantified bands were normalized to GFP-TOC159GM in GFP-Toc159GM:*ppi2* without MG132. Error bars indicate ± SEM ($n = 3$). Student's t test; $**p < 0.01$.

Low GA reduced the accumulation of photosynthesis-associated proteins to similar levels in both the TOC159GM:ppi2 and TOC159GM-K/R:ppi2 mutant lines

The results so far demonstrated that non-SUMOylatable TOC159GM-K/R is significantly more susceptible to UPS-mediated degradation than wildtype TOC159GM under low GA. To determine whether this has an effect on the accumulation of photosynthesis-associated proteins (the presumed TOC159 cargo proteins), western blotting was carried out comparing GFP-TOC159GM-K/R:*ppi2* to GFP-TOC159GM:*ppi2*. It has been shown that the expression of most photosynthesis-associated genes did not change significantly in the presence of moderate and low PAC concentrations (5 and 1 μM). To eliminate the possibility of gene expression effects on protein accumulation, GFP-TOC159GM:*ppi2* and GFP-TOC159GM-K/R:*ppi2* seedlings were grown in the presence or absence of an intermediate 2μM PAC concentration. The western blot analysis reveals that, while it appeared to trend lower, the accumulation of the two photosynthesis-associated proteins (PSBO1 and PSBA) were not significantly impacted in TOC159GM-K/R:*ppi2* when compared to Toc159GM:*ppi2* (**Figure 4A and B**) (**Figure 4 - Figure Supplement 1**). These results suggest that preprotein abundance and import into chloroplasts is regulated to similar levels in both GFP-Toc159GM:*ppi2* and mutant GFP-Toc159GM-K/R:*ppi2*.

