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ABA-dependent differential regulation of chloroplast protein import pathways during the seed-to-seedling transition

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Abstract

Chloroplast biogenesis defines the transition of non-photosynthetic proplastids to photosynthetically active chloroplasts in the embryonic cells of germinating seeds ("seed-to-seedling transition"). It is the key to photoautotrophic growth in plants. The chloroplast requires around 2500 nuclear-encoded proteins for its function. These are synthesized in the cytoplasm as preproteins with an N-terminal transit peptide. During chloroplast biogenesis, two classes of preproteins namely photosynthesis-associated and non-photosynthesis-associated ones, are imported by translocons at the outer and inner membrane of the chloroplast, called TOC and TIC, respectively. Two types of TOC complexes exist and accommodate the respective preproteins. They are trimeric and composed of TOC159, TOC33, TOC75 (for photosynthesis- associated proteins, termed pTOC) or TOC132/-120, TOC34, and TOC75 (for non- photosynthesis-associated proteins, termed nTOC). TOC159 and TOC33 (as well as their homologs TOC132/-120 and TOC34) contain GTPase domains and function in preprotein recognition. They assemble with the β-barrel protein TOC75, resulting in a functional trimeric translocon. Notably, the C-terminal domain of TOC159 (as well as TOC132/-120, predictably) also takes on a β-barrel structure and, together with that of TOC75 forms a hybrid protein translocation channel.

Seed germination is promoted by the plant hormone gibberellic acid (GA) and negatively regulated by abscisic acid (ABA). However, there is still a lack of understanding of how these hormones, and especially ABA, impact chloroplast biogenesis and its synchronization with plant development. In this study, I first investigated the global effects of 0.5 µM ABA on the proteome and transcriptome of 36-hour-old seedlings using RNA-seq and mass spectrometry methods. I then analyzed various concentrations of ABA on 72-hour-old seedlings to validate the omics results, with a particular focus on TOC and TIC components and their substrates. mRNA expression and protein accumulation were analyzed by real-time PCR and western blotting, aiming to elucidate the underlying regulatory mechanisms. My transcriptome-wide RNA-seq analysis indicates the downregulation of photosynthesis-related GO terms and the upregulation of non-photosynthesis/stress-related ones in response to ABA treatment. Proteome-wide mass spectrometric analysis revealed that most corresponding proteins exhibited similar behavior. Under ABA also, components of pTOC were downregulated, whereas those of nTOC were upregulated at the protein

level. However, RNA levels of both pTOC and nTOC were up. The data indicate that ABA represses chloroplast biogenesis by specific down-regulation of photosynthesis-associated proteins together with pTOC components, whereas non-photosynthetic/stress-related proteins are upregulated together with components of nTOC. The results demonstrate a key role for p- and n-TOC in controlling chloroplast biogenesis under ABA. Thereby, my data provide new insight into the ABA-dependent synchronization of chloroplast biogenesis during the seed-to-seedling transition.

Keywords: Chloroplast biogenesis, TOC, TIC, ABA, seed-to-seedling.

Résumé

La biogenèse des chloroplastes définit la transition des proplastides non photosynthétiques vers les chloroplastes photosynthétiquement actifs dans les cellules embryonnaires des graines en germination (“transition de la graine à la plantule”). C'est la clé de la croissance photoautotrophe des plantes. Le chloroplaste a besoin d'environ 2500 protéines codées par le noyau pour fonctionner. Celles-ci sont synthétisées dans le cytoplasme sous forme de préprotéines avec un peptide de transit N-terminal. Au cours de la biogenèse du chloroplaste, deux classes de préprotéines, à savoir celles associées à la photosynthèse et celles non associées à la photosynthèse, sont importées par des translocons au niveau des membranes externe et interne du chloroplaste, appelées respectivement TOC et TIC. Deux types de complexes TOC existent et accueillent les préprotéines respectives. Ils sont trimériques et composés de TOC159, TOC33, TOC75 (appelé pTOC pour les protéines associées à la photosynthèse) ou de TOC132/-120, TOC34 et TOC75 (appelé nTOC pour les protéines non associées à la photosynthèse). TOC159 et TOC33 (ainsi que leurs homologues TOC132/-120 et TOC34) contiennent des domaines GTPase et jouent un rôle dans la reconnaissance des préprotéines. Ils s'assemblent avec la protéine “β-barrel” TOC75, ce qui donne un translocon trimérique fonctionnel. Notamment, le domaine C-terminal de TOC159 (ainsi que TOC132/-120 selon les prédictions) adopte également une structure β-barrel et, avec celui de TOC75, s'assemble en canal hybride.

La germination des graines est favorisée par l'hormone végétale acide gibbérellique (GA) et régulée négativement par l'acide abscissique (ABA). Cependant, la manière dont ces hormones, et en particulier l'ABA, influencent la biogenèse des chloroplastes et sa synchronisation avec le développement de la plante est encore mal comprise. Dans cette étude, j’ai tout d’abord examiné les effets globaux de 0,5 µM d’ABA sur le protéome et le transcriptome de plantules âgées de 36 heures, en utilisant les approches RNA-seq et la spectrométrie de masse. J’ai ensuite analysé différentes concentrations d’ABA sur des plantules âgées de 72 heures afin de valider les résultats omiques, en portant une attention particulière aux composantes TOC et TIC ainsi qu’à leurs substrats. L’expression des ARNm et l’accumulation des protéines ont été évaluées par PCR en temps réel et par western blot, dans le but d’élucider les mécanismes régulateurs sous-jacents. Mon analyse RNA-seq à l'échelle du

transcriptome indique la régulation à la baisse des termes GO liés à la photosynthèse et la régulation à la hausse des termes GO non liés à la photosynthèse/au stress en réponse au traitement par l'ABA. L'analyse des protéines à l'échelle du protéome a révélé que la plupart des protéines correspondantes présentaient un comportement similaire. Sous ABA également, les composants du pTOC ont été régulés à la baisse, tandis que ceux du nTOC ont été régulés à la hausse au niveau des protéines. Cependant, les niveaux d'ARN du pTOC et du nTOC étaient en hausse. Les données indiquent que l'ABA réprime la biogenèse du chloroplaste par une régulation spécifique à la baisse des protéines associées à la photosynthèse ainsi que des composants du pTOC, tandis que les protéines non associées à la photosynthèse/au stress sont régulées à la hausse ainsi que les composants du nTOC. Les résultats démontrent le rôle clé des p- et n-TOC dans le contrôle de la biogenèse des chloroplastes sous ABA. Ainsi, mes données donnent un nouvel aperçu de la synchronisation de la biogenèse du chloroplaste dépendante de l'ABA pendant la transition de la graine à la plantule.

Mots-clés: Biogenèse des chloroplastes, TOC, TIC, ABA, de la graine à la plantule.

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Abbreviations

ATP: Adenosine Triphosphate

ABA: Abscisic Acid

cpDNA: Chloroplast DNA

NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

OEM: Outer Envelope Membrane

LEAs: Late embryogenesis abundant proteins

LHC: Light-harvesting complex

HSPs: heat shock proteins

TOC: Translocon at the Outer Chloroplast membrane

pTOC: TOCs for photosynthesis-associated proteins

nTOC: TOCs for non-photosynthesis-associated proteins

TIC: Translocon at the Inner Chloroplast membrane

tRNA: Transfer ribonucleic acid

rRNA: ribosomal RNA

IEM: Inner Envelope Membrane

SPP: Stromal Processing Peptidase

GTP: Guanosine-5'-triphosphate, is a purine nucleoside triphosphate

LC-MS: Liquid Chromatography Mass Spectrometry

Cdc48: cytosolic AAA+ chaperone

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

UPS: Ubiquitin-proteasome system

SP1: SUPPRESSOR OF PPI1 LOCUS1

Chapter 1: General Introduction

1.1 Arabidopsis, a small plant with a significant impact on biological research

Arabidopsis thaliana, a model organism in plant biology research, is a plant from the mustard family (*Cruciferae* or *Brassicaceae*), which can frequently be found in nature. Johannes Thal first described the species in 1577 in the Harz Mountains of Northern Germany. In 1842, it was renamed by Gustav Heynhold and has been called *Arabidopsis thaliana* since. The use of *A. thaliana* as a model organism gained widespread acceptance in the 1980s (Krämer, 2015). The natural history of *A. thaliana* differs wildly compared to sister species *A. lyrata*, *A. arenosa*, and *A. crotia*, as well as the other three species *A. halleri*, *A. cebennensis*, and *A. pedemontana* (Hohmann et al., 2015). Columbia and Landsberg ecotypes are universally accepted as the standard of *A. thaliana* for genetic and molecular studies. These two ecotypes complete their entire life cycle of seed germination, formation of a rosette plant, bolting of the main stem, flowering, and the first seed maturation, in six weeks. *A. thaliana* is relatively small in terms of botanical anatomy, its flowers are 2 mm long, which open and self-pollinate and can then be crossed by placing pollen on top of the stigma surface. At maturity, the seeds are produced in the subsequent slender fruits called siliques which are small, approximately 0.5 cm long. The genome of Arabidopsis is one of the most explored genomes among higher plants. In 2000, the Arabidopsis reference genome sequence was published as the first nuclear genome of a flowering plant (Arabidopsis Genome Initiative 2000, Somerville & Koornneef, 2002). The nuclear genome is relatively small, 125 Mb, comprising 5 chromosomes (2n = 10), and is believed to contain ~20,000 genes (Meinke et al., 1998).

Its small size, short life cycle, and well annotated genome, make it an ideal model organism for plant biology studies.

1.2 Plastids are essential organelles in plant cells

Plant cells share many types of organelles with animals. But plastids are double-membrane organelles found only in the cells of plants (as well as other photosynthesising organisms) and have several important functions and exist in several different variations. The functions include fundamental cellular processes, from photosynthesis to lipid, hormone, amino acid, pigment and phytochromobilin biosynthesis as well as the assimilation of nitrates and sulfates. Plastid function is reflected in plastid structure and composition. Differentiated plastids such as chloroplasts contain thylakoid membranes, amyloplasts have enlarged starch granules, and chromoplasts accumulate carotenoid-rich bodies. These plastid types are associated with distinct functions of different cell types and organs (leaves, roots, and flowers, respectively) (Finkemeier & Leister, 2010). Chloroplasts are probably the best known of plastids, as they are involved in photosynthesis, from which plant cells derive energy for growth and metabolic processes. Chromoplasts synthesise and store pigments that give fruits and flowers their colour. Leucoplasts are storage plastids for starch, oil, and protein and are strongly associated with non- photosynthetic tissues such as plant roots and tubers. Leucoplasts can also further differentiate into two sub-types: amyloplasts that primarily synthesise and store starch and oleoplasts that synthesise and store lipids. Other plastids, including elaioplasts and proteinoplasts, represent types of plastids that each play a unique role in plant cells. Plastids have exceptional metabolic plasticity and can change types to meet the plant's demand (Figure 1). This is referred to as plastid differentiation, a process that allows plants to adapt and change metabolic processes to accommodate the changing conditions of the environment. For example, chloroplasts may differentiate to chromoplasts in fruit cells to facilitate pigment synthesis as fruit ripens (Jarvis & López-Juez, 2013).

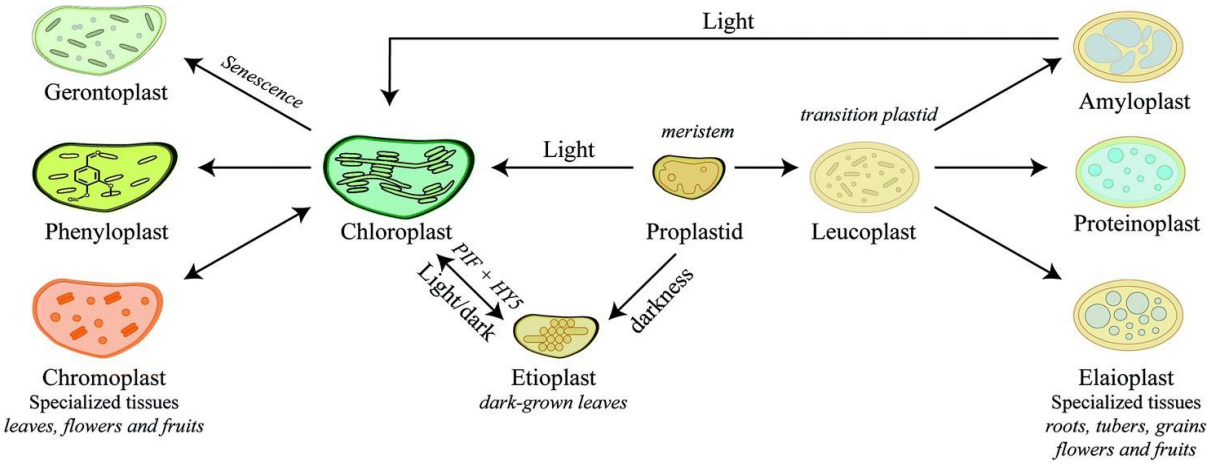

Figure 1: Plastids undergo differentiation and diversification in conjunction with tissue type and developmental stage. The arrows indicate transitions between plastid types. The proplastid, which precedes, becomes either an etioplast or chloroplast, depending on whether it is dark or light. Proplastids can also develop into leucoplasts, which can then differentiate into amylo-, proteino-, or elaioplasts that will accumulate starch, proteins, or oils. Chloroplasts can further differentiate into chromoplasts, amyloplasts, or gerontoplasts, again dependent on environmental signals and cell types. Chromoplasts and phenyloplasts will accumulate carotenoids and phenylpropanoids, respectively, while gerontoplasts, the plastid formed during leaf senescence, can recycle and redistribute thylakoid-derived compounds (Taken from Knudsen et al., 2018).

1.2.1 Insights into the evolution history of plastids

Endosymbionts describe the organelles derived from prokaryotic cells that live inside eukaryotic cells. It has been determined that plastids originated from a sequence of endosymbiotic events. More than 1.6 billion years ago, the primary endosymbiotic steps occurred when a cyanobacterium was engulfed by a heterotrophic mitochondriate eukaryote (heterotrophic protist) and over time, transformed into an intracellular organelle. Through this evolution (subsequent secondary and tertiary endosymbioses between plastid-bearing eukaryotes also occurred), plastids extend out into an enormous collection of photosynthetic and some “non- photosynthetic” organisms. Consequently, the existing plastids in almost all present-day plastid-bearing organisms descended from a single primary endosymbiosis (Zimorski et al., 2014; Keeling, 2010). Plastids derived from endosymbiosis to this day maintain a functional genome (the plastome) together with a transcription and translation system derived from the cyanobacterial endosymbiont (Jarvis & López-Juez, 2013).

1.2.2 Chloroplast Anatomy

The chloroplast is specifically responsible for photosynthesis in plant cells. Like all

plastid types, it is enclosed by a double membrane consisting of an outer and inner membrane, separated by an intermembrane space. Inside, the stroma is a protein-rich soluble phase that contains enzymes for carbon fixation, ribosomes, DNA, and starch granules. Contained within the stroma are stacks of thylakoid membranes called grana, which host the photosynthetic pigments and associated protein complexes for light-dependent reactions, including light-harvesting complexes (LHC), photosystems II and I, cytochrome b6f complex and chloroplast ATP synthase. The thylakoid membranes within plant chloroplasts do not only consist of stacked grana but also of interconnected unstacked stroma lamellae (Pribil et al., 2014). The thylakoid lumen, enclosed by the thylakoid membranes, provides a specialized environment for proton accumulation during photosynthesis and contains a proteome of around 80 proteins that play critical roles in photosynthesis, photosystem assembly, stress responses, and signaling (Järvi et al., 2013).

1.2.3 The chloroplast genome

The chloroplast genome, also called the plastome, is a circular DNA molecule distinct from the nuclear genome. This independent genome provides evidence for the endosymbiotic theory, which proposes that chloroplasts evolved from ancient cyanobacteria. Over evolutionary time, many chloroplast genes were transferred to the nuclear genome or lost entirely, reducing the plastome's size. Research has pointed out that plastid genomes' gene order and content have remained relatively stable for approximately 800 million years (Lemieux et al., 2000). Its size usually ranges between 120 and 170 kb, and its structure and gene content show remarkable conservation throughout the plant kingdom. The complete sequence and characterization of the chloroplast genome from *Arabidopsis thaliana* (Col-0) was published in 1999, with its size of roughly 154,500 base pairs (Sato et al., 1999). As chloroplasts contain their own DNA, replication, transcription, and translation occur inside the chloroplast, facilitated by chloroplast- specific DNA and RNA polymerases and ribosomes. Plastome replication shares similarities with bacterial DNA replication due to the endosymbiotic origin of plastids and is essential for plastid function, development, and by consequence for the productivity of the plant. The process is independent of the cell cycle and relies on nuclear-encoded proteins that are targeted to plastids. These proteins include plastid-specific DNA polymerases (PolIA and PolIB), single-stranded DNA-binding proteins (SSBs), primases, helicases, and topoisomerases, which

together ensure accurate and efficient replication of the plastid genome (Oldenburg & Bendich, 2015). Plastome replication is believed to occur through a combination of mechanisms, including D-loop replication, where replication begins at a specific origin and proceeds unidirectionally, creating a displacement loop, and rolling circle replication, which involves the generation of a single- stranded DNA intermediate serving as a template for complementary strand synthesis. Nuclear- encoded factors regulate the replication process by coordinating DNA synthesis with plastid biogenesis and development (Nielsen et al., 2010). Actively dividing cells in meristems may have 10 to 100 plastome copies per plastid, while mature chloroplasts in photosynthetic tissues typically maintain high genome copy numbers to support plastid-encoded protein synthesis. In photosynthetic cells, the total plastome copy number per cell can reach thousands, with mature leaf cells containing approximately 2,600 to 3,300 plastome copies (Greiner et al., 2019).

1.2.4 Chloroplast function

Chloroplasts are multifunctional organelles central to plant metabolism and environmental responsiveness which are vital in plant physiology and survival. Chloroplasts use light energy to produce chemical energy through photosynthesis, producing glucose and oxygen from carbon dioxide and water. Chloroplasts also produce essential metabolites such as amino acids, fatty acids, and secondary metabolites and store excess glucose as starch granules (Finkemeier & Leister, 2010). They are the site of chlorophyll synthesis as well as that of other photosynthetic pigments such as carotenoids (like beta-carotene, xanthophylls, and lycopene), which are vital for light absorption and photochemistry (Dobrogojski et al., 2020). In addition, chloroplasts play a significant role in nitrogen and sulfur assimilation, reducing nitrite to ammonia and sulfate to sulfide (Neuhaus & Emes, 2003). Biosynthesis of certain plant hormones, such as jasmonates and abscisic acid, which regulate growth and stress responses, start in the chloroplast. Chloroplasts produce reactive oxygen species (ROS) and support calcium-mediated signaling pathways, which are essential for defending against pathogen attacks, also chloroplasts play a significant role in plant defense (Lu & Yao, 2018). In addition, chloroplasts are involved in cellular signaling, enabling plants to respond to environmental stimuli and adapt to changes in light quality and quantity (Yoo et al., 2019).

1.3 Photosynthesis in plants

Photosynthesis is a crucial process for plant survival and Earth's ecosystems as it synthesizes organic molecules for nearly all living organisms. During photosynthesis, energy derived from light is used to reduce CO_2 (carbon dioxide) to carbohydrates. Photosynthesis in green plants takes place in the chloroplast and consists of two series of reactions. The light-dependent photosynthetic reactions take place within chloroplast thylakoid membranes. During this process, water molecules undergo oxidation to form oxygen, while electrons move through a series of protein complexes, generating NADPH and indirectly ATP (Nelson & Ben-Shem, 2005). In the chloroplast stroma, ATP and NADPH are used to convert CO_2 into carbohydrates as part of the light-independent reactions, also known as the Calvin cycle (Heineke & Scheibe, 2009). To improve crop yields and tackle the global issues of food security and climate change, photosynthesis and its efficiency are important research topics.

1.3.1 Components and mechanisms of the photosynthetic light reactions

The light reactions of photosynthesis, the first stage of converting solar energy into chemical energy, occur in the thylakoid membranes of chloroplasts. Within the thylakoid membrane of plants, proteins are organized into five complexes that facilitate the light reactions of photosynthesis, which include light-harvesting complexes, photosystems I and II containing reaction centers using captured energy to liberate excited electrons, cytochrome b6f complex and ATP synthase (Figure 2). Photosynthetic pigments in green plants are bound to light- harvesting complexes, photosystem I, and photosystem II (PSI and PSII). The unstacked and stroma-exposed membrane contains mostly PSI, while the grana stacks contain mostly PSII. The two photosystems are preferentially energized by different wavelengths of light: PSII is most responsive to light at 680 nm, while PSI absorbs light most efficiently at 720 nm.

Additionally, the PSI antenna generally exhibits higher energy conversion efficiency than the PSII antenna. PSII is made up of core proteins (D1 and D2 proteins), small molecular-weight proteins (<10 kDa including PsbH, PsbK, PsbM, PsbTc, and PsbW), proteins of the oxygen- evolving complex (OEC) including PsbO (33 kDa), and light-harvesting complexes (CP47 and CP43) which bind chlorophyll pigments and play a crucial role in capturing light energy and transferring it to the reaction center for photosynthesis (Croce & van Amerongen, 2011). The core of PSI consists of a

heterodimer of two polypeptides (PsaA and PsaB) (Lu, 2016). PSII oxidizes water, generating oxygen and reducing plastoquinone, initiating the electron transport chain (ETC) toward NADP+. Cytochrome b6f receives electrons from PSII and transfers them to PSI. The transfer of electrons between PSII and cytochrome b6f is facilitated by plastoquinone (a lipid-soluble quinone), while the transfer between cytochrome b6f and PSI involves plastocyanin (a water-soluble copper protein in the thylakoid lumen). The cytochrome b6f complex facilitates the movement of electrons from plastoquinol to plastocyanin; during this electron transfer, protons are pumped across the thylakoid membrane into the lumen, establishing an important proton gradient which is used by ATP synthase to generate ATP. PSI, as the terminal electron acceptor, transfers electrons to NADP+ through the redox protein ferredoxin. The redox potential of ferredoxin can be alternatively used for nitrate assimilation and fatty acid desaturation (Ben-Shem et al., 2003). In the following, both ATP and NADPH as energetic molecules will be used in the Calvin cycle to form sugars.

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

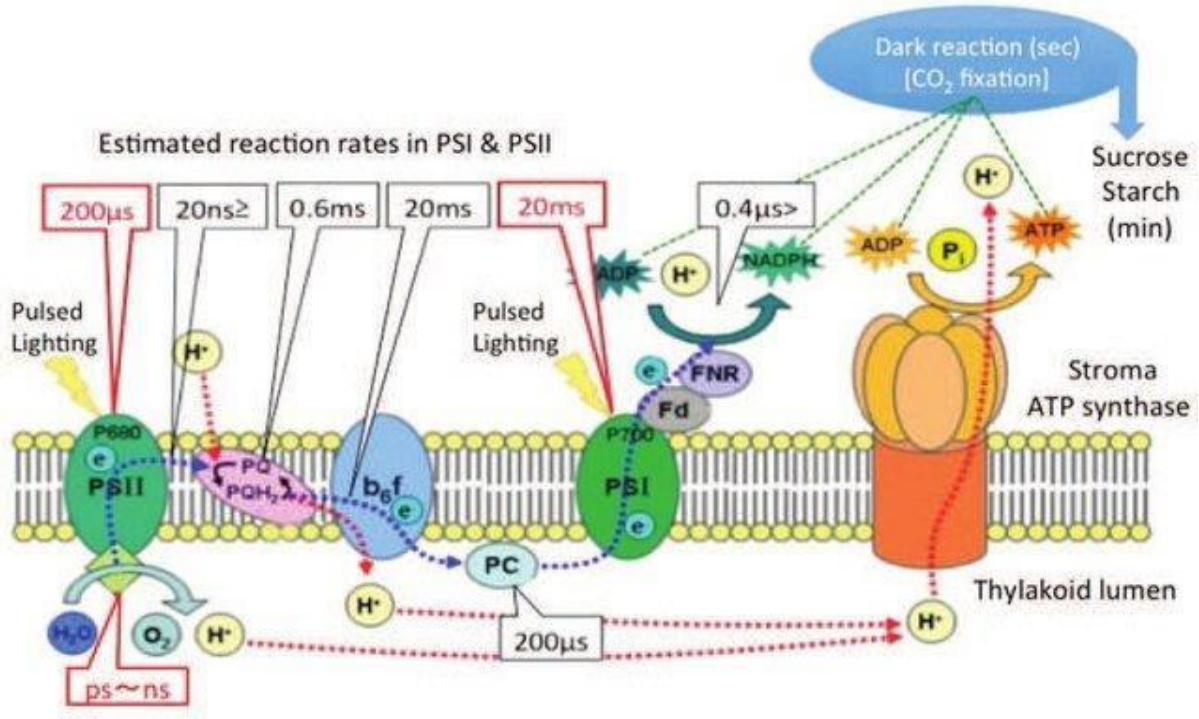

Figure 2: The thylakoid membrane in chloroplasts houses an electron transport chain (ETC) that links the photochemical reactions of photosynthesis between Photosystem I (PSI) and II (PSII), along with ATP synthase. The ETC consists of several components: the PSII reaction center (P680), plastoquinone (PQ), cytochrome b6f complex (b6f), plastocyanin (PC), PSI reaction center (P700), ferredoxin (Fd), and ferredoxin-NADP+ reductase (FNR) (Taken from Kanechi, 2018).

1.3.2 Photosynthetic carbon fixation by the Calvin Cycle

The Calvin-Benson cycle, also referred to as the light-independent reactions of photosynthesis, fixes carbon dioxide into carbohydrates in plants by using the ATP and NADPH generated in the light-dependent reactions. This cycle has three stages, which takes place in the chloroplast stroma: carboxylation, reduction, and regeneration (Adam, 2017). During the carboxylation phase, carbon dioxide enters the leaf through the stomatal openings and diffuses into thechloroplast, where carboxylation of ribulose-1,5-bisphosphate (RuBP) is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This results in two molecules of 3-phosphoglycerate (3-PGA). In the reduction phase, ATP and NADPH are used to convert 3-PGA into glyceraldehyde-3-phosphate (G3P), a triose phosphate that serves as a precursor for glucose and other carbohydrates. Finally, during the regeneration phase, the remaining G3P molecules are recycled to regenerate RuBP, a process that requires additional ATP, ensuring the continuity of the cycle.

The Calvin cycle is configured so that one carbon atom is fixed for every turn of the cycle, and to produce a single six-carbon glucose molecule, six carbon dioxide molecules must be fixed, requiring six turns of the cycle. The ATP and NADPH produced in the light-dependent reactions provide the energy and reducing power needed to drive these processes. G3P molecules produced in the cycle are used to form glucose. These glucose molecules can be further converted into other sugars or utilized in various metabolic processes within the plant. The regeneration of RuBP is essential for maintaining the cycle, utilizing additional ATP and molecular intermediates to ensure continuous carbon fixation (Raines, 2022).

Carboxylation reaction catalyzed by rubisco:

$RuBP + CO_2 + H_2O \rightarrow 2\ 3\text{-}PGA$

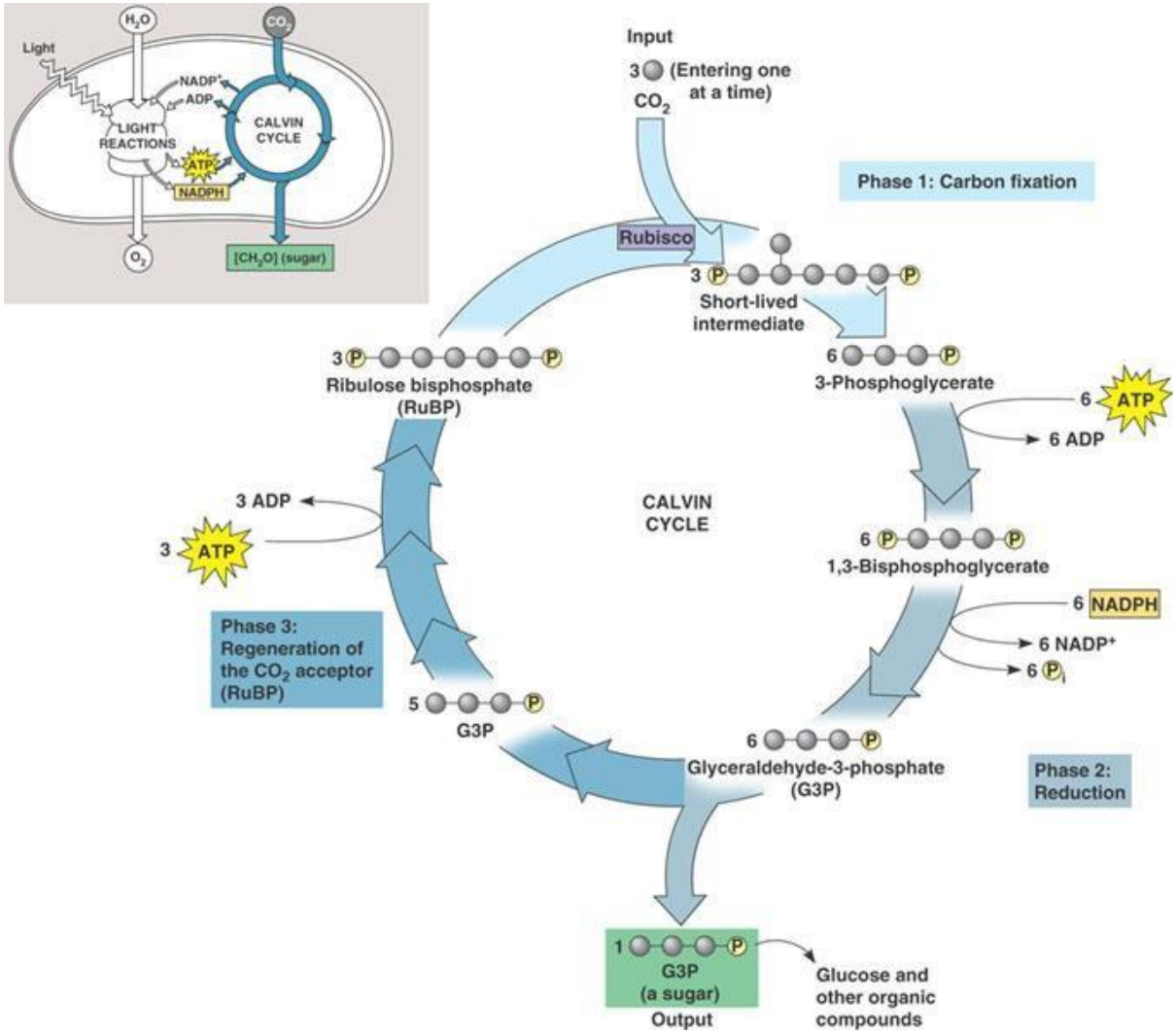

Figure 3: The Calvin cycle (also known as the Benson- Calvin cycle) is a set of chemical reactions that take place in chloroplasts during photosynthesis. The cycle is light-independent because it takes place after the energy has been captured from sunlight (Taken from https://cambridgecapp.wordpress.com/improving-photosynthesis/photosynthesis/light-independent-reactions-of-photosynthesis/).

1.4 Plastoglobules

Chloroplasts and other plastids contain specific lipid-rich structures known as plastoglobules (PGs), structurally resembling lipid droplets. PGs are contiguous with the outer lipid droplet of the thylakoid membrane and play key roles in lipid metabolism, stress responses, and the storage of lipophilic molecules. These PGs are not static entities; instead, they change dynamically in size and composition with the occurrence of environmental stressors and during developmental changes (Bréhélin et al., 2007). PGs are essential structures in prenylquinone metabolism, facilitating the production and accumulation of tocopherols, plastoquinone, and phylloquinone (Eugeni Piller et al., 2012; Ytterberg et al., 2006). PGs also participate in the breakdown of chlorophyll, metabolism of carotenoids, and modification of lipids during senescence and stress responses (Shanmugabalaji et al., 2022; Lundquist et al., 2013).

Furthermore, PGs regulate redox processes, proteolysis within plastids, and adaptation to environmental conditions (van Wijk & Kessler, 2017). In chloroplasts and chromoplasts, the PG proteome contains enzymes involved in the metabolism of isoprenoids, lipids, and carotenoids. Fibrillin, the initial PG protein discovered (Deruère et al., 1999), was subsequently followed by identifying of a PG proteome consisting of roughly 30 proteins that fluctuate based on plastid type and environmental factors. Approximately one-third of these proteins are enzymes involved in lipid metabolism, while several belong to the ABC1K kinase family.

1.4.1 ABC1K family

The ABC1K (ACTIVITY OF BC1-LIKE 1 KINASE) family, which are a typical protein kinases, has undergone expansion in photosynthetic organisms and is essential for plant stress resistance and development (Lundquist et al., 2013; Gao et al., 2011). In Arabidopsis, there are 17 ABC1Ks, with 8 likely found in mitochondria and 9 in plastids. Among the plastid ABC1Ks, 6 out of 9 are located in PG (ABC1K1, -3, -5, -6, -7, -9) (Lundquist et al., 2013). The mechanisms of action for ABC1K family members, such as their potential roles in phosphorylating proteins or metabolites, remain largely unclear. Studies showed that ABC1K7 contributes to cadmium tolerance, oxidative stress response, iron distribution, and lipid metabolism, as well as facilitating crosstalk between abscisic acid and ROS signaling (Manara et al., 2013). ABC1K1 was also identified as PGR6 (Proton Gradient Regulation 6) due to its strong photosynthetic

phenotype. The phenotype has been attributed to a defect in “plastoquinone homeostasis,” which uses plastoquinone stored in the PGs to maintain plastoquinone levels in the thylakoids under light stress (Pralon et al., 2019).

1.4.2 Fibrillins (FBN)

The fibrillin (FBNs) family comprises the majority of the PG proteome's mass and has been associated with roles in PG structure and maintenance (Kessler et al., 1999; Singh et al., 2012). While most FBNs are found in plastoglobules, the presence of some of them have also been detected in the stroma and stromal lamellae thylakoids. FBNs can be categorized into 12 phylogenetic groups and are involved in various functions, including abiotic stress tolerance, growth and development, hormone signaling, and lipid transport between thylakoid membranes and plastoglobules. Group 1 members, which include Arabidopsis FBN1a and FBN1b, have been linked to plastoglobule formation and thylakoid maintenance (Singh & McNellis, 2011).

1.4.3 VTE1

VTE1 (VITAMIN E DEFICIENT 1) has also been identified as a component of the PG proteome. VTE1 functions as tocopherol cyclase and plays a crucial role in the synthesis and metabolism of tocopherols and other tocochromanols, which are fat-soluble antioxidants commonly referred to as vitamin E. In the tocochromanol pathway, VTE1 catalyzes the cyclization of 2,3-dimethyl- 5-phytyl-1,4-benzoquinol (DMPBQ), resulting in the formation of the typical chromanol ring (Sattler et al., 2004). Physiologically, VTE1 is vital for sustaining the antioxidant capacity of chloroplasts by protecting thylakoid membranes from lipid peroxidation harm caused by reactive oxygen species (Munne-Bosch & Alegre, 2002). Mutations in the VTE1 gene lead to a lack of vitamin E, which substantially affects plant seeds' longevity and their ability to withstand stress during germination (Sattler et al., 2004).

1.5 Chloroplast biogenesis: From undifferentiated proplastids to photosynthetic chloroplasts

The development of chloroplasts is essential for the photosynthetic growth of plants. Chloroplast formation and growth in young plants involve the transformation of a proplastid, which is the precursor to a mature chloroplast that is active in photosynthesis, either directly or through an intermediate form called an etioplast that is formed in the dark (Pogson et al., 2015) (Figure 4). As chloroplasts can be located

in various parts of plants, such as cotyledons, leaves, stems, fruits, and flowers, the formation and growth of chloroplasts vary between organs and among different plant species due to the specialization of tissues. In mature leaves, chloroplasts continue to multiply through fission, similar to the process observed in bacteria (Glynn et al., 2007). When young dark-grown, etiolated seedlings are exposed to light, photomorphogenesis occurs, which triggers the conversion of etioplasts into chloroplasts. During this transformation, grana and stroma thylakoids are generated from paracrystalline tubular membranes and flat porous membranes of prothylakoids (Pipitone et al., 2021). Furthermore, chlorophyll is generated from Pchlide (protochlorophyllide) and the synthesis of proteins associated with photosynthesis; particularly thylakoid proteins rise sharply. Light perception and subsequent nuclear and plastid gene expression, lipid and pigment biosynthesis, the import of photosynthesis-associated proteins like chlorophyll a/b-binding proteins into developing chloroplasts, their insertion into thylakoid membranes, and protein assembly into functional complexes all occur during chloroplast differentiation (Rudowska et al., 2012).

Most chloroplast proteins are encoded in the nucleus and synthesized in the cytosol. The import of hundreds or even thousands of different preproteins into the chloroplast requires the chloroplast protein import machinery consisting of the TOC and TIC complexes. Chloroplast biogenesis is an intricate process requiring an orchestrated set of cellular and environmental factors. Hence, many mysteries still remain.

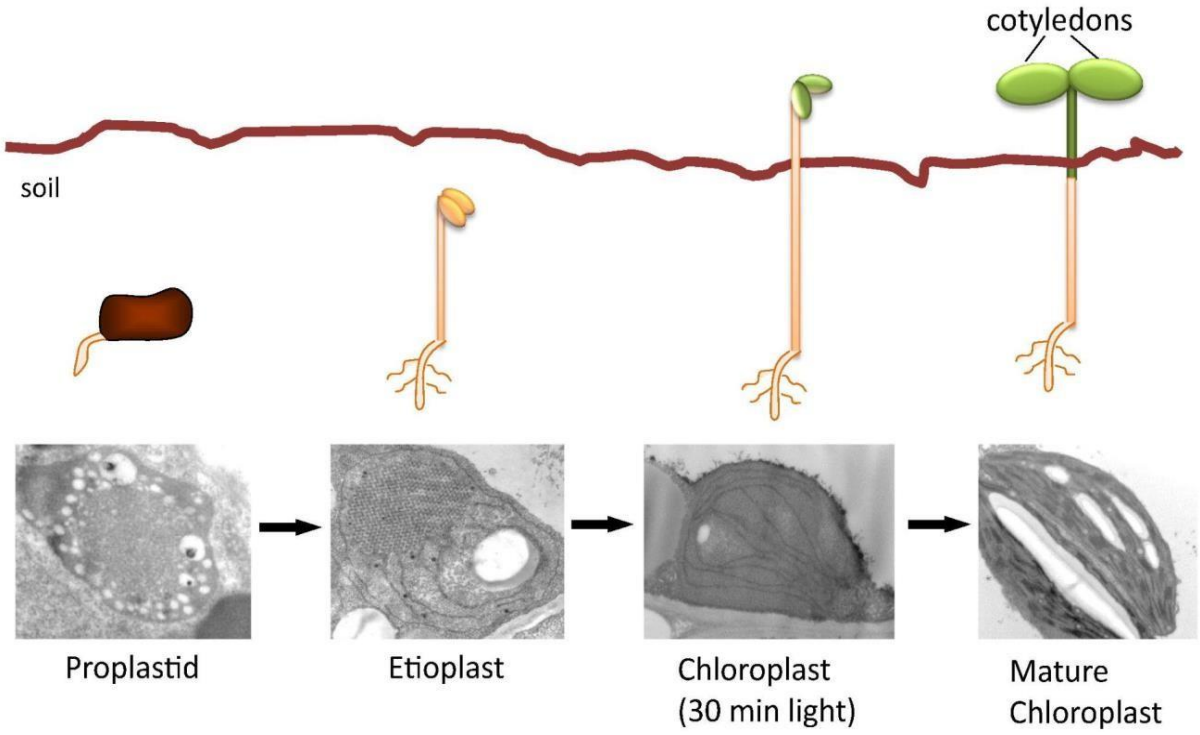

Figure 4: Chloroplast biogenesis occurs during seed germination (Taken from Pogson et al., 2015).

1.5.1 Protein targeting to chloroplasts

During plant evolution and endosymbiosis, a significant portion of the endosymbiont's protein- coding sequences were either lost or transferred to the host nuclear genome (Timmis et al., 2004). However, approximately 150 genes remained in the chloroplast genome, and these encoded core protein components (Leister, 2003). The remaining 2000 or so chloroplast proteins are encoded in the nucleus and are imported post-translationally from the cytoplasm into the chloroplast. The nuclear genome-encoded proteins of the chloroplast are synthesized in the cytoplasm and have N-terminal transit peptides. The transit peptide acts like a molecular zip code or as a tag to facilitate the targeting to the chloroplast and transport of these preproteins across the chloroplast membranes. Two hypotheses regarding cytosolic systems for guiding preproteins have been proposed, although further research is necessary for consolidation. Hsp90, Hsp70/Hsp90-organizing protein (Hop) and the immunophilin FKBP73, has been suggested to guide preproteins to the outer envelope membrane (Fellerer et al., 2011).

Additionally, Hsp70 has been shown to partner with an unidentified 14-3-3 protein to recognize phosphorylated TPs and deliver them to translocon complexes (May & Soll, 2000). In the following, the transit peptide sequentially interacts with translocon complexes on the chloroplast's outer and inner membranes (TOC and TIC), facilitating

the preprotein's passage through membranes (Figure 5). Once the preprotein reaches the stroma, the transit peptide is no longer needed. A specific protease called stromal processing peptidase snips it off. In some proteins, this exposes additional targeting information, allowing further sorting to the thylakoid membrane or lumen (Troesch & Jarvis, 2011).

1.5.2 Diversity, evolution, and specificity of chloroplast transit peptides

Chloroplast transit peptides exhibit diversity and complexity in their structure, function, and evolutionary development (Ivy et al., 2000). There presumably was significant development and diversification of the first ancestral transit peptides since their emergence, mainly due to random insertions, deletions, and alternative splicing in duplicated genes (Christian et al., 2020). There are two hypotheses for the origin of the transit peptide: one is believed to be traced back to ancient cyanobacterial virulence factors, and another is to the antimicrobial peptides (Christian et al., 2020). The ability of proteins to be imported is crucial for distinguishing between different types of plastids, and it relies on the specific sequence found in the transit peptide. It has been demonstrated that the presence of a twin-positive motif in the transit peptide (that is two consecutives positively charged amino acids) specifies that the protein is more likely to be imported into root leucoplasts rather than into leaf chloroplasts (Chu et al., 2020). Transit peptides commonly display species-specific characteristics. For instance, *Arabidopsis thaliana* is incapable of directing certain preproteins containing transit peptides from rice to the plastid. Similarly, the expression of preproteins with Arabidopsis-derived transit peptides within rice resulted in the loss of the plastidial specificity, even suggesting that these preproteins are misdirected to other cellular compartments. This difference may be attributed to variations in the TOC/TIC translocons between the two species (Eseverri et al., 2020).

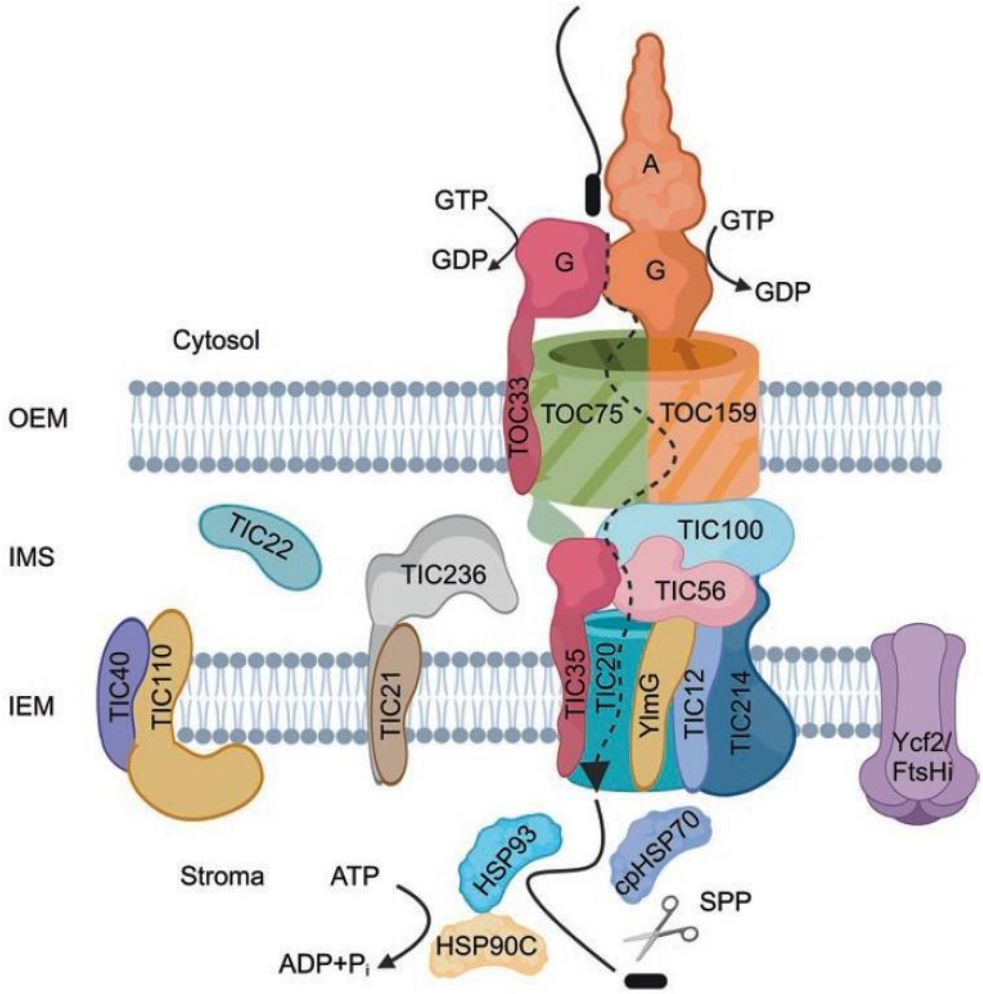

Figure 5: Structure and pathway of protein translocation in chloroplasts. The chloroplast protein translocation mechanism is mediated by two crucial complexes: the TOC and TIC complexes. The TOC complex is in the OEM of the chloroplast, composed of GTP-dependent transit peptide receptors TOC159 and TOC33, with TOC75 forming a hybrid channel together with the C- terminus of TOC159. The receptors possess a GTPase domain in the cytosol necessary for the recognition of transit peptides and initiation of import. From the IEM, the TIC complex mediates the transfer of proteins from the TOC complex into the stroma. There are several models for the TIC translocon. One model involves a 1-MDa TIC complex, which is comprised of plastome- encoded TIC214 or Ycf1 and nucleus-encoded components that include TIC100, TIC56, TIC35, YlmG, TIC20, and TIC12. TIC110 and TIC40 presumably recruit and regulate a set of stromal chaperones, including cpHsp70, Hsp90C, and Hsp93. It has been proposed that these provide energy through ATP hydrolysis for protein import and folding. TIC22 faces the intermembrane space (IMS), while TIC236 lies between the IEM and IMS. Moreover, TIC21, which is present at IEM, also shows interaction with TIC236, but it is not a subunit of the 1-MDa TIC complex. Another model shows a 2-MDa motor complex made of the plastome-encoded Ycf2 and five FtsH-like ATPase subunits (Liang et al., 2024). However, the question regarding the force generating factors has not been completely resolved. Transit peptide cleavage occurs by stromal peptide protease (SPP) upon pre-protein delivery into the stroma (Taken from Venkatasalam & Kessler, 2024).