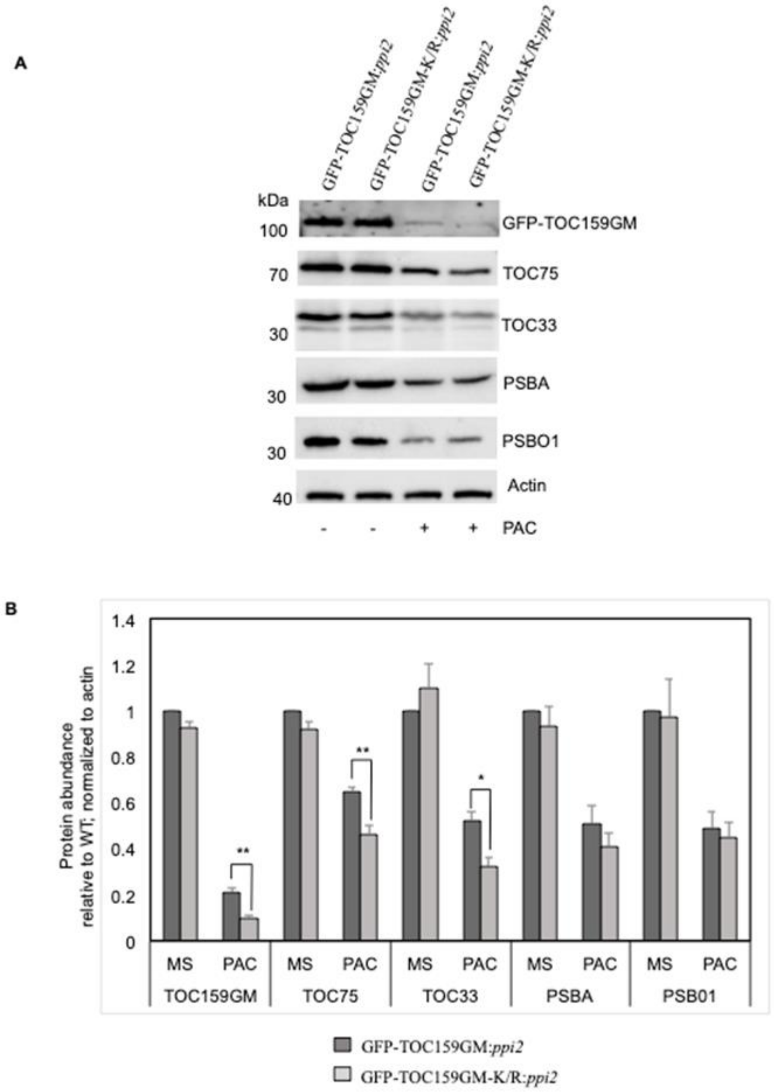


Figure 4. The TOC159 SUMOylation-deficient line Toc159GM-K/R:*ppi2* accumulates two photosynthesis-associated proteins to the same extent as Toc159GM:*ppi2* under low GA conditions. (A) Immunoblotting of total protein extracts from three day old seedling of GFP-Toc159GM:*ppi2* and GFP-Toc159GM-K/R:*ppi2* grown in the presence or absence of PAC (2 µM). The blot was probed with anti-GFP, -TOC75, -TOC33, -PSBA and -PSBO1 antibodies. Anti-actin was used as a loading control. **(B)** The specific bands corresponding to GFP, TOC75, TOC33, PSBA, PSBO1and actin were quantified (A). The quantified bands were normalized to GFP-Toc159GM in GFP-TOC159GM:*ppi2* grown in the absence of PAC. Error bars indicate ± SEM (n = 3). Student's t test; $*p < 0.05$; $**p < 0.01$.

Discussion

An earlier study showed that TOC159 physically interacted with the SUMO E2 enzyme in a yeast two-hybrid screen. An *in vitro* SUMOylation assay in E. *coli* indicated that the SUMO3 isoform was the one ligated to TOC159 (Elrouby and Coupland, 2010). Here, the GPS-SUMO algorithm was used to predict SUMOylation sites and SUMO-interacting motifs (SIMs) in TOC159. It predicted a highly conserved SUMOylation site ("TGV**K**LED") containing a lysine at position 1370 (K1370) in the M-domain of TOC159. We confirmed SUMOylation at K1370 using an *in planta* SUMOylation assay (**Figure 1F**). In addition, the GPS-SUMO algorithm identified a conserved non-covalent SUMO-interacting motif (SIM) ("VKVLP") in the G-domain of TOC159. A yeast two hybrid analysis revealed that the G-domain specifically interacted with the SUMO3 isoform and presumably did so via the predicted SIM (**Figure 1D**). Expression of non-SUMOylatable GFP-TOC159GM-K/R (containing the K1370R substitution) restored a wildtype green phenotype in the GFP-TOC159GM-(K/R):*ppi2* plants, *ppi2* mutant plants normally having an albino phenotype. Based on the presence of wildtype levels of TOC75 and TOC33 it appeared that the TOC complex assembled normally in the GFP-TOC159GM-(K/R):*ppi2* plants (**Figure 2A and B**). Using confocal laser microscopy, GFP-TOC159GM-(K/R) localized to the envelope of the chloroplast (**Figure 2D**). These results indicate that despite the K1370R mutation in the M-domain, outer membrane insertion, Toc complex assembly as well as chloroplast biogenesis take place normally under standard growth conditions despite the inability to SUMOylate.

We previously demonstrated that low GA concentrations in the presence of paclobutrazol promote the DELLA (RGL2)-dependent TOC159 degradation via the UPS (Shanmugabalaji *et al.*, 2018). Here we establish a connection between TOC159 SUMOylation and the UPS-mediated TOC159 degradation under low GA during early developmental stages in seed germination. Under low GA, GFP-TOC159GM-K/R accumulated to significantly (< 50%) lower levels than wildtype GFP-TOC159GM in the respective overexpression lines. In addition, the TOC159-interacting TOC-complex core proteins TOC75 and TOC33 also accumulated to considerably lower levels in the GFP-TOC159GM-K/R:*ppi2* line under low GA (**Figure 3A and B**). The reduced levels under low GA of GFP-TOC159-K/R when compared to the wildtype protein were attributed to increased UPS-mediated protein degradation as both proteins recovered to the same protein levels in the presence of the proteasome inhibitor MG132 (**Figure 3D and E**). We conclude that SUMOylation partially stabilizes TOC159 against UPS-dependent degradation under low GA. The photosynthesis-associated proteins are presumed the preferred transport cargoes of TOC159 because they fail to accumulate in the *ppi2* mutant lacking TOC159. They appeared to accumulate to a slightly lesser degree in GFP-TOC159GM-K/R:*ppi2* plants under low GA, but the difference compared to "wild type" GFP-TOC159GM:*ppi2* was not significant.