1.6 Chloroplast Import machinery

The process of importing chloroplast proteins, which is essential for the proper functioning of chloroplast, is facilitated by TOC and TIC complexes. The TOC complex is composed of TOC159, TOC34, TOC75. Two proteins containing GTPase domains, namely TOC159 and TOC34, play a role in protein recognition. TOC34 and TOC159 associate with the channel- forming β-barrel protein TOC75 to create the complete and functional TOC complex and act as a transit peptide receptor. A feature recently discovered by cryo-EM is the hybrid channel at the core of the TOC complex consisting of the TOC75 and TOC159 C-terminal β-barrel domains.

The cryo-EM map reveals that the TOC complex in *Chlamydomonas reinhardtii* consists of TOC90 (a TOC159 homolog), TOC75, and TOC34 in a 1:1:1 stoichiometry (Liu et al., 2023). In contrast, earlier studies on plant TOC complexes proposed stoichiometries of TOC159:TOC75: TOC34 as either 1:4:4–5 or 1:3:3 (Schleiff et al., 2003; Kikuchi et al., 2006). Alternatively, 1:1:1 TOC159–TOC75–TOC34 complexes may coexist with TOC75–TOC34 complexes that lack TOC159, explaining the observed higher molar ratios of TOC75 and TOC34 compared to TOC159 (Liu et al., 2023).

The TIC complex includes TIC22, TIC110, TIC40, TIC20, TIC21, TIC62, TIC55, and TIC32 (Shi & Theg, 2013; Gutensohn et al., 2006). The TIC complex is associated with a proposed ATP- driven import motor composed of a Ycf2-FtsHi heteromeric AAA-ATPase complex (Kikuchi et al., 2018; Liang et al., 2024).

In a recent study, the structure of the Ycf2-FtsHi complex was studied using cryo-electron microscopy. This complex is composed of 19 subunits, including Ycf2, FtsHi, and other associated proteins. Ycf2 is a large, membrane-integrated protein that forms the core of the complex, while FtsHi is an ATPase associated with various cellular activities (AAA) protein, contributing to the energy-dependent translocation process. The structural analysis reveals that Ycf2 forms a channel-like structure, facilitating the passage of preproteins, with FtsHi providing the necessary ATPase activity for translocation (Liang et al., 2024).

In addition, TIC110 and TIC40 are suggested to recruit stromal chaperones, such as

cpHsp70, Hsp90C, and Hsp93, that may assist in protein translocation and proper folding through ATP hydrolysis (Inoue et al., 2013; Flores-Pérez & Jarvis, 2013). TOC and TIC components have evolved to mediate the import of specific classes of plastid proteins, and their activities are regulated to coordinate plastid biogenesis with developmental and physiological events (Richardson & Schnell, 2020).

1.6.1 TOC159: Structure, Function, and Isoforms

TOC159 has a central role in recognizing and transporting photosynthetic proteins from the cytoplasm into the chloroplast. It functions as a receptor for specific targeting signals of pre- proteins to import into the chloroplast. Multiple genes encode TOC159 homologs, resulting in four isoforms present in Arabidopsis: TOC159, TOC132, TOC120, and TOC90 (the naming convention is based on the molecular masses in kilodaltons). Unlike TOC159, these other three isoforms are associated with the import of non-photosynthetic proteins. This indicates precise regulation of protein import to control protein composition and activities within the chloroplast (Bauer et al., 2000; Jarvis & López-Juez, 2013). TOC159 possesses a high degree of structural complexity. Its N-terminal intrinsically disordered acidic (A) domain might be involved in protein-protein interactions or signal recognition (Agne et al., 2010; Inoue et al., 2010). The C-terminal domain of TOC159 is a membrane anchor (M-) domain and forms a 14-stranded β-barrel structure. This domain associates with the β-barrel-forming domain of TOC75 to create a hybrid channel at the outer membrane chloroplast. The central GTPase (G-) domain has crucial role in recognizing and interacting with preproteins destined for import, and binding and hydrolyzing GTP (Wang et al., 2008). The three homologs of TOC159 have analogous structure, the A- domains being the most divergent.

TOC159 is specific to eukaryotes and has no any known homologs in cyanobacteria (Kalanon & Mc Fadden, 2008). The G, M, and A domains of TOC159 are derived from a common ancestral gene most likely through duplication of an ancient protein domain involved in GTP binding. The knockout mutant of TOC159, *ppi2*, exhibits an albino phenotype, highlighting its important role in the chloroplast import machinery and consequently in chloroplast biogenesis (Bauer et al., 2000). While individual mutation of AtTOC90, AtTOC120, and AtTOC132 do not cause any obvious visible defects but double mutant lines of TOC120 and TOC132 develop an albino phenotype, similar to the TOC159 mutant (Kubis et al., 2004). The requirement of non- photosynthetic

proteins to assemble the photosynthetic machinery may explain this.

1.6.2 Targeting and insertion of TOC159 at the outer membrane

The assembly of TOC159 takes place in a series of steps, including protein-protein interactions, domain-specific functions, and a putative transition from a soluble to an integral membrane- binding state. First, soluble TOC159 has to be directed to the chloroplast outer membrane via chaperone binding similar to that of a transit peptide for targeting. The G and M domains then bind to TOC34 and TOC75, respectively, and this interaction may position TOC159 for membrane insertion. It has been proposed that nucleotide-binding (most likely GDP) occurs at the G-domain and is important for membrane insertion of the M-domain. Upon successful integration into the membrane, TOC159 becomes an integral part of the functional TOC complex (Lung & Chuong, 2012; Richardson et al., 2014).

1.6.3 TOC33: Structure, Function, and Isoforms

TOC33 consists of a GTP binding domain and a short C-terminal transmembrane domain forming an alpha-helix, which is critical for chloroplast protein import and interaction with other TOC components. Recognition of preproteins is essential and involves the GTP-binding domain. Although the function of GTP hydrolysis in the process of preprotein recognition and translocation is not fully defined, another possibility may be the involvement of the G-domain of TOC33 either in its own targeting or even assembly within the TOC complex (Jarvis & Lopez- Juez, 2013; Kessler et al., 1994; Sun & Jarvis, 2023). In Arabidopsis, two homologs, AtTOC33 and AtTOC34 exist. These variations in isoforms are believed to have similar substrate specificities for preproteins involved in photosynthetic (TOC33) and non-photosynthetic (TOC34) processes; this guarantees the import of different types of preproteins and hence the functionality of the chloroplast as well as other plastid types. AtTOC33 is mainly expressed in rapidly growing photosynthetic tissues, while AtTOC34 is expressed at low levels throughout development. Knockout mutations in AtTOC33 (*ppi1*) of Arabidopsis result in a palegreen, non- lethal phenotype characterized by decreased levels of photosynthetic proteins relative to non- photosynthetic proteins. Knockout of AtTOC34 (*ppi3*) mainly caused slowed root growth, with no other noticeable phenotype. Efforts to obtain a homozygous double mutant of AtTOC33 and AtTOC34 were unsuccessful due to lethal effects on embryo development. The mutant analyses

suggest overlapping functions of AtTOC33 and AtTOC34 (Kubis et al., 2004). Similar to TOC159, TOC34 appears absent in cyanobacterial genomes, indicating a eukaryotic origin for this protein and supporting the idea of the evolution of these components of the chloroplast protein import machinery from eukaryotic organisms (Shi & Theg, 2013). The expression and activity of TOC33 are tightly regulated by environmental conditions and the plant's developmental stage. This regulation enables the fine-tuning of chloroplast protein import to adapt to the plant's shifting physiological demands (Demarsy et al., 2014; Richardson & Schnell, 2020).

1.6.4 Targeting and insertion of TOC34 at the outer membrane

Newly synthesized TOC34 is delivered to the chloroplast outer membrane by two cytosolic factors, AKR2 and Hsp17.8, which function as chaperones by specifically recognizing its C- terminal transmembrane domain (TMD). Unlike many other proteins, TOC34 and similar outer membrane proteins with TMD do not rely on a cleavable targeting signal to reach their destination. Instead, their targeting instructions are TMD and the nearby amino acid residues. These regions together act as the "address label", guiding the proteins to the outer membrane of the chloroplast where they belong (Lee et al., 2001; Dhanoa et al., 2010). There are three proposed pathways explain TOC34's targeting and insertion: (1) an outer membrane receptor recognizes the complex and transfers TOC34 to TOC75, (2) the complex binds directly to TOC75 without additional receptors, or (3) TOC75-mediated insertion is facilitated by interactions between TOC34’s G-domain and existing TOC34. In all pathways, TOC75 inserts the TMD into the membrane, forming a stable TOC34-TOC75 association (Richardson et al., 2014).

1.6.5 TOC75: Structure, Function, and Isoforms

TOC75, a beta-barrel structured translocon, associates with the C-terminus of TOC159 to form a hybrid channel within the outer envelope of the chloroplast, enabling the membrane translocation of precursor proteins essential for chloroplast biogenesis and function. In addition, TOC75 has a crucial role in the targeting and insertion of the TOC GTPases (Eckart et al., 2002; Schnell et al., 1994). TOC75 is encoded by a single gene across all plant species (Inoue & Keegstra, 2003) and belongs to the Omp85 superfamily, allowing to trace its ancestry to gram- negative bacteria (Voulhoux et al., 2003). TOC75 consists of a C-terminal beta-barrel beta-barrel domain (~45 kDa) with 16 transmembrane strands for channel formation and an N-terminal domain (~30 kDa)

containing three POTRA (polypeptide transport associated) repeats extending into the intermembrane space (Paila et al., 2016). The POTRA domain of TOC75 plays a critical role in recognizing preproteins and exhibits chaperone-like functionality to prevent misfolding of preproteins as they traverse the intermembrane space pass through the IMS (Kouranov & Schnell, 1997; Sánchez-Pulido et al., 2003; Shanmugabalaji & Kessler, 2024). Recent cryo- electron microscopy (cryo-EM) studies from *Chlamydomonas reinhardtii* have provided detailed insights into the structure of TOC75 in association with the C-terminus of TOC159, revealing it as a voltage-sensitive channel with a pore diameter of approximately 26 Å (Liu et al., 2023).In Arabidopsis, there are three isoforms of TOC75 (which are named according to their chromosomal locations): AtTOC75-III, the orthologue of pea TOC75, functions as an essential gene contributing to the protein import channel; mutants lacking this isoform exhibit arrest at the two-cell stage of embryogenesis (embryo lethality). AtTOC75-IV is expressed at lower levels but considered non-essential. The changes in etioplast ultrastructure and reduced efficiency of de- etiolation suggest that atTOC75-IV may play a role during the dark growth phase of plants (Baldwin et al., 2005). AtTOC75-I is classified as a pseudogene, which suggests it does not have any functionality due to the insertion of gypsy/Ty3 transposon (Baldwin et al., 2005).

1.6.6 Targeting and insertion of TOC75 at the outer membrane

The precursor of TOC75 is synthesized with a mass of approximately 89 kDa and a bipartite targeting signal that contains an N-terminal transit peptide followed by a poly-glycine region. Once synthesized in the cytoplasm, the N-terminal transit peptide of pre-TOC75 is recognised by TOC GTPase receptors, which leads to the translocation of pre-TOC75 into the TOC channel. Subsequently, the transit peptide is cleaved by the stromal processing peptidase, indicating the involvement of the TIC complex in the partial translocation of pre-TOC75 across the inner membrane. The intermediate form of TOC75 (iTOC75) spans the intermembrane space between the outer and inner envelopes, where it is processed by a type I signal peptidase (SPase 1), producing the mature and functional TOC75 protein. Infact, the glycine-rich segment following the transit peptide of pre-TOC75 plays a crucial role in preventing the complete translocation of iTOC75 into the intermembrane space (Baldwin & Inoue, 2005), effectively pausing the process at this stage leads to create two models for insertion of TOC75 into the membrane. In the first model the TOC complex directly mediates the

insertion of iTOC75 into the outer membrane without need for a separate translocase (Inoue et al., 2001). The second model proposes that iTOC75 is engaged in the intermembrane space by a second translocase which specifically integrates beta-barrel proteins into the outer membrane (Huang et al., 2011; Richardson et al., 2014).

1.6.7 Regulation at the TOC complex

The activity of the TOC complex is regulated at multiple levels. The primary regulation relies on different GTPase isoforms that are thought to control the selectivity for preproteins. The TOC33/34 and TOC90/120/132/159 isoforms have preferences for specific classes of preprotein substrates (Bauer et al., 2000; Jarvis & López-Juez, 2013). The UPS (Ubiquitin-Proteasome System), serves as a crucial regulatory mechanism for TOCs. Ubiquitination is a post- translational modification process that involves the attachment of one or more copies of the 8.5 kDa ubiquitin protein to lysine residues on target proteins (Vierstra, 2009). The addition of polyubiquitin chains designates the protein for degradation by the 26S proteasome. The process of ubiquitination entails an enzyme cascade that comprises the activation and targeted conjugation of ubiquitin. To initiate this process, an E1 ubiquitin activase forms a thioester bond with ubiquitin in an ATP-dependent reaction. This ubiquitin moiety is then transferred to an E2 ubiquitin conjugase, which subsequently transfers it to an E3 ubiquitin ligase (Sako et al., 2014). At the outer membrane of chloroplast there is an E3 ligase called SP1 (Suppressor of PPI1 Locus 1), that is responsible for regulating TOC33, TOC75, and TOC159 homolog turnover (Ling et al., 2012). Under stress, SP1 ubiquitinates the TOCs and targets them for extraction from the outer membrane and subsequent degradation by the 26S proteasome. In this pathway, SP2 forms a channel at the outer membrane of chloroplast to assist in the extraction of membrane proteins and CDC48 provides motive force to pull out the ubiquitinated TOCs from the outer membrane. Interestingly, SP2 shares sequence homology with TOC75, suggesting a potential functional relationship. The three proteins SP1, SP2, and CDC48, constitute the chloroplast- associated protein degradation pathway which is called CHLORAD (Ling et al., 2019; Shanmugabalaji and Kessler, 2019). Reducing the import of photosynthesis-related proteins through a decrease in TOC levels by CHLORAD, reduces production of reactive oxygen species under stress and indicates a role of SP1 in stress tolerance. In addition, it has been observed that higher expression of SP1 leads to accelerated ripening in tomato fruit. This is consistent with a model in which

turnover of TOC receptors facilitates rapid transitions from chloroplasts to chromoplasts, leucoplasts or other plastid morphotypes. Conversely, Arabidopsis mutants lacking sp1 exhibit highly inefficient developmental transitions (Reiland et al., 2011; Barsan et al., 2012; Ling et al., 2012). Another role of ubiquitination in the regulation of TOCs emerges during early seed germination when gibberellic acid (GA) levels are low. In this condition, the protein DELLA, which acts as a negative regulator of GA signaling, facilitates the targeting of TOC159 for degradation by an unknown E3 ligase and 26S proteasome. This process leads to a delay in chloroplast biogenesis at an early stage of development and has been demonstrated to be independent of SP1 or CHLORAD pathway (Shanmugabalaji et al., 2018). Moreover, SUMOylation provides some protection for TOC159, helping to prevent its degradation through ubiquitination under stressful conditions (Accossato et al., 2020). Recent research has revealed that phosphorylation at serine 260 of TOC33 by the kinases CTR1, and potentially M3Kδ7 is crucial for its stability, and protection from ubiquitination and degraded by CHLORAD pathway and is facilitating proper chloroplast development (Chien & Yoon, 2025).The redox state might influence the preprotein translocation efficiency by mediating TOC complex interactions (Hirohashi & Nakai, 2000; Küchler et al., 2002; Stengel et al., 2009). Based on in vitro experiments, oxidizing conditions such as those found in stressed or senescent chloroplasts trigger the formation of disulfide bridges between cysteine residues within TOC proteins. These bridges occur both intra- and intermolecularly among TOC159, TOC34, and TOC75, resulting in the formation of a heteromeric TOC complex (Seedorf et al., 1995). This bulky complex can hinder protein import by physically blocking the channel, thereby preventing the entry of precursor proteins. Furthermore, an additional mechanism suggests that the binding capacity of receptor proteins for preproteins is also compromised. This is due to the location of cysteine residues within the preprotein-binding GTPase domain, which undergoes conformational changes under oxidizing conditions (Stengel et al., 2009). Consequently, both channel obstruction and impaired receptor function lead to a significant reduction in the overall efficiency of protein import into chloroplasts, rendering TOCs less effective in their role (Stengel et al., 2009; Sjuts et al., 2017).

1.6.8 TIC complex

After proteins pass through the TOC complex and enter the intermembrane space, they encounter the translocon at the inner chloroplast membrane, which facilitates their

movement across the inner membrane into the stroma or further to the thylakoid membranes and lumen. The TIC (translocon on the inner chloroplast membrane) complex plays a crucial role in regulating preprotein import. While the TIC complex is not as thoroughly understood as the TOC complex, there has been debate about the core TIC channel identity due to its attribution to both TIC110 and TIC20. Initially, TIC110 was proposed a potential protein importing channel because of its abundance and demonstrated activity (Kessler & Schnell, 2006), recent evidence suggests that TIC110 may not function as a channel protein in vivo based on its crystal structure and the apparent absence of interactions with other TIC components (Tsai et al., 2013). Chromatography experiments indicated that only a small percentage of TIC110 is associated with other TIC components (Kikuchi et al., 2013; Nakai, 2015), contradicting earlier assumptions about its role as a channel protein adjacent to TOC. Current scientific knowledge suggests that the core of TIC complex is built around a channel formed by the protein TIC20 and is exceptionally large. Other proteins like TIC21, TIC56, TIC100 and TIC214/YCF1 assist in enabling this channel function of this so-called 1-MDa TIC complex (Kikuchi et al., 2013). In this model, TIC40 and TIC110 do not form the core channel and the relationship between TIC110-based system and 1-MDa TIC during protein import remains unclear. TIC40 and TIC110 might recruit chaperones to assist with protein movement and folding (Kikuchi et al., 2009; Inoue et al., 2013) or they function as docking stations for proteins exiting the TIC channel on their way to the stroma, with TIC40 helping release the proteins (Inaba et al., 2008). TIC110 and TIC20 proposed two independent TIC channels TIC110 acts as a general channel however TIC20 is specialized for importing the substrates (Kovács-Bogdán et al., 2011).

TIC20, as a crucial component of the translocon channel at the inner membrane of the chloroplast, was first identified through chemical crosslinking of importing precursor proteins in pea chloroplasts and initially called IAP21 or TIC21 (Kouranov & Schnell, 1997). The structure of TIC20 is composed of four membrane-spanning α-helices and its N- and C-terminal are exposed to the stroma (Kovács-Bogdán et al., 2011; Kouranov & Schnell, 1997). Arabidopsis possesses four isoforms of TIC20: AtTIC20-I, -II, -IV, and -V with varying functions (Kasmati et al., 2011). Suppression of TIC20-I levels in Arabidopsis results in pale leaves, reduced chloroplast protein levels, and defects in protein import (Chen et al., 2002). TIC20 is an ancient protein that originated

cyanobacteria lineages (Töpel & Jarvis, 2011). Among these, TIC20-I plays a crucial role for chloroplast development and protein import in *Arabidopsis thaliana* (Hirabayashi et al., 2011). Plants lacking these proteins have severe albino phenotypes, and their survival beyond the seedling stage is impossible. Thylakoid membranes do not develop, and photosynthetic proteins are not accumulated (Kasmati et al., 2011; Chen et al., 2002). Conversely, group 2 of TIC20 members (TIC20-II and TIC20-V) are not essential for chloroplast development and exhibit no overlapping functions in protein import. Interestingly, they are expressed at relatively high levels throughout development (Kasmati et al., 2011).

TIC12 is a key part of the central TIC complex, and is likely situated on the inside wall of the channel. It works with TIC20 to interact with the targeting signal on proteins entering the chloroplast (Zhao et al., 2022). TIC236 extends into the space between the inner and outer chloroplast membranes and has been proposed to function in the formation of the TOC/TIC supercomplex (Chen et al., 2018). Plants lacking TIC236 show embryo lethality and reduced amounts of TIC236 caused significant difficulty in importing TIC22, which relies on the TOC-TIC system to reach its location between the membranes (Chuang et al., 2021). Furthermore, TIC236 association with TIC20, a possible core channel protein, suggests a role in supporting the channel structure.

TIC56 acts as a scaffold within the 1 MDa TIC complex, facilitating protein channel stabilization (Agne et al., 2017). The Arabidopsis *tic56* mutant revealed problems with ribosome assembly and protein production within the chloroplast (Köhler et al., 2016). Mutations in the ribosome- associated endonucleases CSP41a and CSP41b contribute further to this defect, impacting chloroplast ribosomal RNA metabolism (Beligni & Mayfield, 2008). TIC100 plays a dual role within the 1 MDa TIC complex, which is involved in importing photosynthetic and non- photosynthetic proteins. TIC100 helps to stabilize the channel that allows proteins to pass through the chloroplast. Additionally, it appears to moonlight in RNA editing within the chloroplast, and its absence might also affect retrograde signaling TIC100 is crucial for both chloroplast biogenesis and embryo development (Loudya et al., 2022). TIC214 stands out as the only protein involved in the chloroplast import machinery that is encoded by the chloroplast DNA. It acts like a bridge between the inner and outer chloroplast membranes. Its C-terminus anchors it in the inner membrane, while the other large section of the intermembrane space domain reaches out and connects to TOC. This IMS

domain of TIC214 intimately connects with other proteins such as TIC100, TIC56, TIC35, and TOC52 and thus contributes to formation of the mega-complex (Jin et al., 2022; Liu et al., 2023). In a green alga, *Chlamydomonas reinhardtii*, temporarily reducing the amount of TIC214 led to problems importing proteins into the chloroplast, disrupting the production of chloroplast ribosomes, protein folding, and the organism's ability to respond to stress (Ramundo et al., 2013). Among eukaryotes and different plastid types, TIC110 is conserved across eukaryotes (Shi & Theg, 2013). It consists of two transmembrane helices at its N-terminus and the greatest part (90 KDa) of it is in the stroma for the recruitment of stromal chaperones for precursor protein import. It also serves as a docking site for precursor proteins during the late stages of translocation (Jackson et al., 1998; Kessler & Blobel, 1996). Mutants display various phenotypic effects, ranging from embryo-lethality (homozygous mutant) to chlorotic phenotypes and defective protein import (heterozygous mutants) and down-regulation leading to reduced plastid protein levels and impaired translocation (Inaba et al., 2005). Thus, TIC110 is integral to biogenesis of plastids and the viability of plants. Compared to the more studied TOC complex, less is known about the regulation of the TIC complex. Whereas the isoform composition was important for the regulation of the TOC complex, it seems that, instead, the TIC complex is influenced by the general physiological state of the chloroplast (Balsera et al., 2010).