The results suggest that SUMOylation at the M-domain of TOC159 serves to fine tune preprotein import under low GA.

Based on the results we propose a hypothetical model for the role of SUMOylation during early developmental stages, when environmental conditions are unfavorable and GA concentrations are low. As previously demonstrated, the chloroplast import receptor TOC159 is ubiquitylated prior to outer membrane insertion by an unknown E3-ligase other than SP1 and degraded via the UPS. In addition, its G-domain interacts with SUMO3, the physiological consequences of which remain unknown. The M-domain may be SUMOylated by SUMO3 protecting to some extent against UPS-dependent degradation. When the environmental conditions become more favorable, GA levels increase, and the GA receptor-DELLA complex is degraded by the UPS and TOC159 liberated for outer membrane insertion. The non-ubiquitinated and de-SUMOylated TOC159 is assembled into the TOC complex thus allowing proplastids to differentiate into chloroplasts (**Figure 5**). Our data extends the molecular framework to implicate SUMOylation and probably SUMO-interaction in the control of proplastid to chloroplast transition by regulation of TOC159 levels.

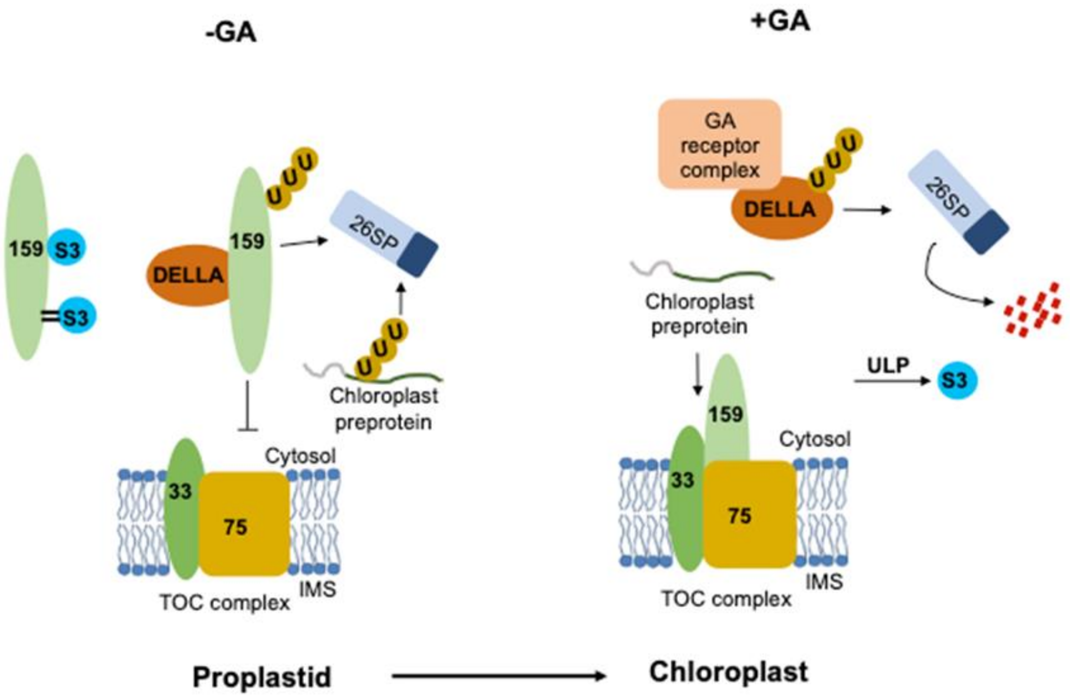
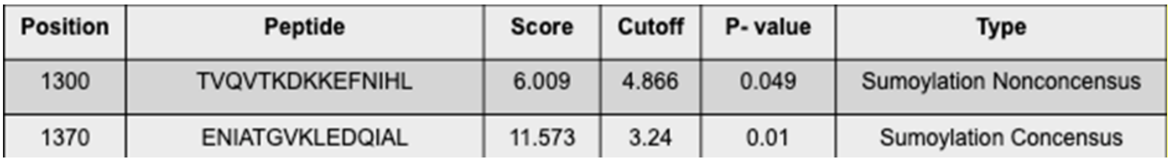