1.7 Chloroplast biogenesis and seed development work together to support plant growth

Chloroplast biogenesis and seed development are intricately connected processes essential for plant growth and reproduction. During seed development and maturation, plastids within the developing embryo undergo differentiation into specialized forms like etioplasts, which later develop into fully functional chloroplasts upon germination and light exposure (Pogson et al., 2015). This transition is tightly regulated, as chloroplast biogenesis ensures the establishment of photosynthetic capacity necessary for seedling growth (Dubreuil et al., 2018). The coordination of chloroplast biogenesis and seed maturation is mediated by complex genetic and hormonal networks (including ABA and GA signaling), environmental cues and developmental signals. Thus, the interplay between chloroplast biogenesis and seed development ensures the successful establishment of the next generation of plants.

1.7.1 The physiological process of seed development and maturation

Seed development in angiosperms, or flowering plants, can be divided into two primary phases: embryogenesis and maturation. The process begins with "double" fertilization of the embryo sac, which triggers the development of three genetically distinct tissues that comprise the seed coat (testa) of maternal origin, the triploid endosperm, and the diploid embryo.

In Arabidopsis, embryogenesis is initiated when the zygote divides asymmetrically, yielding a large basal cell that contributes to the root meristem and a smaller apical cell that forms the shoot apical meristem (Bayer et al., 2017). In the early phase of embryogenesis, the globular stage, the embryo forms a roughly spherical structure composed of a few dozen cells. During this stage, extensive cell divisions occur, and precursor cells for various tissues are established, though they remain largely undifferentiated. This stage sets the foundation for later steps of formation and tissue specification. The heart stage follows the globular stage in which distinct tissue patterns begin to develop, and the initial organization of the shoot and root apical meristems becomes apparent for further organ development. Hence, during the embryogenesis the transition from globular to heart stage is important (Lau et al., 2012; Wendrich & Weijers, 2013). In Arabidopsis, two important events occur at the globular stage: chloroplast differentiation from proplastids and chlorophyll biosynthesis (Tejos et al., 2010). Seed coat differentiation is initiated by ovule fertilization. The endosperm then undergoes a syncytial phase characterized by rapid nuclear divisions without cytokinesis, resulting in a multinucleate cell which this phase is considered crucial for seed size determination and viability (Day et al., 2009). Next is cellularization, in which membranes or walls develop around each nucleus to form separate cells. Further differentiation takes place, enabling separate cells to specialize in their function. Auxin signaling acts as the key regulator for embryogenesis, while ABA orchestrates many aspects of seed maturation that include accumulation of storage reserves and induction of seed dormancy. After cell division and differentiation are completed, the seed develops to maturity phase. During maturation, seeds develop desiccation tolerance and can tolerate severe loss of moisture and then rehydrate without further damage. Molecules accumulate in the embryo include low molecular weight antioxidants, oligosaccharides, such as raffinose and stachyose, late embryogenesis abundant proteins (LEAs), and heat shock proteins (HSPs) (Ballesteros et al., 2020).

Besides, the seeds accumulate several thousand mRNAs, which are translatable into proteins during germination (Sano et al., 2020; Bai et al., 2019). Structural changes during seed maturation include folding of the cell wall, chromatin condensation, and breakdown of the thylakoid membrane in chloroplasts that transiently develop during the later stages of embryogenesis. These events further reduce metabolic-cell shrinkage stresses and increase seed longevity (Verdier et al., 2013).

1.7.2 The physiological process of seed germination

Seed germination is the critical physiological transition from a dormant state into an actively developing seedling, whereby there is reprogramming of a great number of its biological processes (Han & Yang, 2015). Germination is defined as the penetration of the seed coat by the elongation of the embryonic root that involves multiple genetic and physiological pathways working together to allow for the resumption of embryo growth that has been dormant during seed maturation (Holdsworth et al., 2008). Seed germination is divided into three phases. Phase I is marked by a dry seed quick water absorption, resulting in swelling and shape transformation (Bewley, 1997). This rapid rehydration of the membrane leads to release of low molecular weight metabolites and cellular solutes from the seed; afterwards, the membrane structure is repaired following a brief period of hydration. During the phase of water uptake, some physiological processes are initiated, including protein synthesis from existing mRNA and resumption of respiratory activities such as glycolytic and oxidative pentose phosphate respiratory pathways, which cause increasing oxygen consumption and release of carbon dioxide within minutes of imbibition (Bewley, 1997). Following imbibition, DNA ligase is activated to repair DNA, which is damaged through the desiccation time of seed development (Bewley, 1997; Weitbrecht et al., 2011). Dry seeds contain a low amount of adenosine triphosphate (ATP). Another vital event of phase I is mitochondria repair to provide more ATP for the germination process. The germinating seeds enter phase II when the seed's water uptake rate decreases and stabilizes. During this phase, the seed continues to repair its existing DNA and mitochondria and synthesize new mitochondria and proteins from newly transcribed mRNA copies. Subsequently, the seed's embryo expands and seed-covering layers become fragile, leading to radicle protrusion through the testa/seed coat. Finally, germination occurs and phase II terminates. Phase III is marked by mobilization of storage reserves in the seed and a higher intake of water and promotion of seedling

growth. Cell division, DNA synthesis, and radicle cell elongation take place at this stage. As the seedling emerges, chloroplast biogenesis ensures the optimal rate of photosynthesis, preventing harmful oxidation in the early stages of seedling emergence (Pogson et al., 2015).

1.7.3 Gibberellic acid regulates chloroplast biogenesis during early plant development

Gibberellic acid (GA) is a tetracyclic di-terpenoid compound and functions as a plant growth and development hormone. (Schwechheimer, 2012). Moreover, GA plays a critical role in chloroplast development and in enhancing the photosynthesis of mature chloroplasts. In fact, GA inhibits de-etiolation (greening) in darkness by regulating light signaling pathways. In addition, GA triggers the degradation of DELLA proteins (which are encoded by RGL1; RGL2; RGL3; RGA; and GAI genes) and the biogenesis of functional young chloroplasts during de-etiolation (Jiang et al., 2012). It has been studied how GA, as one of the important plant hormones, influences chloroplast biogenesis during early plant development, and pre- germination. At the early stages of seed germination, the level of DELLA protein is high and that of GA low, DELLA interacts with cytosolic TOC159, prior to its assembly into the TOC complex, and triggers its degradation by the 26S proteasome implicating an unknown E3 ligase. Under conditions favorable to germination, GA levels increase causing the ubiquitination and degradation of DELLA. As a consequence, TOC 159 is able to assemble to become a functional receptor to import the pre-proteins into the chloroplast and also in chloroplast biogenesis (Shanmugabalaji et al., 2018). Also, in separate work, it has been revealed that SUMOylation partially stabilizes TOC159 against UPS-dependent degradation under low GA during early development (Accossato et al., 2020) (Figure 6).

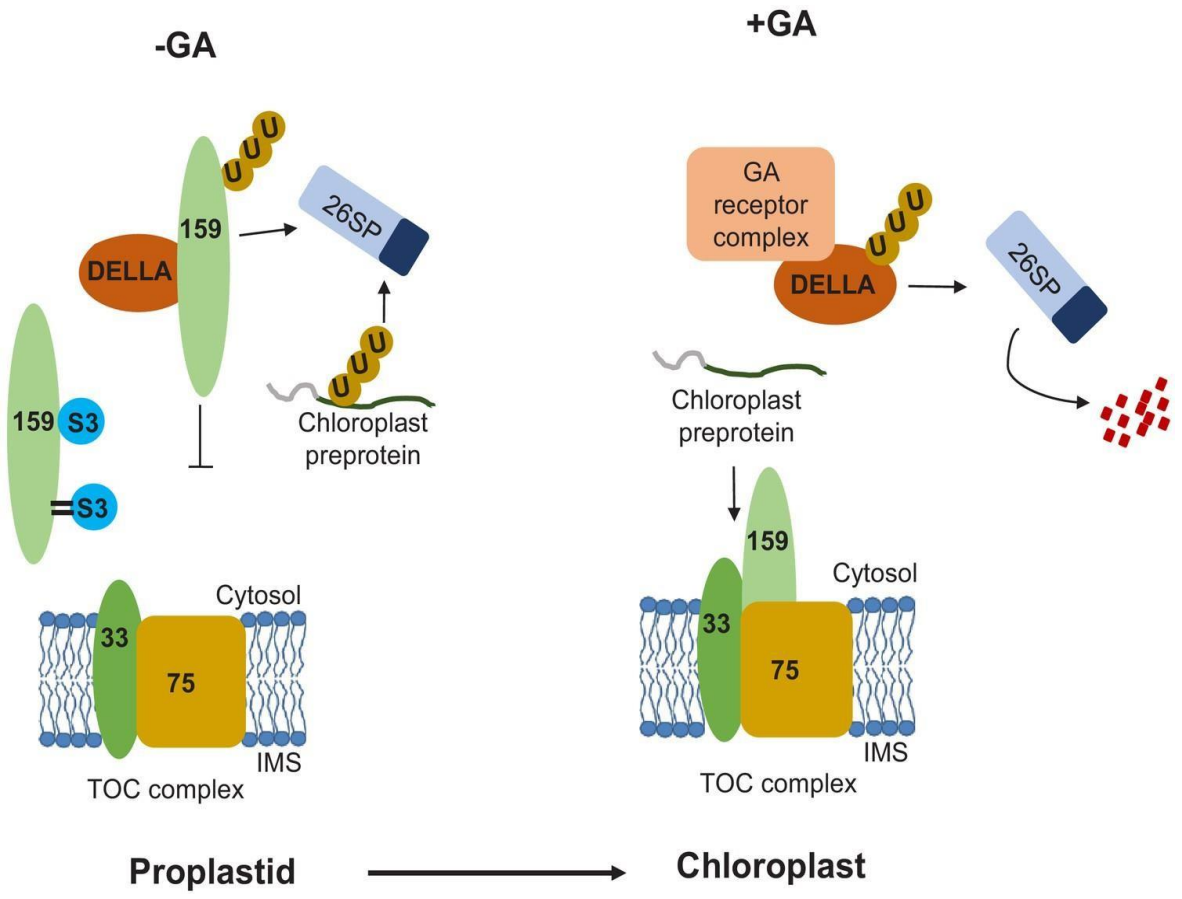

Figure 6: The GA-dependent ubiquitin-proteasome and SUMO systems regulate chloroplast biogenesis at the level of the TOC159 import receptor in early plant development. The levels of GA that accumulate when seeds absorb water are influenced by environmental factors. In stressful conditions where active GA is reduced, DELLA (RGL2) accumulates and binds to TOC159, resulting in its degradation via the UPS. Simultaneously, TOC159 interacts with and is covalently modified by SUMO3. This SUMOylation shields TOC159 from UPS-mediated breakdown and promotes the accumulation of proteins associated with photosynthesis in the chloroplast. Any preproteins that fail to be imported are broken down in the cytosol through the UPS (taken from Accossato et al., 2020).

1.7.4 Abscisic acid regulates chloroplast development

Abscisic acid (ABA) is an isoprenoid-derived phytohormone that is essential for suppressing seed germination and enhancing stress resilience. ABA also controls crucial processes in plant development, especially the synthesis of seed storage proteins and lipids, seed desiccation tolerance and dormancy, and more than this, suppresses the transition from embryonic to germinative growth and from vegetative to reproductive growth (Rohde et al., 2000). ABA inhibits germination in Arabidopsis by repressing genes involved in cell elongation and energy mobilization, while enhancing stress resistance genes, independently of storage reserve mobilization (Pritchard et

al., 2002). During seedling growth, ABA contributes to the regulation of developmental processes such as root architecture and stomatal closure, enabling seedlings to adapt to abiotic stresses such as drought and salinity. ABA has a critical role in water homeostasis by controlling stomatal aperture and stress-responsive genes that lead to a reduction of the entry amount of CO_2 into the leaf and results in restrictions of photosynthesis as well as root tolerance to unsuitable conditions (Finkelstein et al., 2002). In roots, ABA is involved in processes stimulating water uptake, including hydrotropism (Miao et al., 2021) and xylem formation (Ramachandran et al., 2021). Deposition of hydrophobic suberin layers and primary root growth is promoted by ABA (Ramachandran et al., 2021). Although ABA was originally considered a root-derived signal sent to the shoot, ABA can also be synthesized in the shoot and plays a critical role in stomatal regulation and consequently respiration (Christmann et al., 2007). The main components of ABA perception and signaling include the ABA receptor (PYR/PYL/RCAR), protein phosphatase 2C (PP2C) proteins, and subclass III sucrose non- fermenting protein (SNF1)-related kinases 2 (SnRK2). In the absence of ABA, PP2Cs deactivate SnRK2s via dephosphorylation. However, when ABA is present, PYR/PYL/RCARs prevent the phosphatase activity of PP2Cs. Subsequently, SnRK2s are active and phosphorylate the downstream transcription factors which are essential for the ABA-responsive expression of hundreds or even thousands of nuclear genes (Ma et al., 2009; Park et al., 2009; Cutler et al., 2010; Raghavendra et al., 2010). The SnRK (sucrose non-fermentation-related protein kinase) is a Ser/Thr protein kinase (SnRK1, SnRK2, SnRK3), which is abundant in plants. There are some findings that revealed that the SnRK2 subfamily plays important in abiotic stresses including salt and osmotic stresses through phosphorylation of the target proteins to regulate the interconnection of various signaling pathways including ABA signaling and sugar metabolism (Wang et al., 2018) (Figure 7). It has been demonstrated that under ABA treatment TOC159 was phosphorylated by SnRk2.6 specifically at Thr692 in its A-domain. Pre-protein import in a mutant deficient in ABA biosynthesis (*aci1* and *aci2*) is affected (Zhong et al., 2010). These observations suggest that there is a close relation between ABA signaling and chloroplast protein import regulation potentially through TOC159 A-domain phosphorylation. How ABA influences chloroplast biogenesis during the early plant development stage, will be the central question to be addressed in this thesis.

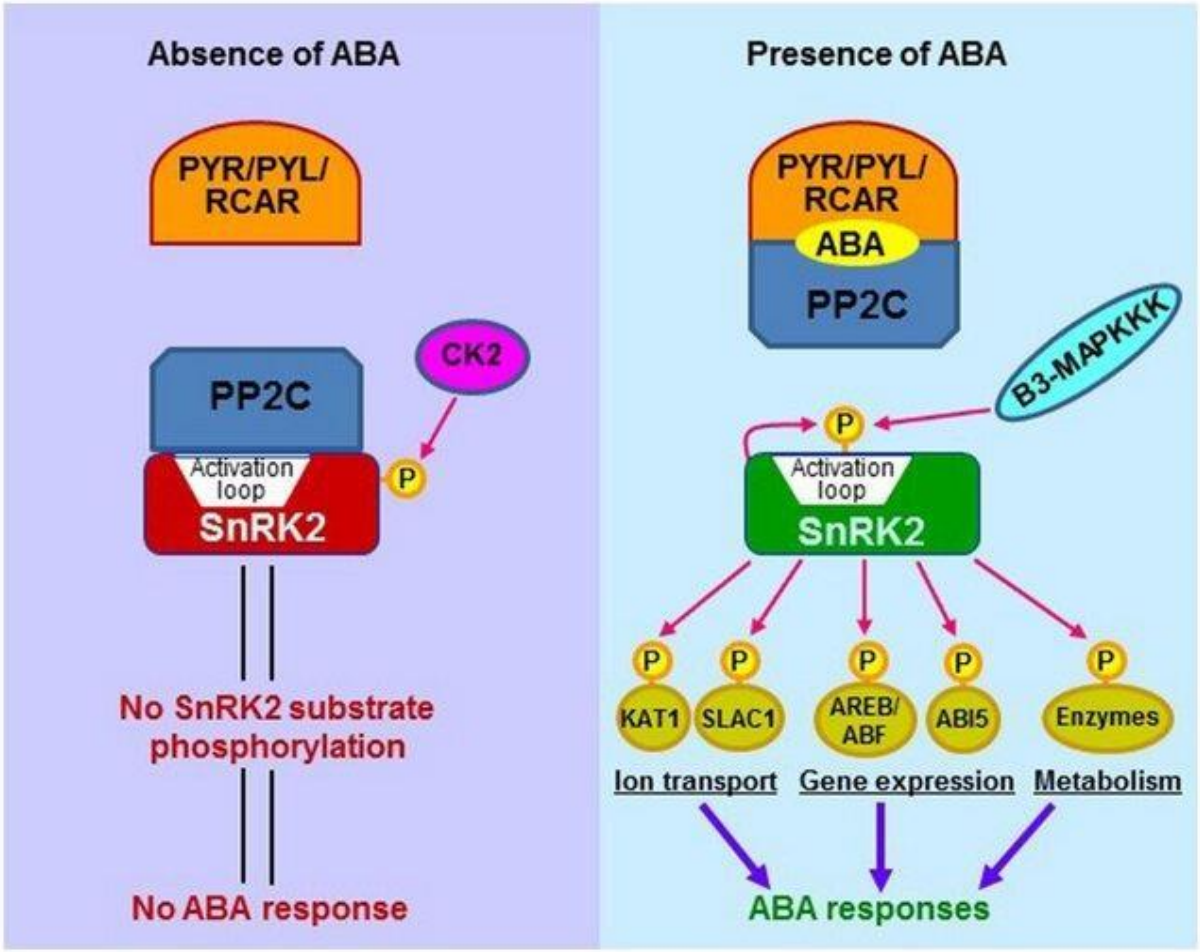

Figure 7: The major ABA signaling pathway in plants, with and without ABA, involves ABA receptors (PYR/PYL/RCAR), PP2C phosphatases (negative regulators), and SnRK2 kinases (positive regulators). Without ABA, PP2Cs bind to SnRK2s, preventing their activation and subsequent phosphorylation of downstream substrates, halting signal transduction. With ABA, PYR/PYL/RCAR receptors bind to ABA and interact with PP2Cs, releasing SnRK2s, which then auto phosphorylate and activate. Active SnRK2s phosphorylate downstream proteins, including transcription factors, ion channels, and enzymes like NADPH oxidases, initiating ABA responses. SnRK2s are regulated by other kinases: Raf-like kinase (B3-MAPKKK) activates SnRK2 by phosphorylating the activation loop, while casein kinase 2 (CK2) phosphorylates SnRK2's C-terminal serine residues, enhancing SnRK2-PP2C interaction and inactivating SnRK2. Active SnRK2 is depicted in green and inactive SnRK2 in red. Key components include ABF (ABA-responsive element binding factor), ABI5 (ABA insensitive 5), AREB (ABA-responsive element binding protein), B3-MAPKKK (B3-group Raf-like MAP kinase kinase kinase), KAT1 (potassium channel in *Arabidopsis thaliana* 1), PP2C (Protein phosphatase 2C), PYR (pyrabactin resistance), PYL (PYR-related), RCAR (regulatory component of ABA receptor), SLAC1 (slow anion channel 1), and SnRK2 (sucrose nonfermenting-1-related protein kinase 2) (Taken from Sah et al., 2016).

1.8 The aims of this PhD thesis

Chloroplast biogenesis, the process by which proplastids transform into functional, photosynthetic chloroplasts, is a pivotal event during germination. This PhD thesis aims to investigate the impact of ABA on the biogenesis of chloroplasts, with a focus on the components of the chloroplast import machinery and their substrates, during the transition from seed to seedling in *Arabidopsis thaliana*. The hypothesis of this PhD project is that ABA modulates chloroplast biogenesis during seed-to-seedling transition in *Arabidopsis thaliana* by regulating the expression and accumulation of components of the chloroplast import machinery and their substrates.

Therefore, this thesis aims to uncover the impact of ABA on the chloroplast import machinery and its substrates at both the gene expression and protein accumulation levels during the seed- to-seedling transition *in Arabidopsis thaliana*. Furthermore, the thesis aims to demonstrate that ABA promotes the import of non-photosynthesis-associated proteins via the non- photosynthesis-associated translocons (nTOC) while simultaneously inhibiting the import of photosynthesis-associated proteins via photosynthesis-associated translocons (pTOC).

The project adopts a multifaceted approach, proceeding with the following goals and aims:

- Regulation of the Arabidopsis transcriptome under ABA: genome-wide analysis of the entire transcriptome of ABA-treated Arabidopsis seedlings in order to identify both upregulated and downregulated genes through RNAseq with a focus on genes encoding chloroplast proteins.

- Regulation of the Arabidopsis proteome under ABA: quantitative mass spectrometric analysis (LC-MS) of the proteome of ABA-treated seedlings to identify both upregulated and downregulated proteins with a focus on chloroplast proteins.

- ABA-dependency of chloroplast protein import and biogenesis: determine the effects of increasing ABA concentrations on Arabidopsis seedlings, examine accumulation of components of nTOC and pTOC as well as their transport substrates.

Preferential protein import: address the hypothesis that ABA treatment promotes the preferential import of non-photosynthetic proteins through non-photosynthetic translocons.This research will provide insight into the impact of ABA on chloroplast

biogenesis and the role of (nTOC) and (pTOC) regulation in the process. This will engender a more profound understanding of plant developmental processes during the seed-to-seedling transition.

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Chapter 2: The journey of preproteins across the chloroplast membrane systems

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2.1 Abstract

The photosynthetic capacity of chloroplasts is vital for autotrophic growth in algae and plants. The origin of the chloroplast has been explained by the endosymbiotic theory that proposes the engulfment of a cyanobacterium by an ancestral eukaryotic cell followed by the transfer of many cyanobacterial genes to the host nucleus. As a result of the gene transfer, the now nuclear-encoded proteins acquired chloroplast targeting peptides (known as transit peptides; transit peptide) and are translated as preproteins in the cytosol. Transit peptides contain specific motifs and domains initially recognized by cytosolic factors followed by the chloroplast import components at the outer and inner envelope of the chloroplast membrane. Once the preprotein emerges on the stromal side of the chloroplast protein import machinery, the transit peptide is cleaved by stromal processing peptidase. In the case of thylakoid-localized proteins, cleavage of the transit peptides may expose a second targeting signal guiding the protein to the thylakoid lumen or allow insertion into the thylakoid membrane by internal sequence information. This review summarizes the common features of targeting sequences and describes their role in routing preproteins to and across the chloroplast envelope as well as the thylakoid membrane and lumen.

Keywords: chloroplasts, preprotein, transit peptides, TOC-TIC, thylakoid.

2.2 Introduction

The chloroplast is a member of the plastid organelle family known mostly for its photosynthetic activity though it does perform a vast array of other metabolic activities essential to plant survival, development, and stress responses (Jarvis and López-Juez, 2013). Plastids are the result of an endosymbiotic process that started over a billion years ago (Zimorski et al., 2014). Since that time, most plastid genes have either been lost or transferred to the nucleus (Timmis et al., 2004). Of the 2,000 plus chloroplast proteins only about 10% remain encoded by the chloroplast genome. The nuclear-encoded chloroplast preproteins contain an N-terminal transit peptide (TP). The TP can be compared to a molecular zip code of preproteins to be targeted to the chloroplast and imported via the chloroplast protein import machinery (Lee and Hwang, 2021). The import mechanism involves multiple steps at different (sub-) organellar locations. Initially, the preprotein is guided through the cytosol accompanied by a chaperone complex until it is handed off at the outer envelope of the chloroplast where the transit peptide makes first contact with the TOC (Translocon at the Outer envelope of the Chloroplast) complex (Flores-Pérez and Jarvis, 2013). This involves the action of the two GTP-binding receptors TOC159 and TOC34. In a process that requires GTP and low concentrations of ATP (0.1 mM), the preprotein is inserted across the large hybrid outer membrane protein-conducting channel that consists of the C-terminal ß-barrel membrane (M-) domain of TOC159 and that of TOC75 (Richardson et al., 2014; Schnell, 2019). At this stage already, the transit peptide is in contact with the intermembrane POTRA-domains of TOC75 and initiates contact with components of the TIC (translocon at the Inner envelope of the chloroplast) complex, namely, TIC22 and TIC20, however, without traversing the inner membrane (Kouranov et al., 1998). In the presence of high concentrations of ATP (> 1 mM) the preprotein crosses the TIC20 inner membrane protein-conducting channel and enters the chloroplast stroma assisted by ATP- dependent motor components (Richardson et al., 2018). Once inside the stroma, the transit peptide is cleaved by the Stromal Processing Peptidase (SPP) (Richter and Lamppa, 1998). Many imported, mature proteins remain in the stroma and are folded with the help of chaperones. Some proteins, however, are targeted further to the thylakoid membrane or lumen. Thylakoid lumen targeted proteins possess bipartite targeting sequences

consisting of a transit peptide followed by a thylakoid targeting signal that engages one of two pathways leading to the thylakoid lumen: The ΔpH-dependent TAT (twin arginine targeting) and SEC (secretory) pathways. However, the Signal Recognition Particle (SRP) pathway inserting proteins into the thylakoid membrane relies on targeting information residing within the mature sequence. Each of these pathways relies on a distinct set of protein components. Thylakoid targeting signals of preproteins are removed by a thylakoid processing peptidase (TPP) which promotes final assembly of the mature proteins leading to functional chloroplasts (Mori and Cline, 2001; Albiniak et al., 2012; Teixeira and Glaser, 2013).

2.3 Protein translocation into the chloroplast

2.3.1 The primary structures of transit peptides are highly diverse

“Signal Peptide” refers to an endoplasmic reticulum targeting sequence, “pre-sequence” to a mitochondrial one, and “transit peptide” is specific for chloroplast-targeted proteins (Bruce, 2000). In the late 1970s, after the signal hypothesis had been proposed, a study showed that in vitro translated Rubisco small unit (RbcS) protein had a higher molecular mass than mature RbcS in plant extracts. It was therefore considered a putative precursor (Dobberstein et al., 1977). The RbcS cDNA was cloned and revealed an N-terminal extension that was not present in the mature RbcS. It was identified as the chloroplast targeting sequence and coined “transit peptide” (Broglie et al., 1981; Coruzzi et al., 1983). Later studies demonstrated that the putative precursor of RbcS was transported into isolated chloroplasts and processed to its mature form (Highfield and Ellis, 1976; Chua and Schmidt, 1978). It has been proposed that transit peptides evolved from antimicrobial amphipathic peptides derived from host cells during endosymbiotic events, an intriguing hypothesis that is supported by experimental evidence (Caspari et al., 2023). A motif study has shown that transit peptides contain three regions, a N-terminal region lacking charged amino acids, a central one containing hydroxylated amino acids and C-terminal one containing an arginine rich motif. This domain structure may be common to most preproteins (Karlin-Neumann and Tobin, 1986; von Heijne et al., 1989; Bruce, 2001). A later study, reporting extensive mutagenesis of the RbcS transit peptide, provided clues to the existence of FP/RK and MLM motifs in the transit peptide and their vital role in chloroplast protein import (Lee et al., 2006). Site- specific cross-linking experiments with the

RbcS transit peptide, demonstrated that the FP/RK motif is important for interaction not only with components of the TOC complex, but also with the TIC20 component of the TIC complex (Richardson et al., 2018). In addition, FGLK is a transit peptide motif that has been characterized as being recurrent in transit peptides and playing an important role in the preprotein recognition by TOC34. The deletion of the FGLK sequence by mutagenesis prevented the preprotein from being translocated into the chloroplast (Chotewutmontri et al., 2012; Holbrook et al., 2016).

Based on a synthetic transit peptide, a study demonstrated that FGLK and FP/RK motifs are essential for RbcS transit peptide function and preprotein targeting of the chloroplast (Lee et al., 2015). Moderate hydrophobicity at the N-terminal region of the transit peptide is important for preprotein recognition, (Bhushan et al., 2006; Lee et al., 2006; Lee et al., 2008). Exchange of basic amino acids (N-terminal region) to acidic amino acids negatively affected preprotein import into chloroplasts (Razzak et al., 2017; Lee and Hwang, 2019). Twin-positive (positively charged amino acids) motifs in the TP appear to play a key role in preprotein import into old versus young chloroplasts (Teng et al., 2012). In addition, large scale in silico analysis and experimental evidence revealed that the twin-positive motif is important for preprotein import into leucoplasts (Chu et al., 2020).

The importance of proline residues in transit peptides has been demonstrated by comparing the import of preproteins containing proline-rich transit peptides with those lacking proline residues. The mutation of transit peptides by the replacement of prolines by alanines resulted in reduced efficiency of translocation into the chloroplast, specifically concerning transmembrane proteins and proteins prone to aggregation (Lee et al., 2018; Jeong et al., 2021). Proline is an amino acid that tends to disrupt the secondary structures of polypeptides (Guzzo, 1965). As preproteins are believed to be translocated across the TOC complex an unstructured transit peptide as described by the “perfect random coil hypothesis” may be advantageous to initiate the early stages of protein import (von Heijne and Nishikawa, 1991).

2.3.3 Energetics of translocation across at the chloroplast envelope membranes

The energy requirement of preprotein transport across the chloroplast envelopes was first analyzed in an in vitro import assay using isolated chloroplasts that were either light- or dark- adapted. The study showed that import into dark-adapted chloroplasts was compromised (Grossman et al., 1980). Exogenously added ATP rescued imports into dark- adapted chloroplasts, demonstrating that ATP was the primary energy source (Cline et al., 1985). Later studies demonstrated that import of preproteins into chloroplasts was driven by the hydrolysis of ATP inside the chloroplast (Flügge and Hinz, 1986; Pain and Blobel, 1987; Theg et al., 1989). It was then revealed that distinct concentrations of ATP in different compartments defined separate steps of chloroplast protein import. Low concentrations of ATP (50–100 μM) were sufficient for preprotein binding to the surface of the chloroplast, whereas high concentrations of ATP (1 mM or more) were required for protein translocation across the chloroplast envelope (Olsen et al., 1989). The RbcS preprotein could be chemically crosslinked to chloroplast envelope component in an ATP-dependent manner (Perry and Keegstra, 1994).

The energetics findings were exploited to generate preprotein translocation intermediates and isolate the first components of the protein import machinery from isolated pea chloroplasts (Schnell and Blobel, 1993). In these experiments, recombinant preprotein of RbcS fused to two IgG-binding domains of Staphylococcus aureus ProteinA (resulting in pS-ProtA) was used as a tool. When incubated at low ATP concentrations, pS-ProtA is stably bound to isolated chloroplasts. pS-ProtA remained sensitive to exogenous protease and the transit peptide was not cleaved. This state defines the “early translocation intermediate”. When incubated at high ATP concentrations, pS-ProtA was fully imported. However, its import could be arrested by chilling on ice. At this stage, pS-ProtA was both accessible to exogenous protease and the transit peptide partially cleaved resulting in mature S-ProtA. Thus, pS-ProtA and S-ProtA had traversed and were now spanning both the outer and inner envelope membrane. This state defines the “late translocation intermediate”. It is important to note that the formation of both the “early” and “late” translocation intermediates critically depended on the presence of the transit peptide in pS- ProtA (Schnell and Blobel, 1993). The production of “early” and “late” translocation intermediates was

upscaled from analytical to biochemical quantities allowing their isolation by IgG-affinity chromatography. The “early” translocation intermediate pS-ProtA was associated with three visible bands on a SDS-PAGE gel. These first three proteins were molecularly cloned and sequenced and are now known as TOC159, TOC75 and TOC34 (Kessler et al., 1994; Schnell et al., 1994). The three form the core of the TOC-complex as it is widely accepted today. In addition to the three core components of the TOC-complex, the “late” translocation intermediate pS-ProtA and S-ProtA associated with two more bands. One is known today as TIC110 while the second one, named IAP36 at the time, was never identified (Schnell et al., 1994). To this day, the role of TIC110 in chloroplast protein import remains contested and is notably absent from algal protein import complexes (Ramundo et al., 2020).

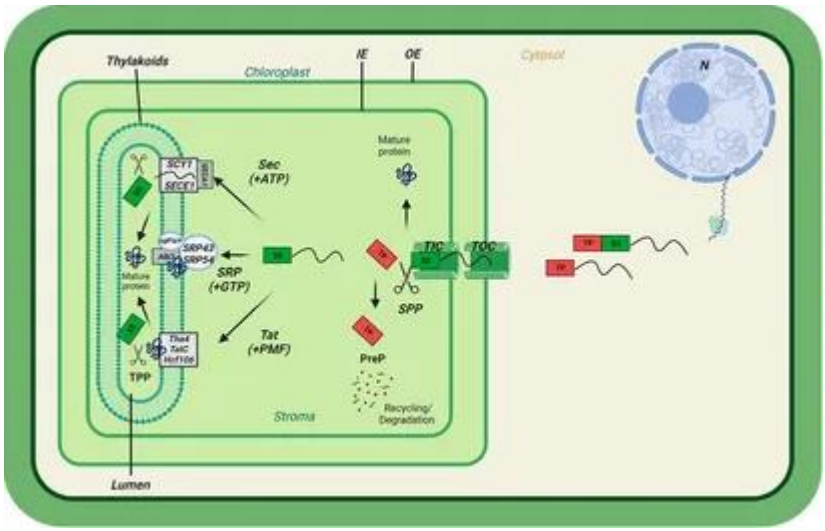

Figure 1: Preproteins translocation into chloroplast membrane systems. General scheme of chloroplast import of nuclear-encoded preproteins containing a transit peptide (TP) followed by a thylakoid Signal sequence (SS). Preprotein translocation passes through the Translocons at the Outer envelope (OE) of the Chloroplast (TOC) and the Inner envelope (IE) of the chloroplast (TIC). Upon entry, the transit peptide is cleaved by the Stromal Processing Peptidase (SPP) and processed by the PreP protease for recycling/degradation. The thylakoid-targeted proteins pass through either the Twin Arginine Transport (Tat) pathway requiring the Proton Motive Force (PMF), the Sec requiring ATP, or the Signal Recognition Particle (SRP) pathway. Upon thylakoid membrane insertion, the thylakoid signal sequence (SS) is cleaved by the Thylakoid Processing Peptidase (TPP), completing the final import step.

2.3.4 Translocon complexes at the inner and outer chloroplast membranes

The large majority of chloroplast proteins are imported via the TOC-TIC complexes (Figure 1). The first components were identified in the beginning of 1990s as a result of studies on isolated pea chloroplasts and revealed three components of the outer and one at the inner envelope membranes (namely, Import intermediate Associated Proteins or Outer Envelope Protein), IAP/ OEP34, IAP/OEP75, IAP/OEP86 and Inner Envelope Protein IAP100/IEP110 (Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994). These translocon components were renamed according to the TOC–TIC nomenclature as Toc34, Toc75, and Toc159 (for IAP/OEP86) and Tic110 (Schnell et al., 1997). The initial characterization revealed that TOC159 and TOC34 were homologous GTP- binding proteins exposed at the chloroplast surface. They were both sensitive to the addition of exogenous thermolysin protease fulfilling an important criterium for preprotein receptors at the chloroplast surface (Kessler et al., 1994). TOC75 was insensitive to exogenous thermolysin, bore homology to cyanobacterial ß-barrel solute channels related to the β- barrel assembly machinery A (BamA) family fulfilling criteria for a protein-conducting channel at the outer chloroplast membrane. TIC110 had two N-terminal alpha-helices and a large stromal domain suggesting that it may function as scaffold coordinating late translocation functions such as recruitment of chaperones for protein folding and assembly (Kessler and Blobel, 1996).

The presence of GTP-binding proteins in the TOC complex encouraged further energetics experimentation. Preprotein binding to chloroplasts does not only require low concentrations of ATP but also implicates GTP as non- and slowly-hydrolyzable GTP analogs inhibited import. These findings supported the importance of the role of TOC GTPase receptors (Olsen and Keegstra, 1992; Kessler et al., 1994). Apart from the irreversible energy- dependent interactions, the transit peptide is also reversibly bound to TOC159 and TOC75 in an energy-independent way as demonstrated by chemical cross-linking (Ma et al., 1996). In Arabidopsis as well as other species, both of the GTP-binding TOCs are encoded by multigene families and consequently several isoforms of each have been discovered. The structure of TOC34 as well as those of its homologs consists of two main features, a N- terminal GTPase domain and a single C-terminal alpha-

helical membrane-spanning domain followed by a short hydrophilic tail (Jarvis et al., 1998). TOC159 and its three homologs in Arabidopsis (atTOC120, −132, −90) possess a central GTPase (G-) domain, a C-terminal membrane-anchoring (M-) domain, and a N-terminal acidic (A-) domain at the N-terminus (Kubis et al., 2004). The M-domain has now been shown to take on a ß-barrel structure and associate with TOC75 to form a large hybrid channel at the outer chloroplast membrane (Jin et al., 2022; Liu et al., 2023). The A-domains in the four Arabidopsis isoforms of TOC159 are much more divergent than the G- and M-domains and appear to play a role in pre- protein specificity (Agne et al., 2010). It, however, is not clear how the various A-domains distinguish the transit peptides of different classes of preproteins (i.e., photosynthesis- associated versus house-keeping) (Bauer et al., 2000; Ivanova et al., 2004). TOC75 belongs to the BamA family with homologs in Gram-negative bacteria as well as mitochondria and plastids (Schleiff and Becker, 2011). Based on these similarities, TOC75 was proposed to function as the protein-conducting channel at the outer membrane of the chloroplast. TOC75 is encoded by a single orthologous gene in the genomes of all plant species sequenced so far. In addition to forming a ß-barrel channel, TOC75 has three N-terminal POTRA (polypeptide transport-associated) domains (Sánchez-Pulido et al., 2003; Srinivasan et al., 2023). The POTRA domain contributes to preprotein recognition and has chaperone- like activity to guide the incoming preprotein across the intermembrane space (Kouranov and Schnell, 1997; Paila et al., 2016; O'Neil et al., 2017).

At the inner envelope membrane, at least two models have been proposed for the TIC complex, the first consisting of the TIC20 (channel) TIC214 (plastid-encoded), TIC100, TIC56, TIC21 and TIC12 forming a 1 MDa complex (Kikuchi et al., 2013) the second consisting of TIC110 and TIC40. Currently, it is not clear whether the second complex functions together with or independently from the 1 MDa TIC complex in land plants. Cryo- EM structures of the Chlamydomonas TOC-TIC holocomplexes, however, did not contain homologs of TIC40 or TIC110 (Jin et al., 2022; Liu et al., 2023). In addition to the aforementioned components the intermembrane space component TIC236 constitutes a physical link between the TOC and TIC complexes (Chen et al., 2018). TIC22, another intermembrane space component, has been proposed to promote preprotein import across both

envelope membranes and the intermembrane space besides its function as a chaperone (Kouranov et al., 1999). As preprotein import requires ATP, the existence of ATP-dependent motors has been proposed. However, the exact nature of such stromal import motor(s) is currently contested. On the one hand biochemical and genetic information provide support for a chaperone network consisting of cpHsp70, Hsp90C, and Hsp93) consuming the ATP and energizing translocation (Su and Li, 2010; Inoue et al., 2013; Huang et al., 2016). On the other hand, an alternative stromal motor has been proposed that consists of a 2-MDa ycf2/FtsH1 complex that also has predicted ATP hydrolysis activity (Kikuchi et al., 2018). However, the respective significance of the two proposed motor systems has not been evaluated so far, and neither of the two systems were observed in the currently available Cryo- EM structures (Jin et al., 2022; Liu et al., 2023).

2.3.5 Transit peptides are cleaved by stromal processing peptidase

Upon entry into the chloroplast stroma and possibly before complete translocation, the transit peptide is cleaved by Stromal Processing Peptidase (SPP) (Figure 1) (Richter and Lamppa, 2002; Richter and Lamppa, 2003). SPP is an M16 metallopeptidase carrying out a function comparable to that of the Mitochondrial Processing Peptidase (MPP), a metalloprotease, involved in the maturation of nuclear encoded proteins imported into mitochondria (Pollock et al., 1988; Braun et al., 1992). SPP, cleaves at a semiconserved motif ((I/V)-X-(A/C)-↓-A (arrow marks cleavage site) at the C-terminus of the transit peptide (von Heijne et al., 1989). Thereby initiating the final steps of preprotein maturation.

After transit peptide cleavage, these may include folding and/or assembly in the stroma or insertion into or translocation across the thylakoid membrane. SPP is an essential component of the import mechanism as demonstrated by the aborted seed phenotype observed in the spp homozygous knockout mutants (Trösch and Jarvis, 2011). Once cleaved, transit peptides are further degraded by presequence peptidases (PrePs) (Figure 1) (Ståhl et al., 2005).

2.3.6 Thylakoid membrane targeting sequences and alternative insertion pathway

The thylakoid membrane is home to the light reactions of photosynthesis. For thylakoid biogenesis, assembly of thylakoid luminal and integral membrane proteins is essential. For a considerable number of proteins, the journey therefore

is not finished upon arrival inside the chloroplast. Cleavage of the transit peptide may expose a secondary targeting sequence that will engage one of at least two entry pathways to the thylakoids. Two routes exist for entering the thylakoid lumen: the twin-arginine translocase (TAT) that may accommodate folded proteins, or the SEC translocase for unfolded proteins (Figure 1) (Yuan et al., 1994; Mori et al., 1999). In addition, integral thylakoid membrane proteins require the signal recognition particle (SRP) pathway for alternative insertion (Figure 1) (Schünemann, 2007). Interestingly, all three pathways have been conserved from the cyanobacterial ancestor and exist in bacteria and, in the case of the SEC and SRP pathways, in animals to this day.

2.3.7 SEC translocation mechanism

The SEC pathway is well-known for its evolutionary conserved mechanism (Dalbey and Chen, 2004). In thylakoid targeting signals, the SEC-specific signal sequence has been described as containing three domains, a charged domain at the N-terminal part, a hydrophobic mid-section and C-terminal cleavage domain containing an A-x-A motif set for interaction with the thylakoid processing peptidase (TPP) (Hsu et al., 2011; Celedon and Cline, 2013). SEC1 is the SEC translocase at the thylakoid membrane (Fernandez, 2018). The SEC1 complex contains SCY1 and SECE1 thylakoid membrane protein channels associated with the stromal motor protein SECA1 (Nakai et al., 1994). Nuclear-encoded lumenal proteins are translocated in an unfolded form across the SEC translocase. The N-terminal part of the signal peptide interacts with SECA1 translocation motor and its ATPase activity provides the energy for translocation across the SCY1/SECE1 channel. Subsequently, the signal sequence is cleaved in the thylakoid lumen (Figure 1) (Albiniak et al., 2012). HSP90C may also assist the SEC1 translocation pathway in translocating thylakoid precursor proteins from the stroma to the lumen (Jiang et al., 2020). Surprisingly, a SEC2 translocase system also exists that is similar to SEC1, but SCY2 and SECE2 are inner envelope membrane protein channels using the stromal motor protein SECA2 (Skalitzky et al., 2011). The known examples of SEC2-dependent translocation of inner envelope proteins are TIC40 and FTSH12 (Li et al., 2017). However, the SEC2 translocase system is poorly understood compared to SEC1 due to a lack of studies.

2.3.8 TAT translocation mechanism

The Twin Arginine Transport (Tat) pathway is so called because the corresponding targeting sequences contain two neighbouring arginine residues (Cline et al., 1992). The TAT pathway is distinct from others in that it is able to transport fully folded protein across the thylakoid membrane and into the lumen. The TAT-specific signal sequence features are similar to those of SEC with the exception of the N-terminal part that contains the twin arginine (RR) motif. The RR motif is responsible for SEC avoidance response in thylakoid targeting (New et al., 2018). The Tat pathway is estimated to be responsible for the import of an estimated 50% of the thylakoid lumen proteins (Robinson and Bolhuis, 2004). The characteristic twin-arginine motif is essential for translocation and is disabled by mutation to other combinations of amino acids. The TAT pathway requires only the proton motive force (pmf) as energy source in order to achieve protein transport and has therefore also been called the ΔpH pathway (Mould and Robinson, 1991). Three proteins named TatC, Hcf106 and Tha4 form a complex that binds to the precursor protein's RRXFLK motif in the N-terminal part of the signal sequence in order to facilitate translocation (Figure 1). Liquid- liquid phase separation by Hcf106-ankyrin-repeat proteins (STT) interaction facilitates the TAT dependent translocation of the luminal proteins (Ouyang et al., 2020). Several models of translocation have been proposed for the plant TAT pathway. However, no proven model exists to date (New et al., 2018).

2.3.9 SRP

The chloroplast signal recognition particle (cpSRP) pathway, which is derived from prokaryotes and known as cpSRP pathway, targets and inserts abundant thylakoid membrane proteins, for example, light-harvesting chlorophyll-binding proteins (LHCPs) (Ziehe et al., 2018). Unlike SEC and TAT pathways, no conserved motif or domain is present at the N-terminal of the protein for thylakoid targeting. Several studies address LHCP recognition by the cpSRP pathway. The L18 motif (18 amino acids within the second and third transmembrane helices) of LHCP is crucial for recognition by cpSRP transit complex (Tu et al., 2000). Once nuclear-encoded LHCP is imported into the chloroplast via the TOC-TIC complex and processed by SPP, it forms the stromal transit complex together with cpSRP54 (GTPase) and cpSRP43 (Schuenemann et al., 1998). The cpSRP transit complex containing

LHCP binds to cpSRP receptor cpFtsY (GTPase) (Kogata et al., 1999; Tu et al., 1999; Nguyen et al., 2011) and docks to Alb3 (insertase at thylakoid membrane) via cpSRP43, promoting precursor/LHCP insertion into thylakoid membrane (Figure 1) (Moore et al., 2000; Bals et al., 2010). cpSRP43 has two distinct chaperone activities for i) LHCP insertion and ii) tetrapyrrole biosynthesis enzymes. The chaperone activity towards tetrapyrolle biosynthesis activity allows to coordinate LHCP insertion with chlorophyll biosynthesis and assembly into LHCP. Interestingly, cpSRP54 activates the cpSRP43 chaperone function towards LHCP insertion and inhibits the chaperone activity towards tetrapyrrole biosynthesis enzymes (Wang et al., 2018; Ji et al., 2021). However, except for LHCP, there is a lack of information about how the SRP targets are recognized by the components of the SRP pathway.