Figure 5. Hypothetical model for the SUMOylation dependent fine-tuning of chloroplast biogenesis at the level of the TOC159 import receptor in early plant development. Environmental conditions influence the concentrations of GA that accumulate upon seed imbibition. When active GA levels are reduced by stress (-GA; left hand panel), DELLA (RGL2) accumulates and sequesters TOC159, which is then degraded via the UPS. In addition, TOC159 interacts and is also covalently SUMOylated by SUMO3. Covalent SUMOylation protects TOC159 against UPS-mediated degradation and supports the accumulation of photosynthesis-associated proteins in the chloroplast. Any un-imported preproteins are degraded in the cytosol via the UPS. When GA concentrations increase (+GA, right hand panel), the GA receptor complex binds to DELLA, which is degraded by the UPS. TOC159 is then free to assemble into the TOC complex. Presumable, DeSUMOylation of TOC159 by a SUMO protease (ULP) assists assembly into the TOC complex. Protein import is thus enabled, allowing proplastids to differentiate into chloroplasts.

Supplemental information



Position	Peptide	Score	Cutoff	P- value	Type
1300	TVQVTKDKKEFNIHL	6.009	4.866	0.049	Sumoylation Nonconcensus
1370	ENIATGVKLEDQIAL	11.573	3.24	0.01	Sumoylation Concensus

Figure S1-table. Predicted SUMOylation sites at TOC159GM. Predicted SUMOylation sites at TOC159GM domain by using GPS SUMO prediction site with high threshold (http://sumosp.biocuckoo.org/online.php).