2.3.10 Thylakoid processing peptidase (TPP)

The thylakoid proteins are translocated into the thylakoid lumen by either the Sec or Tat pathways and, in a final step the N-terminal thylakoid targeting sequence is cleaved by Thylakoid Processing Peptidase (TPP) (Figure 1) (Hsu et al., 2011). TPP is a member of the membrane-bound proteases belonging to the type I signal peptidase (SPase I) family in both prokaryotes and eukaryotes. Plsp1 and Plsp2A/B are the two TPPs present in the thylakoids (Hsu et al., 2011). Plsp1 is known to be involved in the SEC and TAT dependent signal sequence cleavage and, surprisingly also, processing of TOC75 at the envelope membrane, suggesting that at least the Plsp1 protease is found not only in the thylakoid membrane but also in the envelopes. Plsp1 is essential for chloroplast biogenesis, its mutation resulting in a very pale green phenotype (Shipman and Inoue, 2009). Currently, the physiological and functional roles of Plsp2A/B in signal peptide processing are unclear.

2.4 Conclusion and future perspectives

In the last years, significant progress has been made with regard to the understanding of the molecular and mechanistic details of chloroplast import of nuclear-encoded proteins by the TOC-TIC complex. The recent cryo-EM structural studies reveal how the TOC-TIC components are arranged in detail and provide some information on the likely path of the preprotein and its transit peptide across

the chloroplast envelope. It would now be highly interesting to study the cryo-EM structure of the TOC-TIC complex in association with a preprotein and its transit peptide to gain a complete understanding of the import process. Also, the recent cryo- EM structures failed to reveal the cytosolic GTPase domains of the TOC34 and −159 (Jin et al., 2022; Liu et al., 2023) that play a central role in transit peptide recognition. The GTPase domains should remain a key target in future structural work. Recent advances in the chloroplast transit peptide field reveal that specific motifs, i.e., the proline-rich motif, have vital roles in the preprotein interaction with the TOC-TIC translocon. However, fundamental knowledge concerning the recognition and distinction of transit peptides belonging to different classes of preproteins (i.e., photosynthesis- associated vs. nonphotosynthetic housekeeping) is still lacking. In the future, the identification and investigation of particular motifs playing essential roles in tissue- and plastid-specific protein import pathways are predicted to be important research questions. Last but not least, many questions regarding second targeting sequences and their role in processing and assembly of the all-important photosystems remain open and should be addressed in the future.

2.5 Author contributions

FK and VS conceived this review article. GB, MF, FK, and VS participated in the writing of the manuscript. GB prepared the figure. All authors contributed to the article and approved the submitted version.

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2.8 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.9 Publisher’s note

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Chapter 3: ABA-dependent differential regulation of chloroplast protein import pathways during the seed-to-seedling transition

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3.1 Abstract

Chloroplast biogenesis defines the transition of non-photosynthetic proplastids to photosynthetically active chloroplasts in the embryonic cells of germinating seeds (“seed to seedling transition”). It is a key to photoautotrophic growth in plants. Seed germination is controlled by plant hormones and negatively regulated by abscisic acid (ABA). However, there is still a lack of understanding of how ABA impacts chloroplast biogenesis and its synchronization with seedling development. Chloroplast biogenesis requires the import of nuclear-encoded photosynthetic proteins through a translocon that specialises in the import of proteins associated with photosynthesis, called the photosynthesis-associated Translocon of the Outer membrane of the Chloroplasts (pTOC). Meanwhile, an alternative translocon, known as the non- photosynthesis-associated Translocon of the Outer membrane of the Chloroplasts (nTOC), preferentially imports other proteins. Transcriptome-wide RNA-seq analysis indicates the downregulation of photosynthesis-related GO terms and the upregulation of stress-related non- photosynthesis-associated GO-terms in response to ABA treatment. Proteome-wide analysis revealed that most proteins exhibited similar regulation as the corresponding mRNA. Under ABA, pTOC components were downregulated at the

protein level, at least in part due to CHLORAD (chloroplast-associated protein degradation), whereas nTOC components were upregulated. Concomitantly, the import of a number of non-photosynthesis-associated proteins via nTOC is increased. The results indicate specific remodeling of the plastid proteome at the level of photosynthesis-associated proteins and the protein import machinery, which represses chloroplast biogenesis under ABA. At the same time, stress-related non-photosynthesis- associated proteins were upregulated. Our data provide new insight into ABA-dependent synchronization of chloroplast biogenesis with plant development.

Keywords: Chloroplast biogenesis, TOC, TIC, ABA, seed-to-seedling.

3.2 Introduction

Photosynthesis, the nutritional foundation of life on Earth, occurs within the chloroplasts of plant cells. Chloroplast biogenesis is a crucial process for assembling the active photosynthetic apparatus (Jarvis and López-Juez, 2013). A critical part of the biogenesis of chloroplasts from undifferentiated proplastids is the import of hundreds of nuclear-encoded proteins from the cytosol (Schnell, 2019). Many of these are required components of the photosynthetic apparatus. These are encoded in the nucleus and synthesized in the cytoplasm as preproteins, with a targeting sequence (transit peptide, or TP) at their N-terminus. Preproteins are imported through the action of two multi-subunit translocon complexes at the outer membrane (TOC) and inner membrane (TIC) (Ballabani et al., 2023). Earlier research has discovered multiple TOC preprotein receptors, which are believed to possess distinct specificities for various preprotein classes (Bauer et al., 2000; Bischof et al., 2011; Kubis et al., 2004). However, the molecular basis and even the nature of the respective import substrates are not entirely understood. The core of the TOC complex is trimeric and composed of the import receptor GTPases TOC33 and TOC159, which preferentially recognize and facilitate the import of photosynthesis-associated pre-proteins (Paila et al., 2015; Chu and Lee, 2018). The third component, TOC75, was identified as a channel protein and has recently been shown to form a hybrid beta-barrel channel with the C-terminus of TOC159 (Jin et al., 2022; Liu et al., 2023). Additional homologs of TOC159 (TOC 120,

and -132) and TOC33 (TOC34), exist and, together with TOC75, may form alternative trimeric complexes that preferentially enable the import of non-photosynthesis-associated proteins, predominantly present in non-photosynthetic tissues and dark-grown seedlings (Kubis et al., 2004; Ivanova et al., 2004). The switch between one type of trimeric complex and the other is not entirely understood. Still, the cytosolic ubiquitin-proteasome system (UPS) is involved in several different ways: The UPS contributes to the regulation and remodeling of the chloroplast protein import machinery via CHLORAD (chloroplast-associated protein degradation pathway) (Sun and Jarvis, 2023). UPS also eliminates unimported pre-proteins from the cytosol (Lee et al., 2009; Grimmer et al., 2020). It has been shown that the SUPPRESSOR OF PPI1 LOCUS1 (SP1), a RING-type E3 ligase at the outer chloroplast membrane, has a crucial role in mediating TOC components and chloroplast proteome remodeling throughout plant development (Ling et al., 2012). SP1 directly interacts with and ubiquitinates TOC proteins for destruction by the proteasome (Ling et al., 2012). *sp1* mutant plants are hypersensitive to salt, osmotic, and oxidative stresses. This has been explained by SP1 targeting the TOC apparatus and depleting it under stress conditions. This limits the import of photosynthesis-associated proteins responsible for ROS production in the wild type, thereby protecting the plant from photooxidative stress (Ling and Jarvis, 2015).

Chloroplast biogenesis and seed germination are intricately connected processes essential for successful photoautotrophic growth. During the seed-to-seedling transition, proplastids within the embryo differentiate into chloroplasts, enabling photosynthesis (Liang et al., 2018; Shanmugabalaji et al., 2018). The phytohormones abscisic acid (ABA) and gibberellin (GA) antagonistically regulate seed germination. In previous work, we demonstrated that GA controls chloroplast biogenesis during seed-to-seedling transition via DELLA-mediated degradation of TOC159 by the ubiquitin-proteasome system (Shanmugabalaji et al., 2018; Accossato et al., 2020). ABA is a key regulator that suppresses seed germination and implicates master regulator transcription factors ABI4 and-5 (Sano and Marion-Poll, 2021).

Plastoglobules (PGs) are lipid droplet-like compartments in the chloroplast and are associated with stress- and senescence-related pathways (van Wijk and Kessler, 2017; Shanmugabalaji et al., 2022). ABA plays a role in regulating PG by controlling their size and number, and it also affects the expression and accumulation of PG

proteins (Gillet et al., 1998). It has been demonstrated that the PG protein FBN1a was upregulated at both the RNA and protein levels in response to ABA treatment (Yang et al., 2006). Furthermore, it has been shown that long-term ABA treatment impairs chloroplast metabolism and increases chlorophyll degradation, leading to leaf yellowing (Wang et al., 2018). Based on previous studies that showed chloroplast biogenesis starts around 36 hours after imbibition (Liang et al., 2018; Shanmugabalaji et al., 2018), we performed genome-wide RNA-seq analysis and proteome-wide analysis to examine the effects of ABA on the seed-to-seedling stage, focusing on components of the TOC complex and their substrates. We provide new insights into how ABA-induced remodeling of the plastid proteome and transcriptome influences chloroplast development. Additionally, it gives biochemical evidence on how ABA regulates the TOC translocons during the transition from seed-to-seedling.

3.3 Results

3.3.1 Transcriptome-wide RNAseq analysis reveals down-regulation of photosynthesis- associated gene expression

We carried out a transcriptome-wide RNA-Seq analysis of wild type Arabidopsis Col-0 (WT) germinating seeds 36 hours after imbibition to investigate changes in transcripts in response to ABA. At this time point, no visible differences between the control (-ABA) and ABA-treated (+ABA (0.5µM)) seedlings (Figure 1A) can be observed. Principal component analysis (PCA) of the RNAseq data showed that the biological replicates within each treatment group clustered tightly together, demonstrating high reproducibility (Figure 1B). The analysis clearly distinguished between the control and ABA-treated samples, reflecting distinct transcriptional profiles. 17,867 mRNA species were detected in the genome-wide RNA sequencing analysis. 9,474 (53%) of transcripts were found to be regulated by ABA. Up- and down-regulation were determined solely by the sign of the fold change, with positive values indicating up-regulation and negative values indicating down-regulation; no fold-change threshold was applied. Statistical significance was defined using a 5% false discovery rate (adjusted p-value ≤ 0.05). Based on this criteria, 4,435 transcripts were found to be upregulated and 5,039 transcripts downregulated in a statistically significant manner. Among these, 554 nuclear-encoded transcripts of chloroplast proteins were significantly upregulated, while 733 were significantly downregulated. The differential expression analysis is presented in the form of a volcano plot, with nuclear-encoded transcripts of chloroplast proteins

highlighted in green (Figure 1C). Gene ontology analysis (GOCC, cellular compartments; KEGG functional categories) revealed relatively minor changes in the general “chloroplast” term, comparable to those observed for the “mitochondria” and “peroxisomes” terms (Figure 1D). However, GO terms related to photosynthesis-associated pathways, namely photosystems I and -II, Calvin-Benson-Bassham (CBB) cycle, and chlorophyll biosynthetic pathway, exhibited strong downregulation indicating specific repression by ABA treatment (Figure 1E). In addition, genes considered master regulators of chloroplast biogenesis, namely GLK2, MYBS1, HY5, CGA1, and GNC, were downregulated in response to ABA treatment (Figure S1). The GO-terms, PG (stress-related), abscisic acid biosynthesis, chlorophyll catabolism, and starch metabolism were found to be upregulated by ABA (Figure 1E). We further analyzed the significantly up- and downregulated nuclear-encoded transcripts of chloroplast proteins using the STRING database to identify functional association networks that responded similarly to ABA treatment, which corroborates our GO term results (Figure S2-3).

3.3.2 Proteome-wide analysis revealed a decreased accumulation of photosynthesis- associated proteins under ABA

We further performed a proteome-wide analysis on the same plant material used for transcriptome-wide RNA-Seq analysis. PCA showed a clear distinction between control and ABA-treated samples. This distinct clustering indicated substantial differences in the proteome in response to ABA (Figure 2A). A total of 5,888 proteins were identified and 783 proteins were assigned to the chloroplast based on PPDB (http://ppdb.tc.cornell.edu/). Overall, GO analysis showed a moderate down-regulation of the general “chloroplast” term, similar to what was observed for “mitochondria” and “peroxisomes” which was in good agreement with the RNAseq data (Figure 1C). 480 proteins were classified as significantly differentially regulated with p-value <0.05. Volcano plotting highlights this, showing protein abundances that were significantly altered. Amongst these, 33 chloroplast proteins (highlighted in purple) were downregulated, whereas 8 were upregulated under ABA (Figure 2B). In line with the transcriptomic data, GO terms related to photosynthesis, the Calvin–Benson–Bassham (CBB) cycle and the chlorophyll biosynthetic pathway were found to be far more strongly downregulated than the general “chloroplast” term. By contrast, GO terms, related to plastoglobule-associated proteins and the abscisic acid biosynthesis pathway were upregulated under ABA (Figure 2D).

3.3.3 ABA regulates levels of pTOC and nTOC components at the protein level

We further studied the correlation between transcript and protein abundance for specific pathways and GO terms related to chloroplast biogenesis, namely photosynthesis, tetrapyrrole biosynthesis, lipid biosynthesis, and TOC-TIC translocon components (Liang et al., 2018; Pipitone et al., 2021). To these, PG-associated proteins were added as a chloroplast biogenesis- independent term, an example of a GO term upregulated at both the RNA and protein levels. It is known that TOC components can be regulated posttranslationally by UPS and CHLORAD (Kessler, 2012). A dual-axis chart method was used to determine whether the pTOC and nTOC pathways are regulated differently at the levels of RNA abundance and protein accumulation. To do so, we selected specific proteins and analyzed their accumulation patterns under ABA. In addition to p- and nTOC components, this selection included photosynthesis-associated protein components within the thylakoid membrane, namely cytochrome b6f complex (PetC), and photosystem I (PsaD-1, PsaE-1, PSAL, Lhca) and II (PsbO, PSBQ, PsbQ-2, PSB27, Lhcb) as well as ATP synthase (AtpF, AtpC1, AtpD). These photosynthesis-associated components (green-filled circles) were downregulated both at the level of transcript and protein (Figure 3).

The same observation was made for components of the tetrapyrrole pathway, including those involved in chlorophyll biosynthesis, as well as for components of the chloroplast lipid biosynthetic pathway (purple-filled circles) (Figure 3A). By contrast, PG proteins, including FBNs, ABC1Ks, and VTE1, showed increased accumulation (blue-filled circles) and upregulation both at the levels of transcript and protein (Figure 3).

3.3.4 ABA post-transcriptionally remodels the p- and nTOC composition

For further analysis, we selected components of the p- and nTOC and visualized their mRNA and protein accumulation patterns under ABA treatment in a heatmap (Figure S4). This analysis showed that TOC33 and TOC159 displayed upregulated mRNA and downregulated protein levels (Figure 3 and S4). By comparison, the nTOC components TOC132 and TOC120 behaved differently: Although TOC132 was found to be upregulated in the transcriptome-wide RNA-seq analysis, no significant changes were observed at the protein level in the proteome-wide analysis. In the case of TOC120, transcriptome-wide RNAseq analysis revealed no change in transcript level under ABA treatment; however, the protein could not be detected in the proteome-wide analysis

(Figure S4).

We validated these results by semi-quantitative Western blot analysis and investigated the impact of ABA 72 hours after imbibition. Extracts were prepared from seedlings that had been germinated on increasing concentrations of ABA (0.05 to 5 µM) and probed for pTOC and nTOC components using specific antibodies in a Western blot. Above 1 µM ABA seed germination was completely repressed as expected (Figure 4). Starting at an ABA concentration of 0.5µM, the pTOC components TOC159, TOC75, and TOC33 decreased concentration- dependently up to 5µM (Figures 5A and B). By contrast, nTOC components TOC120 and TOC132 increased concentration-dependently up to 5 µM (Figures 5A and C). Next, to investigate the effect of ABA on the accumulation of TOC mRNA, we quantified transcript levels under increasing concentrations of ABA (0, 0.2, 0.5, and 2 µM) using qRT-PCR. Confirming the RNAseq results, TOC33, TOC75, TOC159, and TOC132 showed increased transcript levels in response to ABA (Figure 5D).

3.3.5 Chloroplast outer membrane E3 ubiquitin ligase SP1 contributes to the control of levels of pTOC components in the presence of ABA

It is known that pTOC components are regulated by CHLORAD, together with the ubiquitin- proteasome system (UPS), under stress conditions. First, we addressed the question of whether pTOC regulation under ABA is UPS-dependent. A semi-quantitative Western blot analysis showed that the levels of TOC159 and TOC75 were 20% higher in ABA-treated seedlings incubated with MG132, a proteasome inhibitor while TOC33 was unchanged. This indicates that the UPS contribute to the control of TOC159 and TOC75 but not TOC33 levels (Figures 6A and B). Next, we investigated whether the chloroplast outer membrane E3 ligase SP1 was involved in TOC ubiquitination and degradation by the UPS during the seed-to-seedling stage under ABA. Therefore, we compared TOC protein levels under ABA in the *sp1* mutant to WT by semi- quantitative Western blot (Figure 6C). This experiment revealed that TOC159, TOC75, and TOC33 protein levels increase 10 to 20% in *sp1* compared to WT under ABA (Figure 6D). In support of role for CHLORAD in response to ABA treatment, transcription of the SP1, SP2, and CDC48 components was upregulated (Figure S5).

3.3.6 TOC33 and TOC159 mRNA translation under ABA are not affected by their untranslated regions

Messenger RNAs comprise a protein-coding region and 5´ and 3´ untranslated regions (UTRs). Post-transcriptional regulation by 3´untranslated regions (3′ UTRs) is a well-known mechanism of protein translation regulation (Schwerk and Savan, 2015). To investigate the effects of 3′UTRs on protein accumulation, we used transgenic lines that expressed the TOC33 CDS (NTAP- TOC33) and the TOC159 CDS(NTAP-TOC159). Both CDSs were N-terminally fused to the TAP-tag and expressed under the control of the 35S promoter and nopaline synthase terminator in the respective mutant backgrounds (*ppi1* and *ppi2*). Compared with WT, the semi-quantitative Western blotting analysis of transgenic lines revealed a reduction of TOC protein levels under 0.5 and 2 µM ABA in transgenic lines similar to that in WT (Figure 7). While protein levels of TOC33 and TOC159 were assessed, mRNA quantification for these constructs carrying a non-native 3′ UTR and promoter was not conducted.

3.3.7 ABA differentially regulates substrates of pTOC and nTOC at the seed-to-seedling stage

We wanted to validate the observations made by RNAseq and proteome analyses regarding the p- and nTOC substrates. First, we examined the accumulation of photosynthesis-associated pTOC substrates (PSII components psbA, psbO, LHCB2 and PSI components psaH and psaD) in plants germinated for 72 hours in the presence of increasing ABA concentrations (0, 0.05, 0.1, 0.2 and 0.5 µM) using semi-quantitative Western blotting. The rising concentrations of ABA progressively reduced the accumulation of the photosynthesis-associated proteins up to 60-80% (Figures 8A and B). We then examined the effect of ABA on non-photosynthesis-associated proteins using the plastoglobule-associated fibrillin proteins (FBN1A, FBN2, and FBN4) as models. The FBN1A protein level remained constant under 0-0.5µM ABA, while a slight reduction of FBN2 and FBN4 protein levels was observed at 0.5µM ABA (Figures 8C and D). qRT-PCR analysis of LHCB2, PsaH, PsbO, and FBN1A gene expression revealed that the photosynthesis-associated genes were downregulated at the mRNA level, while FBN1A was upregulated under ABA treatment (Figure 8E). In addition, we have compared the upregulated, as well as non-regulated chloroplast proteins under ABA with previously known TOC159- independent import substrates (Bischof et al., 2011). Nearly 85% of the TOC159-independent import substrates matched with up-regulated, non-regulated chloroplast proteins under ABA (Figure

S6).

3.4 Discussion

The transition from proplastids, undifferentiated plastids in plant embryos, to chloroplasts during seed-to-seedling development is critical for establishing plant autotrophic growth (Pogson et al., 2015). The proper assembly of the photosynthetic apparatus during seed-to-seedling development is a key step in chloroplast biogenesis, and it relies on the chloroplast protein import machinery and the pTOC component TOC159 specifically (Bauer et al., 2000). However, the question of how ABA, which suppresses the seed-to-seedling transition and impairs greening, influences chloroplast biogenesis at the mechanistic level remains largely unexplored. Here, we provide insights into how ABA mediates the arrest of chloroplast biogenesis at the seed-to- seedling transition.

Transcriptome-wide RNA (RNA-seq) analyses indicated that around half of all Arabidopsis genes were differentially expressed under ABA (Figure 1C). The GO-analysis revealed that the general terms “chloroplast”, “mitochondria” and “peroxisomes” were slightly down-regulated overall (Figure 1D). This observation suggests that a small subset of components within the general GO-term chloroplast may contribute disproportionately to chloroplast biogenesis.

We therefore focused on a detailed analysis of the chloroplast term due to identify specific components important for establishing photoautotrophy during the seed-to-seedling stage. This analysis revealed that the more specific GO terms photosynthetic apparatus, Calvin cycle, and chlorophyll biosynthetic pathway were far more strongly suppressed than the general chloroplast term (Figure 1E). Earlier studies have shown that ABA reduces photosynthetic capacity and inhibits the activity of ribulose 1,5-bisphosphate carboxylase (Seemann and Sharkey, 1987). In particular, during the seed-to-seedling stage, ABA represses LHCB and RBCS gene expression by binding of ABI4 to their promoters (Koussevitzky et al., 2007; Hernández et al., 2005). Therefore, our findings are in good agreement with the known negative effects of ABA on the expression of genes linked to chloroplast biogenesis. However, we were surprised by the narrow scope of the downregulated pathways and their close association with photosynthesis whereas the general chloroplast terms were far less affected.

Little is known about the ABA-dependent upregulation of nuclear genes encoding chloroplast- localized proteins. Our GO analysis revealed that the terms PG, ABA biosynthesis, chlorophyll catabolism, and starch metabolism pathways were enriched (Figure 1E). ABA biosynthesis pathway gene upregulation is a well-known response to exogenous ABA (Barrero et al., 2006). ABI5 directly binds to the promoter regions of chlorophyll catabolism genes *SGR1* and *NYC1*, activating their expression (Skubacz et al., 2016). Thus, our findings suggest that ABA, amongst other effects, negatively regulates photosynthesis by promoting chlorophyll degradation.

The proteome-wide analysis showed that only 10% of all proteins differentially accumulated under ABA (Figure 2B). As in the transcriptome-wide RNA analysis, the proteome-wide protein analysis revealed that the general GO term “chloroplast” was only mildly affected and only around 5% of all chloroplast proteins were downregulated. Again, downregulation mainly affected GO terms that included photosynthesis, the Calvin cycle, and the chlorophyll biosynthetic pathway (Figure 2D). This correlation between gene expression and protein accumulation provides compelling evidence that ABA actively inhibits chloroplast biogenesis by decreasing the expression of a remarkably small number of genes and the accumulation of key photosynthesis-associated proteins.