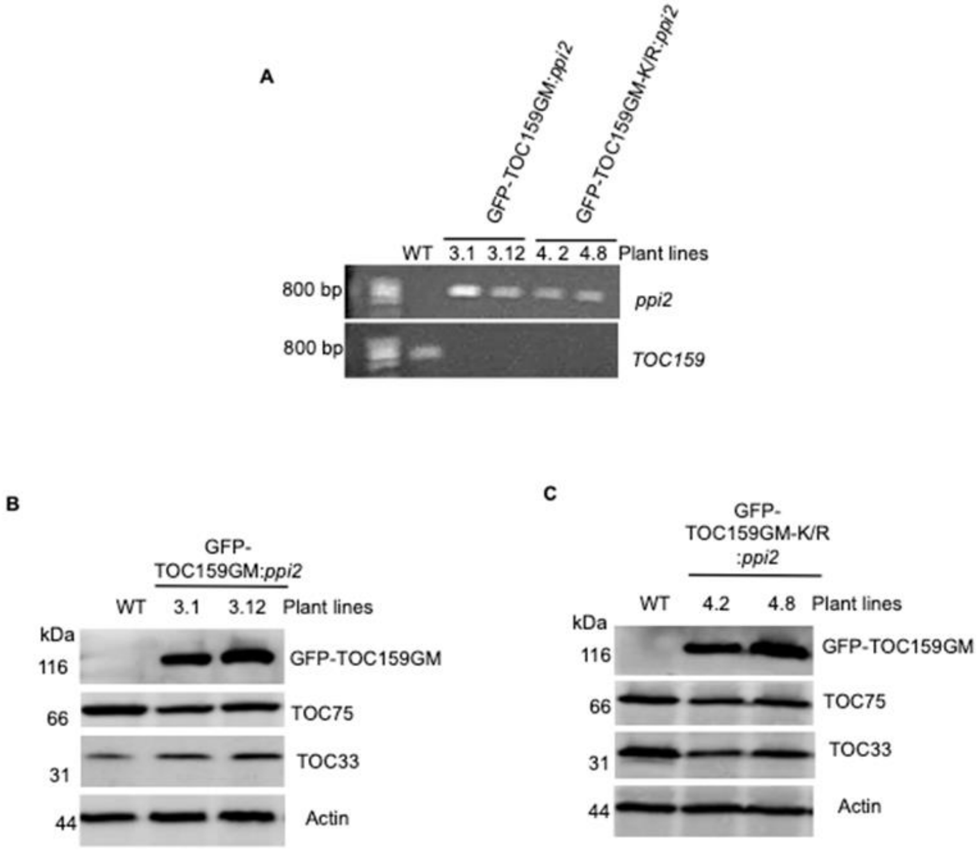


Figure S2. Screening of GFP-Toc159GM and GFP-Toc159GM-K/R complemented plants in *ppi2* background. (A) Genotyping of two independent plant lines (3.1 and 3.12) expressing GFP-Toc159GM in *ppi2* background and another two independent plant lines (4.2 and 4.8) expressing GFP-Toc159GM-K/R in *ppi2* background. WT was used as the control for primers. The following set of primers was used *ppi2* and TOC159. **(B)** Immunoblotting of total protein extracts from two independent plant lines (3.1 and 3.12) seedlings expressing GFP-Toc159GM in *ppi2* background. WT was used as the control for antibody specificity. The following antibodies were used GFP, TOC75, and TOC33 proteins. Actin was used as a loading control. **(C)** Immunoblotting of total protein extracts from two independent plant lines (4.2 and 4.8) seedlings expressing GFP-Toc159GM-K/R in *ppi2* background. WT was used as the control for antibody specificity. The following antibodies were used GFP, TOC75, and TOC33 proteins. Actin was used as a loading control.

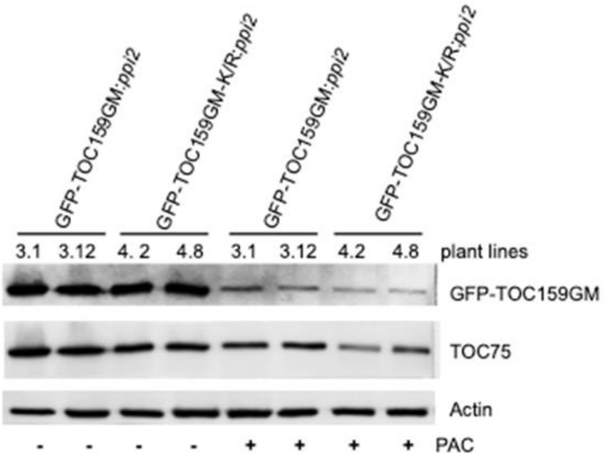


Figure S3-1. Immunoblotting of total protein extracts from two independent plant lines (3.1 and 3.12) expressing GFP-Toc159GM in *ppi2* background and another two independent plant lines (4.2 and 4.8) expressing GFP-Toc159GM-K/R in *ppi2* background. The protein samples extract from three days old seedlings grown in the presence or absence of PAC (5 µM). WT was used as the control for antibody specificity. The following antibodies were used GFP, TOC75, and TOC33 proteins. Actin was used as a loading control.

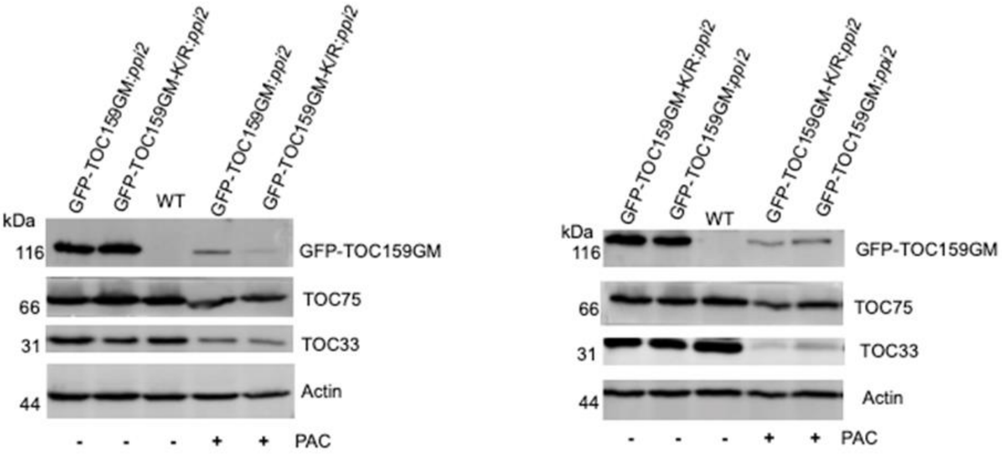


Figure S3-2. Immunoblotting of total protein extracts from *ppi2* seedlings expressing GFP-Toc159GM and GFP-Toc159GM-K/R grown in the presence or absence of PAC (5 µM). WT was used as the control for antibody specificity. The following antibodies were used GFP, TOC75, and TOC33 proteins. Actin was used as a loading control. Both blots were used for quantified bands from antibodies against GFP, TOC75, TOC33 and actin. The quantified bands were normalized to *ppi2* expressing GFP-Toc159GM grown without PAC. These data were used for figure 3.B.

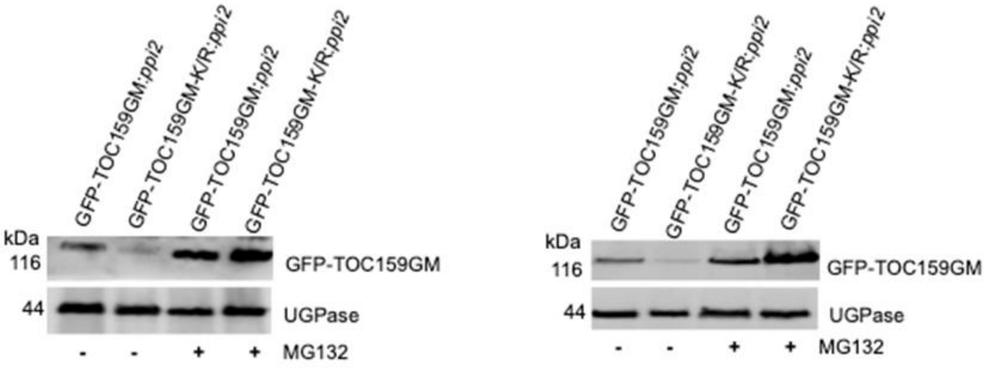


Figure S3-3. Total protein extracts from of three-day-old seedlings from *ppi2* expressing GFP-Toc159GM or GFP-Toc159GM-K/R grown in PAC and further treated with or without MG132, analyzed by immunoblotting using antibodies against GFP. UGPase was used as a loading control. Both blots were used for quantified bands from antibodies against GFP, and UGPase. The quantified bands were normalized to *ppi2* complemented GFP-Toc159GM without MG132. These data were used for figure 3.E.

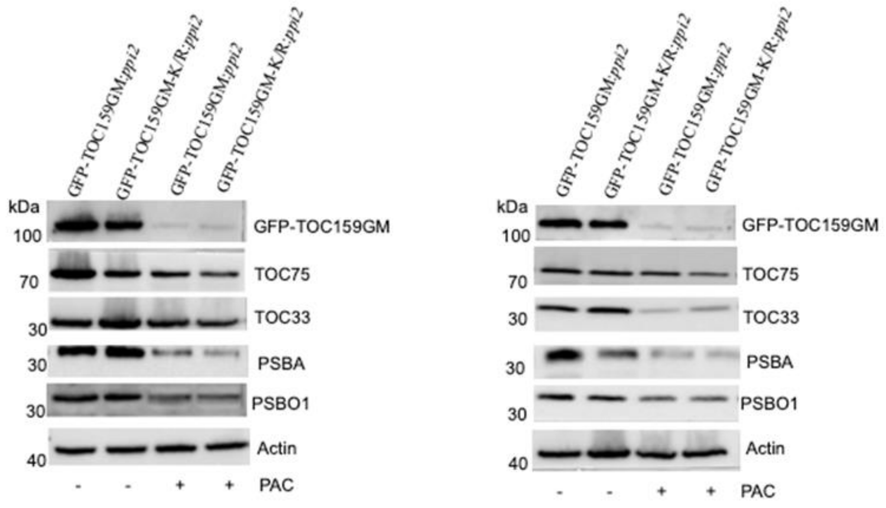


Figure S4. Immunoblotting of total protein extracts from *ppi2* complemented with GFP-Toc159GM and GFP-Toc159GM-K/R seedlings grown in the presence or absence of PAC (5 µM). The following antibodies were used GFP, TOC75, TOC33, PSBA and PSBO1 proteins. Actin was used as a loading control. Both blots were used for quantified bands from antibodies against GFP, TOC75, TOC33, PSBA, PSBO1and actin were quantified. The quantified bands were normalized to *ppi2* complemented GFP-Toc159GM grown seedlings without PAC. These data were used for figure 4.B.