Chloroplast PGs play multiple roles in protecting against oxidative stress (Arzac et al., 2022; Bréhélin et al., 2018). In contrast to the suppression of the photosynthetic apparatus in this study, we observed robust upregulation of PG proteins known to be associated with the alleviation of stress, including FBNs, ABC1Ks, and VTE1, in response to ABA treatment (Figure 2D). This is in line with the well-established role of ABA in plant adaptation to environmental stress (Rai et al., 2024; Parwez et al., 2022; Zhang et al., 2006). In the case of PG-associated proteins, this suggests that ABA actively induces their expression to facilitate stress adaptation by increasing the biosynthesis of the lipid antioxidants tocopherol and plastoquinone, associated with PG. We focused on additional pathways that arose from our analysis and corresponded to those in Pipitone et al. (2021) that had characterized their regulation during chloroplast biogenesis. These were tetrapyrrole biosynthesis, lipid biosynthesis and the TOC-TIC translocons. Our results show that ABA significantly downregulates the tetrapyrrole biosynthesis pathway, particularly chlorophyll biosynthesis at both the transcript and protein levels. This may reflect the role of ABA in

adjusting metabolism under stress: Limiting chlorophyll production at the same as that of chlorophyll-binding proteins could potentially reduce oxidative damage. The downregulation of lipid biosynthesis suggests a delay in thylakoid membrane assembly in the absence of photosynthesis-associated proteins and chlorophyll. Except for the TOC-TIC translocons, a strong correlation existed between transcript levels and protein abundance under ABA (Figure 3).

Photosynthesis-associated preproteins are imported through the TOC159/TOC33 receptors, also known as pTOC. Non-photosynthesis-associated preproteins are thought to be substrates of the TOC132/120/TOC34 receptors (nTOC) and to be imported independently of TOC159 (Ivanova et al., 2004; Inoue et al., 2010; Bischof et al., 2011). The components of the pTOC exhibited an inverse relationship between transcript and protein abundances under ABA i.e. increased transcript versus reduced protein. This suggested control of protein levels by post- transcriptional regulatory mechanisms (Figure 3B). By contrast, TOC132, an isoform of TOC159 and component of nTOC, exhibited both higher transcript and higher protein level under ABA (Figures 5A-C and Figure S4). Together with observed upregulation of stress-related GOs the findings suggest that the import of non-photosynthetic /stress-associated proteins under ABA conditions remained active or was even increased whereas the import of photosynthesis- associated proteins was strongly downregulated.

Studies have shown that TOC33 and TOC75 are targets of SP1 E3 ligase during the seed-to-seedling transition under low levels of GA (Shanmugabalaji et al., 2018). However, in that study, the degradation of TOC159 was not CHLORAD-dependent and involved an unknown E3 ligase (Shanmugabalaji et al., 2018). TOC159, TOC75 and TOC33 levels were increased, albeit slightly, under ABA in the *sp1* mutant suggesting a contribution of SP1 E3 ligase (Figure 6).

To investigate other potential mechanisms of post-transcriptional regulation, we addressed the effects of 5’- and 3’-untranslated regions (UTRs) of TOC33 and TOC159 on protein accumulation. Levels of these pTOC components in transgenic lines expressing transgenic TOC33 and TOC159 without the endogenous UTRs, resembled those of the wild type under ABA (Figure 7). These findings suggest that the endogenous 5' and 3' UTRs of TOC33 and TOC159 mRNA do not affect their translation in response to ABA.

The two types of TOC complexes, pTOC and nTOC, are thought to exhibit distinct substrate preferences during chloroplast development. It has been shown that TOC159 preferentially recognizes transit peptides of photosynthesis-associated proteins, whereas TOC132 has greater affinity for non- photosynthesis-associated proteins (Kubis et al., 2004; Chu and Li, 2018; Chu et al., 2010). In our study, we observed that under ABA treatment, pTOC components TOC33 and TOC159 were downregulated in a posttranscriptional fashion which correlated with a decrease in photosynthesis-associated proteins (Figures 8A and B). In contrast, nTOC components, TOC132 and TOC120, were upregulated, accompanied by an increase in non-photosynthesis-associated proteins, such as FBNs (Figure 8C and D). These results indicate that FBNs as well as other upregulated or unaltered non-photosynthesis-associated proteins (a total of at least 258 proteins) are potential substrates of the nTOC pathway (Figure S6).

In summary, the data in this manuscript indicate that ABA specifically inhibits the pTOC protein import pathway and hence delays chloroplast biogenesis at the seed-to-seedling transition. Simultaneously other essential plastid functions are maintained or even upregulated by the nTOC pathway. This mechanism may enable the independent fine-tuning of plastid functions and photosynthetic activity thereby minimizing harmful stress responses such as reactive oxygen production in response to developmental and environmental cues eliciting ABA- production (Figure 9).

3.5 Materials and methods

3.5.1 Materials and Reagents

Reagent	Source	Identifier
Antibodies		
TOC159	Bauer et al., 2000	N/A
TOC75	Hiltbrunner et al., 2001	N/A
TOC33	Rahim et al., 2009	N/A
TOC120	Kasmati et al., 2011	N/A
TOC132	Ling et al., 2012	N/A
TIC56	Köhler et al., 2015	
PsaD	Agrisera	
PsaH	Agrisera	
PsbO	Agrisera	AS142824; RRID: AB_1031788
LHCB2	Agrisera	AS01003; RRID: AB1832080
FBN1A	Vidi et al., 2006	N/A
FBN2	Shanmugabalaji et al., 2020	
FBN4	Shanmugabalaji et al., 2013	
anti-actin	Sigma	
anti-UGPase	Agrisera	AS05 086; RRID: AB_1031827
Chemicals, Peptides, and Recombinant Proteins		
Abscisic acid	Sigma	A1049
MG132	AbMole	M1902
Protease inhibitors	Sigma	P9599
Murashige and Skoog Basal Medium	Duchefa Biochemie	M022
Triton X-100	Roche	10789704001
Critical Commercial Assays		
RQ1 RNase-Free DNase	Qiagen	
Power SYBR Green PCR master mix	Applied Biosystems	4367659
oligo(dT)15 primer	Promega	C1101
Improm II reverse transcriptase	Promega	A3800
ECL Plus western blotting substrate	Pierce	32132
Experimental Models: Organisms/Strains		
sp1		
NTAP-TOC33/ppi1	Rahim, 2008	
NTAP-TOC159GM/ppi2	Agne et al., 2010	

Gene	Oligo Name	Sequence (5'-3')
Act2 (AT3G18780)	ACT2 F	CTTGCACCAAGCAGCATGAA
	ACT2 R	CCGATCCAGACACTGTACTTCCTT
Toc159 (AT4G02510)	RT Toc159 FW	CAC AGT CTT GCT CTA GCT AG
	RT Toc 159 Rev	CTC CTC TGA CCA CAT ATG CC
Toc75 III (AT3G01015)	RT TOC75 F	CCG AGA TAC AGA GAC TTC C
	RT TOC75 R	CAT TTC CAT CTC CAC CAC CAC
Toc33 (AT1G02280)	Toc33-F-qPCR	GGAAAATTGAAGCAAAAGGA
	Toc33-R-qPCR	AGCTTGGAAAGGACTGACAC
TOC132 (AT2G16640)	qPCR-TOC132-F	AAC TTG CTA TGG TTG CGA TT
	qPCR-TOC132-R	TGC TAC CAG ACA GAT CCT TT
FBN1a (At4g04020)	PG35_q_F	CAAACCATTGATTCCGATAG
	PG35_q_R	AGTCCCTATAACACCTTGCT
PsbO (AT5G66570)	PsbO-qPCR_F	CCA GTC TGA CTT TAA GGA CT
	PsbO-qPCR_R	TTC CAT GTA TGT CTT GCT CT
PsaH (AT3G16140)	PsaH.F	ACACAAAATTCCCACTCACC
	PsaH.R	CACACCATTAGCCCGGATG
LHCB2 (At3g27690)	LHCB2.4 fw	GCCATCCAACGATCTCCTC
	LHCB2.4 rev	TGGTCCGTACCAGATGCTC

3.5.2 Plant materials and growth conditions

Seeds of wild type Arabidopsis Col-0 (WT) were surface-sterilized with 70% ethanol containing 0.05% v/v Triton, and then washed with absolute ethanol. The sp1-3 mutant employed in this research was of the Col-0 ecotype and has been characterized in earlier study (Ling et al., 2012). The transgenic line NTAP-TOC159GM/ppi2, in the Ws (Wassileswskija) ecotype, were previously described (Agne et al., 2009), whereas the NTAP-TOC33/ppi1(Rahim, 2008) transgenic line, which have not yet been published, were also utilized in this study. Seeds were sown on agar plates containing 0.5 Murashige and Skoog salt mixture (MS, Duchefa) without sucrose as a negative control, plus different concentrations of ABA: 0, 0.01, 0.2, 0.5, 1, 2, 5 µABA. Without stratification, seeds were exposed to 16 h of light with a photoperiod of 40 µmol.m- 2s-1 and 8 hours in darkness under the temperature regime of 21°C for 36 and 72 hours, respectively. For proteasome inhibition assays, 72-hour-old seedlings were treated

with 100 µM MG132 in liquid MS medium via vacuum infiltration for 20 minutes. Following infiltration, the seedlings were incubated in liquid MS medium for an additional 12 hours. Control treatments were performed using equivalent volumes of the solvents used for ABA and MG132 (ethanol and DMSO) respectively.

3.5.3 Protein precipitation and immunoblotting

Equal fresh weight samples from each plant were frozen in liquid nitrogen, and proteins were extracted in a buffer containing 100 mM Tris pH 8, 2% b-mercaptoethanol, 4% SDS, 20% glycerol. The proteins were precipitated with acetone. Samples of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were further stained with amido black. The membranes were probed with the following primary antibodies: TOC159 (Bauer et al., 2000), TOC75 (Hiltbrunner et al., 2001), TOC33 (Rahim et al., 2009), TOC132 (Ling et al., 2012), TOC120 (Kasmati et al., 2011), TIC56 (Köhler et al., 2015), FBN1A (Vidi et al., 2006), FBN2 (Shanmugabalaji et al., 2020) and FBN4 (Shanmugabalaji et al., 2013). Photosynthesis- related protein-detecting antibodies included PsaD, PsaH, PsbA, PsbO and LHCB2 from Agrisera, Sweden. Other antibodies against affinity tags or non-photosynthetic housekeeping proteins used included IgG (Cell Signaling Technology) and UGPase (Agrisera). Secondary antibodies used were anti-rabbit IgG conjugated to horseradish peroxidase from Millipore and anti-mouse IgG conjugated to horseradish peroxidase from Sigma. Chemiluminescence detection was done using ECL Plus Western Blotting Detection Reagents from Pierce, and it was visualized on a GE Amersham Imager 600. Band intensities were quantitated using ImageQuant TL software from GE Healthcare. Statistical comparisons between two groups (without and with ABA) were performed using Student's t-tests (two-sided, paired) in Microsoft Excel, based on data from three biological replicates. Significance thresholds are indicated, with asterisks denoting levels of significance ($p < 0.05$; $p < 0.01$; $p < 0.001$).

3.5.4 RNA extraction and gene expression analysis

Total RNA was extracted from the treated seedlings, using the Sigma kit, and were treated with RNase-Free DNase (Qiagen) and reverse-transcribed using Improm II reverse transcriptase (Promega) and oligo(dT)15 primer (Promega) according to the manufacturer's recommendations. cDNA was synthesized from 1 µg of total RNA using the Promega kit following the manufacturer's instructions and samples were stored at -20ºC until used. qPCR was performed with FastStart Essential DNA Green Master kit, using specific primers for TOC33 TOC75 TOC159, TOC132, LHCB2, PsaH, PsbO and FBN1A (Table S1), the actin used as a reference gene to normalize gene expression (Table S1) For each 10 µl reaction, 2 µl of the samples' cDNA was mixed with 5 µl of FastStart SYBR Green Master (ROX; Hoffmann-La Roche, Basel, Switzerland), 0.25 µl of each primer (final concentration 500 nM), and sterile water up to the final volume. Samples were subjected to thermal cycling conditions of 95ºC for 10 min, followed by 40 cycles of 10 sec at 95ºC for annealing and 30 sec at 60ºC for extension. The melting curve was designed to increase from 55ºC to 95ºC to calculate the melting temperatures for each PCR product. Amplification of cDNA serial dilutions was performed to estimate primer efficiencies (E), using the formula E = (10–1/regression line slope − 1) × 100 (Table 1). Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction (CT; Pfaffl, 2001). Relative transcript level was calculated by using the comparative ΔCt method and normalized to the PP2A (At1g69960) gene transcript levels.

3.5.5 RNA-seq pre-processing and mapping

Five independent biological replicates were produced for RNA sequencing. For each sample, total RNA was extracted from whole 36-hour-old treated seedling using the RNA Clean & Concentrator Kits (Zymo Research®, California, U.S.A.). RNA-seq libraries were constructed with 500ng of total RNA by the POPS platform (IPS2) using the QuantSeq 3'mRNA-Seq V2 Library Prep Kit FWD with Unique Dual Indices (12nt) (LeXogen®, for illumina) with UMI according to the supplier's instructions. The libraries were sequenced in single-end (SE) mode with 68 bases for each read on a NextSeq500 (Illumina, California, U.S.A.) to generate between 7.93 and 14.02 million of reads per sample. UMIs were removed and appended to the read identifier with the extract command of UMI-tools (v1.0.1, Smith et al., 2017). Reads with UMI base quality below 10 were removed. To remove adapter sequences, poly(A), poly(G) sequences,

and low-quality nucleotides, raw reads were trimmed with BBduk from the BBmap suite (v38.84, Bushnell and Brian, 2014) with the options k=13 ktrim=r useshortkmers=t mink=5 qtrim=r trimq=10 minlength=30. Trimmed reads were then mapped and counted using STAR (v2.7.3a, Dobin et al 2013), with the following parameters --alignIntronMin 5 --alignIntronMax 60000 --alignMatesGapMax 6000 –alignEndsType Local--outFilterMultimapNmax 20 -- outFilterMultimapScoreRange 0 –outSAMprimaryFlag AllBestScore –mismatchNoverLmax 0,6 on the Arabidopsis thaliana Col-0 reference genome (TAIR10+ARAPORT11+MtCol0). Reads with identical mapping coordinates and UMI sequences were collapsed to remove PCR duplicates using the dedup command of UMI-tools with the default directional method parameter. RSeQC (v2.6.6, Wang et al., 2012) was used to evaluate deduplicated mapped reads distribution. Deduplicated reads were counted using HTSeq version v0.12.4. (Anders et al., 2015) (htseq-count mode intersection-nonempty) based on the A. thaliana TAIR10- ARAPORT11 annotation.

3.5.6 Statistical Analyses of Expression Data

Statistical analyses were conducted on R v3.6.2 (R Core Team, 2020) using the R script-based tool DiCoExpress (Lambert et al., 2020; Baudry et al., 2022) based on the Bioconductor package edgeR (v 3.28.0, (Robinson et al., 2010; McCarthy et al., 2012).

3.5.7 Gene Filtering and Normalization

For each analysis, low counts were filtered using the “filterByExpr” function where the group argument specifies the biological conditions, the min.count value is set to 5 and the min.total.count, large.n and min.prop arguments are set to their default values. Libraries were normalized with the Trimmed Mean of M-values (TMM) method using the function “calcNormFactors” with the default parameter values.

3.5.8 Differential Expression Analysis

The differential analysis was based on a negative binomial generalized linear model. The logarithm of the average gene expression is an additive function of a treatment effect (ABA or no ABA) and a replicate effect (4 modalities). A likelihood ratio test was applied, and raw p-values were adjusted with the Benjamini–Hochberg procedure to control the false discovery rate. The distribution of the resulting p-values followed the quality criterion described by Rigaill et al. (2018). Up- and down-regulation were

determined solely by the sign of the fold change, with positive values indicating up-regulation and negative values indicating down-regulation; no fold-change threshold was applied. Statistical significance was defined using a 5% false discovery rate (adjusted p-value ≤ 0.05).

3.5.9 Annotation enrichment

Enriched annotations within up- or downregulated genes were identified with a hypergeometric test using the genes considered for the differential expression analysis as the reference gene set. The annotation terms associated with GO (Berardini et al., 2004), Kegg ortholog, and Mercator4 (v6, Bolger et al., 2021) were tested. A term was declared differentially enriched if its p-value adjusted with the Benjamini–Hochberg procedure was lower than 0.01.

Hierarchical clustering: A hierarchical tree was made using the ‘hclust’ function from the R package, ‘stats’ (R Core Team, 2013).

3.5.10 Liquid chromatography-mass spectrometry (LC-MS) and data analysis

To extract the proteins from the whole 36-hour-old treated seedling, 10 mg of frozen, powdered sample was mixed with 300 µl extraction buffer (4% [w/v] SDS, 100 mM Tris-HCl pH 6.8, 17.2% [v/v] glycerol, 8% [v/v] β-mercaptoethanol) and incubated at 93°C for 3-5 minutes, followed by centrifugation at 18,000 g for 15 min to separate the supernatant containing the extracted proteins. Protein concentrations were determined using a staining-based dot blot assay adapted from (Helbing et al., 2021). Briefly, 1 µL BSA protein standards and 1 µL protein extracts (in triplicates) were dropped onto a membrane, the dots were allowed to dry and then stained with Amido black. The intensity of each dot was measured using Amesharm and Q10 software. To remove components incompatible with subsequent peptide preparation, 100 µg of protein was precipitated in at least 4 volumes of acetone at -20°C, washed once with 80% (v/v) acetone, and air-dried at room temperature. Peptides were prepared using the iST kit for plant tissues (PreOmics, Germany) following the manufacturer’s instructions. Briefly, the dried protein pellet was resuspended in 100 µl LYSE buffer by boiling and sonication in a Bioruptor, loaded onto the cartridge and digested with Trypsin/LysC mixture for 3 h at 37 °C. The peptides were sequentially washed with the wash buffers X, 0, 1, and 2, then eluted, dried, and stored at -80°C until use. Prior to drying, an aliquot was taken for peptide quantitation by bicinchoninic acid (BCA) assay. Peptides

were re-solubilized in 3% (v/v) acetonitrile, 0.1% (v/v) formic acid by sonication and vortexing and spiked with iRT peptides (Biognosys) as internal standard. Equal peptide amounts (based on peptide quantitation by BCA assay) from each sample were analysed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific) and coupled to an M-Class UPLC (Waters). Peptides were loaded on a nanoEase M/Z Symmetry C18 trap column (100 Å, 5 µm, 180 µm x 20 mm, Waters) followed by a nanoEase M/Z HSS C18 T3 analytical column (100 Å, 1.8 µm, 75 µm x 250 mm, Waters).

For data-independent acquisition (DIA) proteomics of the peptide samples, peptides were separated at a flow rate of 300 nl min-1 by the following 90-min gradient: initial hold at 5% solvent B (0.1% formic acid in acetonitrile) / 95% solvent A (0.1% formic acid in water) for 3 min, followed by a gradient to 25% B / 75% A in 80 min, and to 32% B / 68% A in 10 min, a cleaning step at 95% B for 10 min and re-equilibration at 5% B / 95 % A for 10 min. The full MS was scanned at a resolution of 60,000 with a normalized automatic gain control (AGC) target value of 300% and a maximum injection time of 50 ms. The mass range was set to 400–900 m/z. Peptides were fragmented at a normalized collision energy of high-energy collision dissociation (HCD) of 33%. The normalized AGC target value for fragment spectra was set to 1000%. At the MS2 level, 50 windows of 10 m/z with optimized window placement and an overlap of 1 m/z within the mass range of 400–900 m/z were analysed at a resolution of 30,000. Maximum injection time was set to dynamic with a desired minimum of 6 data points across the peak. Spectra were acquired using internal lock mass calibration of m/z 371.101 and 445.120.

In addition, two pools composed of the five replicates from either the ABA-treated or untreated group were analysed by data-dependent acquisition (DDA) for the subsequent spectral library built. In these runs, peptides were separated at a flow rate of 300 nl/min by the following 90-min gradient: initial hold at 5% solvent B /95% solvent A for 3 min, followed by a gradient to 22% B / 78% A in 80 min, and to 32% B / 68% A in 10 min, a cleaning step at 95% B for 10 min and re- equilibration at 5% B / 95 % A for 10 min. The full MS was scanned at a resolution of 120,000 with a normalized AGS target of 300% and a maximum injection time of 50 ms. The mass range was set to 300-1500 m/z. Precursors were selected for MS/MS based on a minimum intensity of 5,000, and ions were isolated with a 1.6 m/z isolation window and fragmented by HCD using

a normalized collision energy of 33%. Maximum injection time was set to 80 ms. Single, unassigned, and charge states higher than six were rejected. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 25 s, and the exclusion window was set to 10 ppm. Internal lock mass calibration was the same as for DIA. DIA-MS raw files were converted into mzML files using MSConvert version 3.0.2472-63d00b1 (Chambers et al., 2012). The MS data were further processed using MSFragger-DIA implemented in the computational platform FragPipe version 22.0 (Yu et al., 2023). A hybrid spectral library was built from the primary DIA data and the auxiliary DDA data using the DIA_SpecLib_Quant workflow with default settings. Spectra were searched with MSFragger version 4.1 (Kong et al., 2017) against the Araport 11 proteome database concatenated to its reversed decoyed fasta database and common protein contaminants using default settings, i.e. allowing for one missed tryptic cleavage and setting carbamidomethylation of cysteine as fixed modification and methionine oxidation and acetylation of the protein N-terminus after methionine removal as variable modifications. DIA data was analysed using the hybrid spectral library as input in DIA-NN version 1.8.2 beta 8 (Demichev et al., 2020), using the same FragPipe workflow and the same search against the Araport 11 database as above. Fold changes of protein abundances and statistics were calculated using the Amica webserver version 3.0.1 (Didusch et al., 2022).