Primer name	**Sequence (5'-3')**
S1-F	CCTTTCCATATGTCTGCAAACCAGGAGG
S1-R	CCTGGACGAATTCTCAGGCCGTAGCACC
S2-F	GATAATAAAGTCATATGTCTGCTACTCCG
S2-R	CCATTAAAATAAGAATTCCTAAAAGCAGAAGAGC
S3-F	GATAAAAGGACATATGTCTAACCCTCAAGATG
S3-R	CCAAATTATGAATTCTTAAAGCCCATTATGATGG
SUMO3–F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTAACCCTCAAGATGACAAGCC
SUMO3–R(GATE)	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAAGCCCATTATGATGGAAAAGC
TOC159S3F	GCCACTGGGGTCCGACTCGAGGACCAAATAGC
TOC159S3R	GCTATTTGGTCCTCGAGTCGGACCCCAGTGGC
TOC159 INT	GAATAGGGTTTTAATCGGAAG
TOC159 A3R	TGCCACATCAACATGCACTG
LB5	GATGCAATCGATATCAGCCAATTTTAGAC

Table S5-table. Primers used in this study. Related to STAR Methods.

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General Conclusions

Plants use a variety of post-translational mechanisms to control proteins involved during development and growth. To do so, specific polypeptides may act as protein modifiers. An example is the small family of proteins called SUMOs, which were discovered in the past few decades initially in studies on nuclear import in mammalian cells (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). These proteins share sequence similarity to ubiquitin and, like ubiquitylation, are thought to be involved in the degradation pathway of target proteins. Another fundamental consideration is that post-translational modifications of proteins are a centerpiece of the signal transduction pathways, particularly important to regulate gene transcription in response to plant hormones. These endogenous signal molecules control all aspects of plant, from embryogenesis to senescence, using an intrinsic signaling cascades known as hormone crosstalk. One of these plant hormones that has been considered in this work is gibberellic acid (GA). It is produced by the plant itself and known to be required for promoting germination. Not surprisingly, the synthesis of GA is strongly upregulated in seeds. When plants need to adjust their metabolism in response to environmental stimuli, the post-translational modifications may come into play as a consequence of those stimuli. Therefore, with a multitude of modifications generated by proteolytic cleavage and covalent addition of modifying groups to amino acids, they can crucially regulate the fate of the chloroplast.

The first chapter of my thesis regards the relation between TOC159 protein import receptor and the DELLA protein RGL2 in early plant development. From a yeast two-hybrid screening, we found that DELLA specifically interacts with the TOC159 G-domain. Knowing that gibberellic acid (GA) controls germination, we checked the protein level of TOC159 under low GA conditions and noticed that the low concentration of GA increased the level of the DELLA RGL2, which consequently attenuates germination. Remarkably, TOC159 protein level decreased under low GA conditions indicating that the accumulation of TOC159 protein depends on the plant hormone gibberellic acid, which also positively regulates chloroplast biogenesis during early stages of plant development. We showed that when gibberellic acid is low, the protein import receptor TOC159 binds directly to DELLA which targets TOC159 for destruction by the ubiquitin-proteasome system (UPS). When gibberellic acid levels increase DELLA itself is targeted for degradation, liberating TOC159 and promoting chloroplast biogenesis. This to my knowledge is the first report showing plant hormone-dependent regulation of the chloroplast protein import machinery.

The second chapter of my thesis is devoted to post-translational modification by SUMOylation and its potential role in chloroplast biogenesis. SUMOylation is considered a key post-translational regulatory mechanism also in plant cells, A systematic study conducted by Coupland *et al.* identified Arabidopsis SUMOylation substrates amongst them TOC159. I decided to further investigate the role of SUMOylation and -interaction. Initially, I identified a SUMOylation site and SUMO-interacting motif using a prediction algorithm. Following a similar approach to the one used to characterize the interaction between TOC159 and DELLA proteins, I verified the functionality of the SUMO-Interacting Motif (SIM). In a yeast two hybrid assay the TOC159 G-domain interacted specifically with the SUMO3 isoform. The TOC159 M-domain contains a predicted SUMOylation site at K1370. I verified the functionality of this site by mutagenesis and demonstrated SUMOylation with the SUMO3 isoform using co-expression in *N. benthamiana*. The lysine residue at the predicted SUMOylation site was substituted with an arginine residue (K1370R) and introduced into a TOC159 T-DNA vector. The expression of the TOC159K1370R mutant in the *toc159* mutant *(ppi2)* complemented the *ppi2* albino phenotype. This showed that SUMOylation at theTOC159 M-domain is non-essential for TOC159 function. Importantly, I demonstrated that the stability of TOC159K1370R is affected under low GA conditions as TOC159K1370R accumulated to a lesser degree than wild type TOC159. SUMOylation protect TOC159 from degradation by the UPS. When I treated the TOC159K1370R expressing plants with the proteasome inhibitor MG132, TOC159K1370R accumulated to the same level as wild type TOC159 indicating that SUMOylation acts to partially stabilize TOC159 against UPS-mediated degradation. Thus, this second part of my work provided the first evidence for post-translational modification of TOC159 by SUMOylation and demonstrated a role for in TOC159 proteostasis during seed germination.

Clearly, much work remains to be done here. It would be interesting to know, for instance, how SUMOylation at the M-domain affects TOC159 assembly into the TOC-complex. Would this process require de-SUMOylation prior to outer membrane insertion? Although I was able to demonstrate physical interaction of SUMO3 with the G-domain, it’s role currently remains unknown. It’s tempting to speculate that the TOC159 SUMO-interacting motif mediates interaction with other SUMOylated proteins involved in post-translation regulation of TOC159. Among such proteins the DELLAs and components of the gibberellic acid signaling pathway are known to be SUMOylated and therefore prime candidates for a SUMO-mediated interaction with TOC159. I believe these would be examples of interesting projects for future PhD students to work on.

Abbreviations

% v/v	volume per volume [ml / 100 ml]
% w/v	gram per volume [g / 100 ml]
Aa	amino acid
AMP	adenosine monophosphate
AMP	ampicillin
APS	ammonium peroxodisulfate
at	*Arabidopsis thaliana*
ATP	adenosine-5'-triphosphate
atToc / atTic	refers to the respective protein in Arabidopsis
atToc159A	acidic domain of atToc159
atToc159G	GTP binding domain of atToc159
atToc159GM	combined G- and M- domains of atToc159
atToc159M	membrane anchor domain of atToc159
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA from transcribed RNA
Col	Columbia
C-terminus	carboxy terminus
DGDG	digalactosyldiacylglycerol
DMSO	dimethyl sulfoxyde
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
GA	gibberellic acid
GFP	Green fluorescent protein
GID	GA Insensitive
GTP	guanosine-5'-triphosphate
GTPase	guanosine triphosphate hydrolase
His	histidine
Kan	kanamycin
kb	kilobase
kDa	kilodaltons
LB	Luria Bertani
LD	long day
Mb	megabase
MGDG	monogalactosyldiacylglycerol
mRNA	messanger RNA

NAD	nicotinamide adenine dinucleotide
Ni-NTA	nitrilo triacetic acid matrix charged with Ni^{++}
N-terminus	amino terminus
OD	optical density
OEP	outer envelope protein
PCR	polymerase chain reaction
PIC	PML interacting protein
PMSF	phosphatidylglycerol
ppi	plastid protein import
protA	protein A
psToc / psTic	refers to the respective protein in pea
RanGAP1	Ran GTPase-activating protein 1
RGA	Repressor of gal-3
RNA	ribonucleic acid
RT	room temperature
RubisCO	Ribulose-1,5-bisphosphate carboxlase/oxygenase
SAE	SUMO activating enzyme
SCE	SUMO conjugating enzyme
SD	short day
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIZ	SAP and Miz
SPP	Stromal processing peptidase b
SUMO	Small Ubiquitin-like MOdifier
TCA	Trichloroacetic acid
T-DNA	transfer DNA
Tic	Translocon of the inner envelope membrane of chloroplast
Toc	Translocon of the outer envelope membrane of chloroplast
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Ub	ubiquitin
UDP	Ubiquitin domain proteins
ULPs	Ubiquitin-like protein processing enzymes
UV	ultra violet
Ws	Wassilewskjia
wt	wild type
β-MeEtOH	β-mercapto ethanol (2- mercapto ethanol)

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