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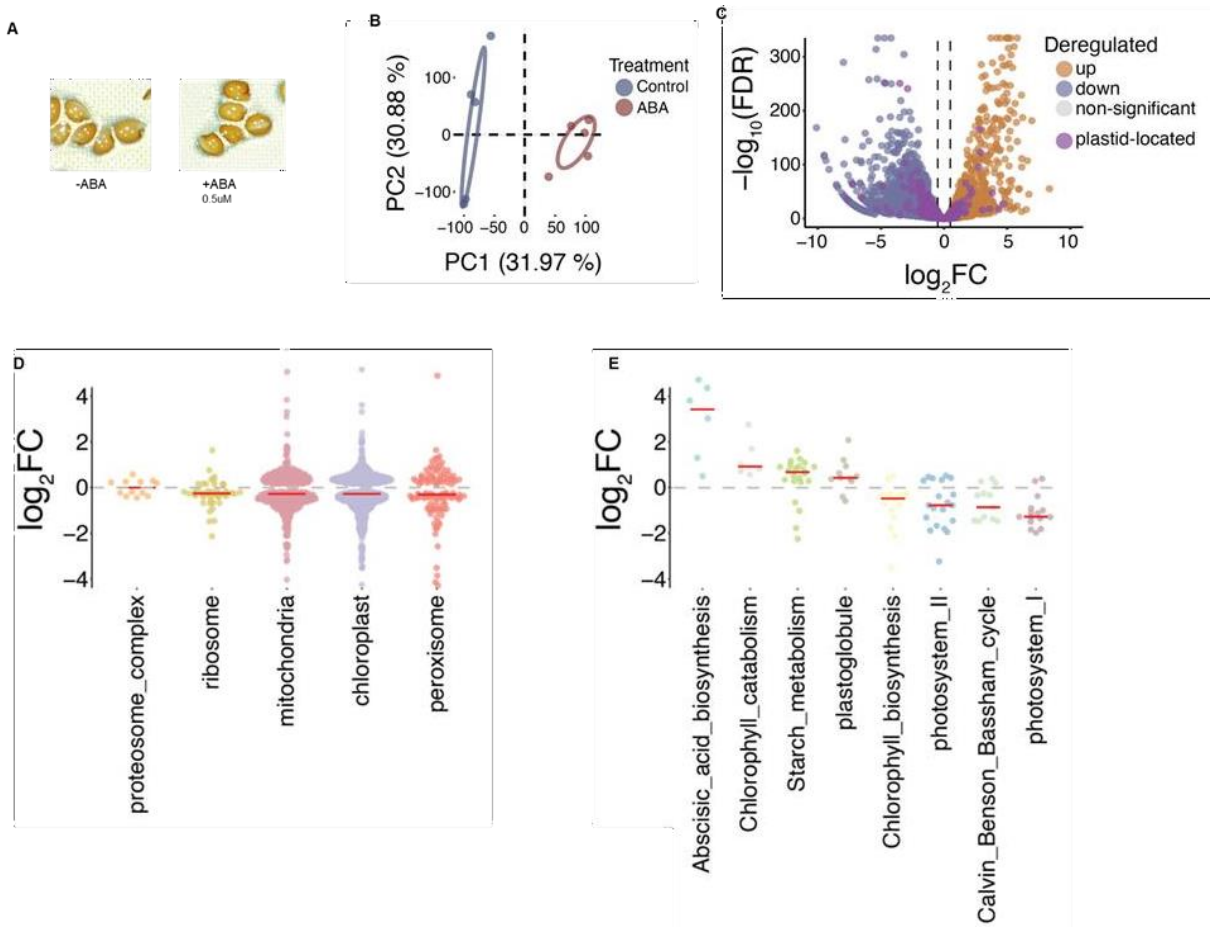

Figure 1: Genome-wide RNAseq analysis shows differential expression of genes under ABA

(A) The phenotype of 36-hour-old WT seedlings in control and 0.5 uM ABA. (B) Principal component analysis (PCA) of transcriptomic data, showing separation of samples based on treatment with ABA versus control. (C) Volcano plot illustrating differentially expressed genes. Genes are categorized as upregulated, downregulated, or non-significant and plastid-located (D). Gene ontology of differentially expressed genes associated with different organelles (mitochondria, peroxisome, chloroplast), proteasome complex, and ribosomes. (E) Gene ontology of differentially expressed genes related to up- and down-regulated pathways, including photosystems and plastoglobules.

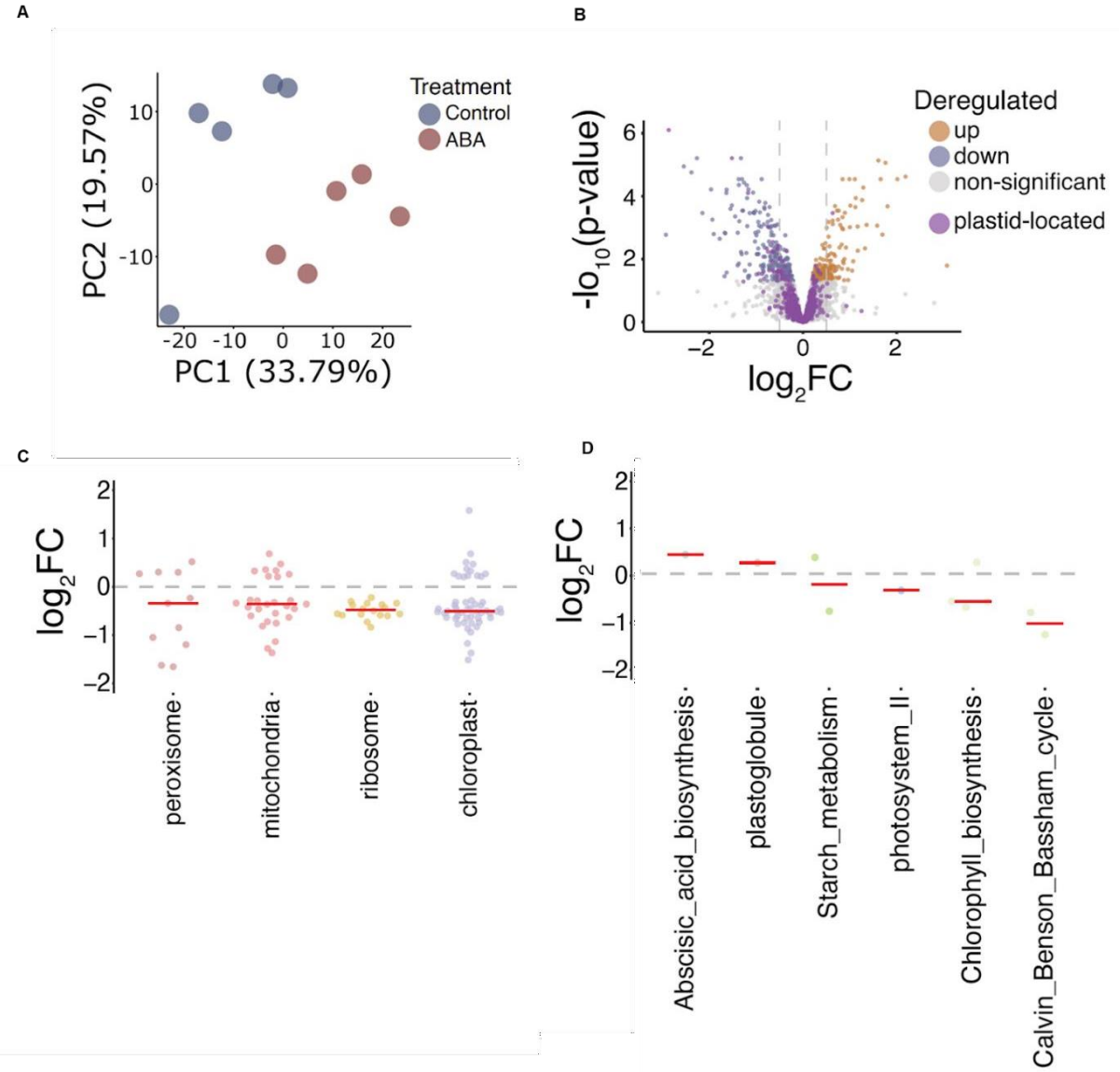

Figure 2: Proteome-wide analysis shows differential accumulation of protein under ABA

(A) Principal component analysis (PCA) of proteomics data, showing separation of samples based on treatment with ABA versus control. (B) Volcano plot illustrating differentially accumulated protein, categorized as upregulated, downregulated, or non-significant, and chloroplast localized. (C) Gene ontology of differentially accumulated protein at different organelles (mitochondria, peroxisome, chloroplast), proteasome complex, and ribosomes. (D) Gene ontology of differentially accumulated protein related to up- and down-regulated pathways, including photosystems and plastoglobule.

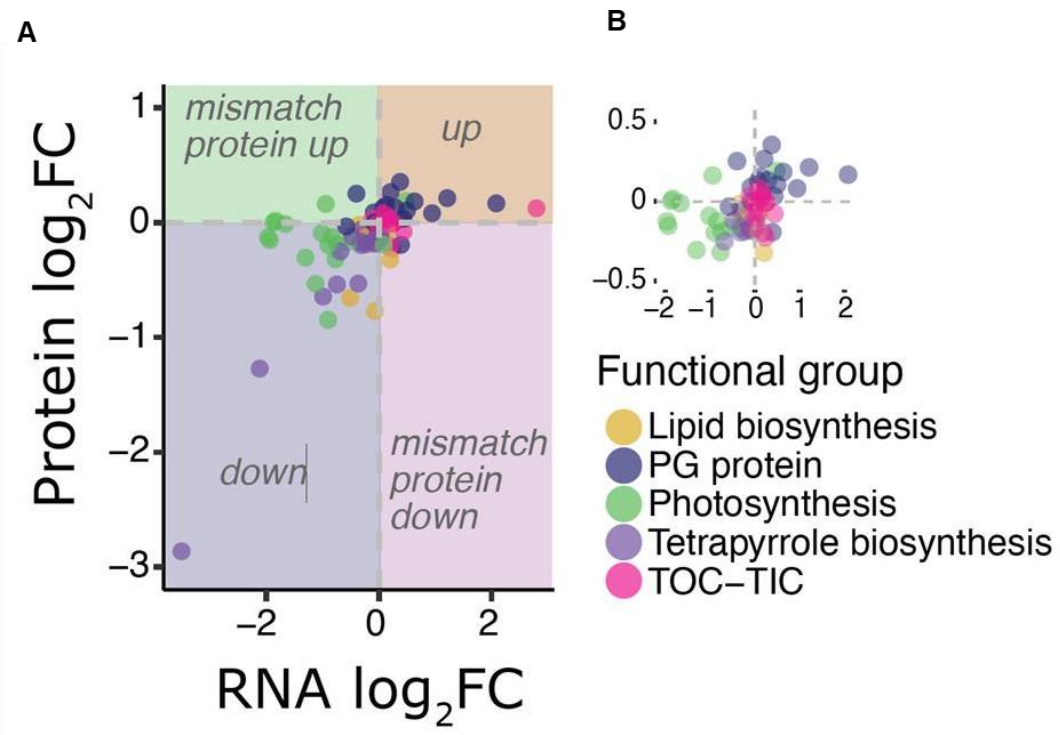

Figure 3: Relative comparison between genome-wide RNAseq and proteome-wide analyses shows mismatched protein down at TOC complex. (A) Two directional map of protein and transcript abundance of selected chloroplast biogenesis pathway (TOC-TIC, Lipid biosynthesis, Photosynthesis, Tetrapyrrole biosynthesis) and PG protein. (B) Magnification of Figure A, scale at -2 to 2 for X axis and -0.5 to 0.5 for Y axis.

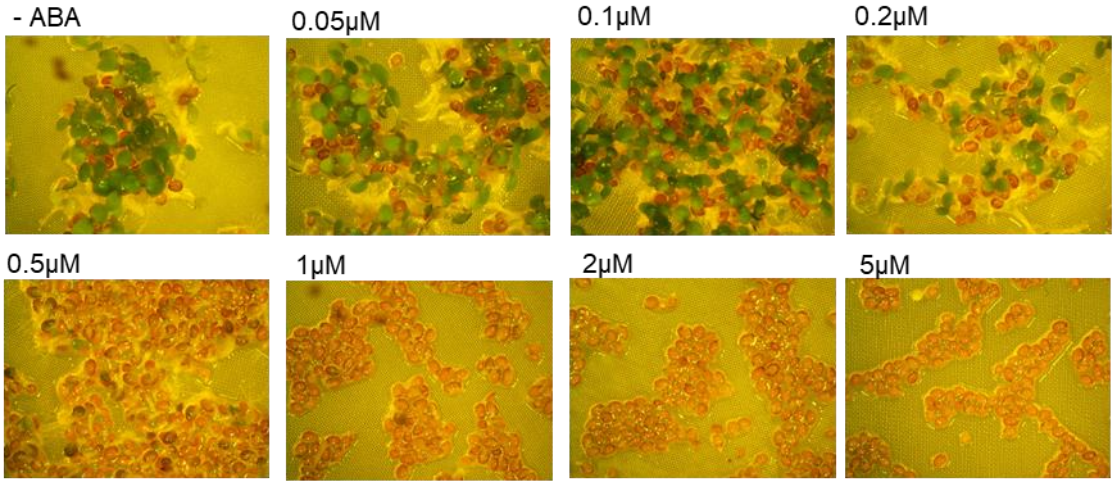

Figure 4: Phenotype of 72-hours old WT Arabidopsis in the presence of ABA.

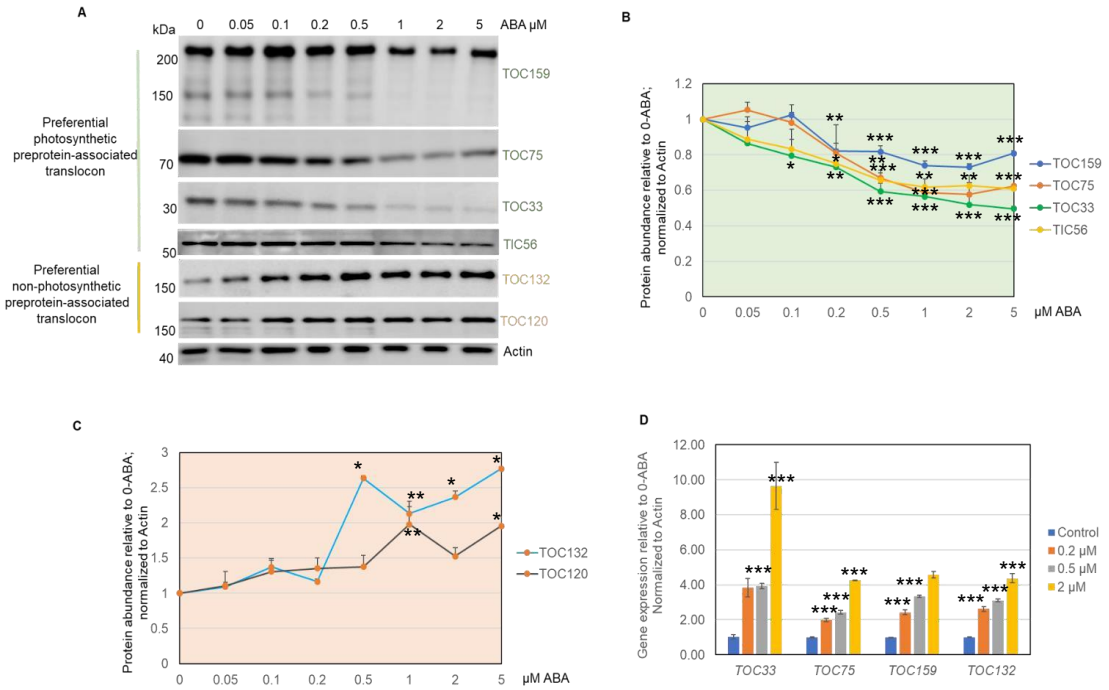

Figure 5: ABA reduced the associated photosynthetic translocon proteins

(A) Total protein extracts from WT seedlings, grown in the absence or presence (0.05 to 5 µM) of ABA treatment, were subjected to immunoblot analysis. Antibodies specific to TOC159, TOC132, TOC120, TOC75, TOC33, TIC56, and actin (used as a loading control) were used for protein detection. (B and C) Quantification of specific bands in western blotting and normalization with actin. Data represent means. Error bars indicate ± SEM (n =3), Student's t-tests (two-sided, paired); ***p < 0.005. (D) Transcript levels of TOC159, TOC132, TOC75, and TOC33 were measured from 72-hour-old WT seedlings grown in the absence or presence of ABA (0.2, 0.5, and 2 µM). Data represent means ± SD of TOCs mRNA level relative to actin, Student's t-tests (two-sided, paired); ***p < 0.005.

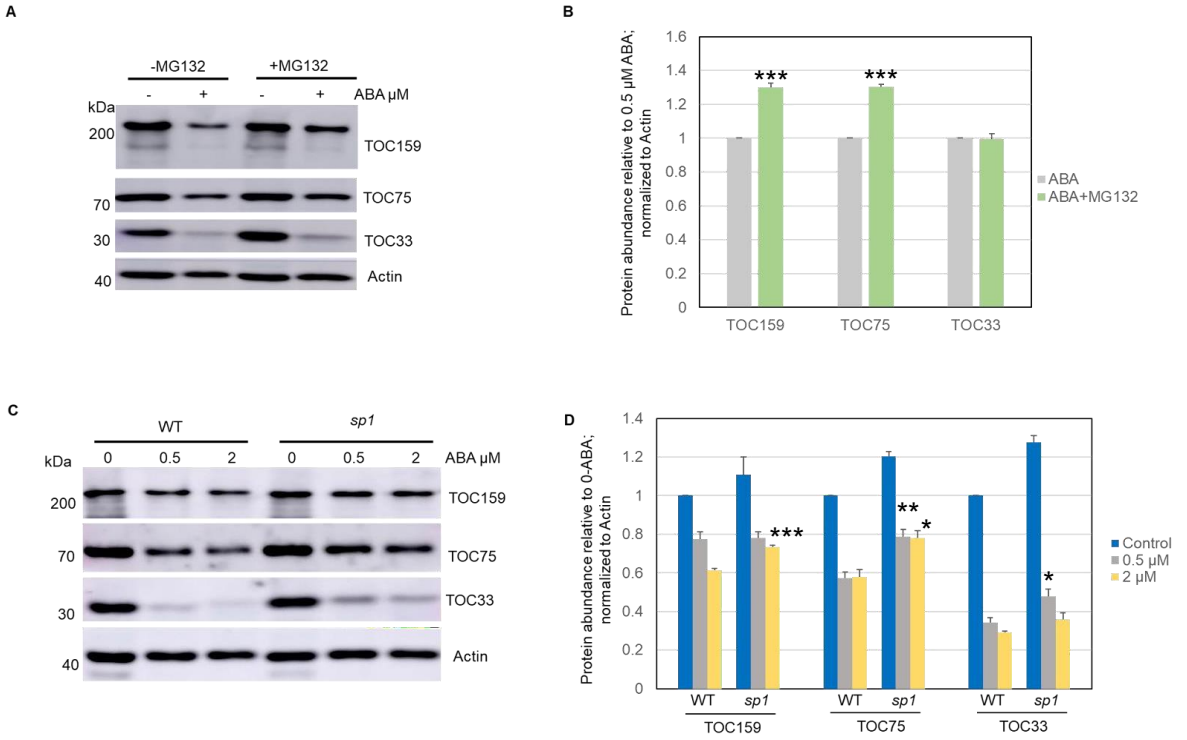

Figure 6: Role of ABA-dependent post-translational regulation of TOC

(A) Western blot analysis of WT seedlings grown in the presence or absence of ABA and further treated with and without MG132 were subjected to immunoblot analysis. Antibodies specific to TOC159, TOC75, TOC33, and actin (used as a loading control). (B) Quantify specific bands in western blotting and normalization with actin. The data represent means. Error bars indicate ± SEM (n =3). Student's t-tests (two-sided, paired); ***p < 0.005. (C) Total protein extracts from WT and *sp1* seedlings, grown in the presence or absence of ABA treatment, were subjected to immunoblot analysis. Antibodies specific to TOC159, TOC75, TOC33, and actin (used as a loading control) were used for protein detection. (D) Quantification of specific bands in western blotting and normalization with actin. The data represent means. Error bars indicate ± SEM (n =3). Student's t-test (two-sided, paired); ***p < 0.05

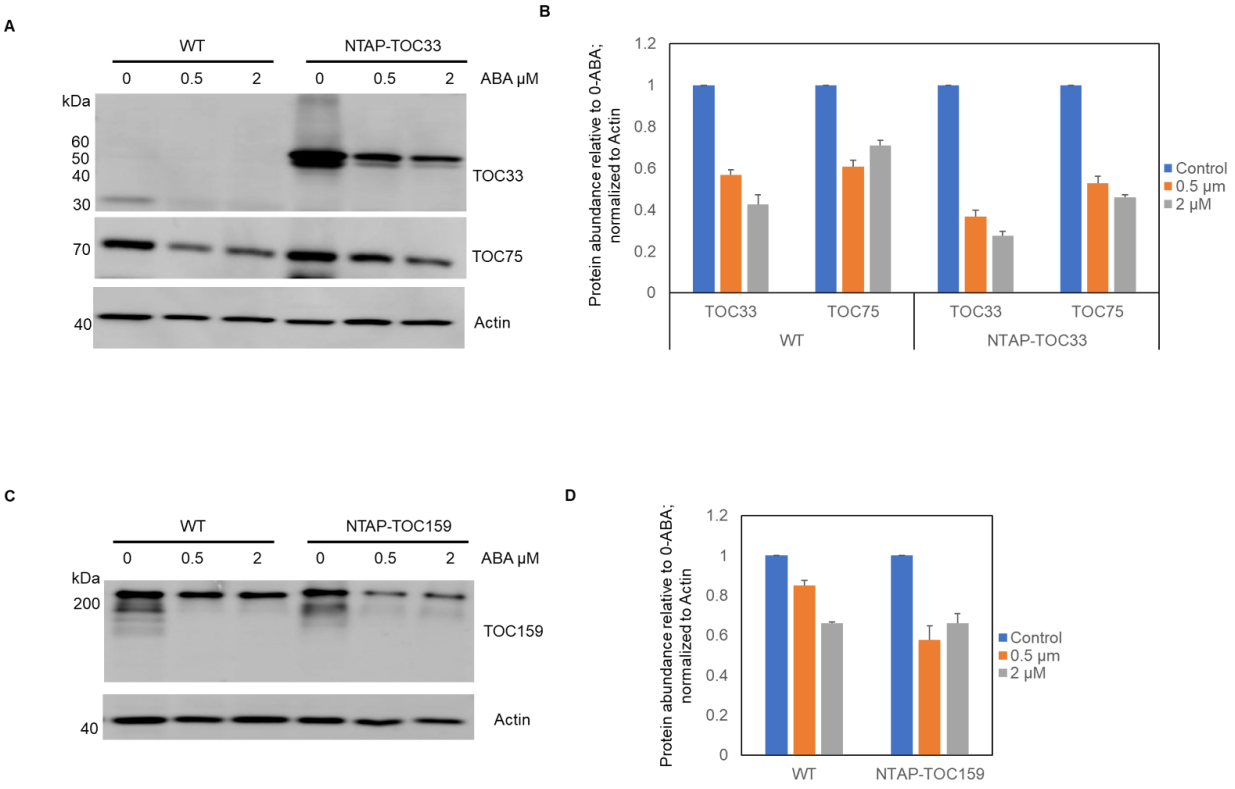

Figure 7: ABA regulation of TOC proteins is not dependent on their UTR

(A) Total protein extracts from WT and NTAP-TOC33/*ppi1* seedlings, grown in the presence or absence of ABA treatment, were subjected to immunoblot analysis. Antibodies specific to TOC75, TOC33, and actin (used as a loading control) were used for protein detection. (B) Quantification of specific bands in western blotting and normalization with actin. The data represent means. Error bars indicate ± SEM (n=3). Student's t-tests (two-sided, paired); ***p < 0.05. (C) Total protein extracts from WT and NTAP-TOC159/*ppi2* seedlings, grown in the presence or absence of ABA treatment, were subjected to immunoblot analysis. Antibodies specific to TOC159 and actin (used as a loading control) were used for protein detection. (D) Quantification of specific bands in western blotting and normalization with actin. The data represent means. Error bars indicate ± SEM (n =3). Student's t-test (two-sided, paired); ***p < 0.05

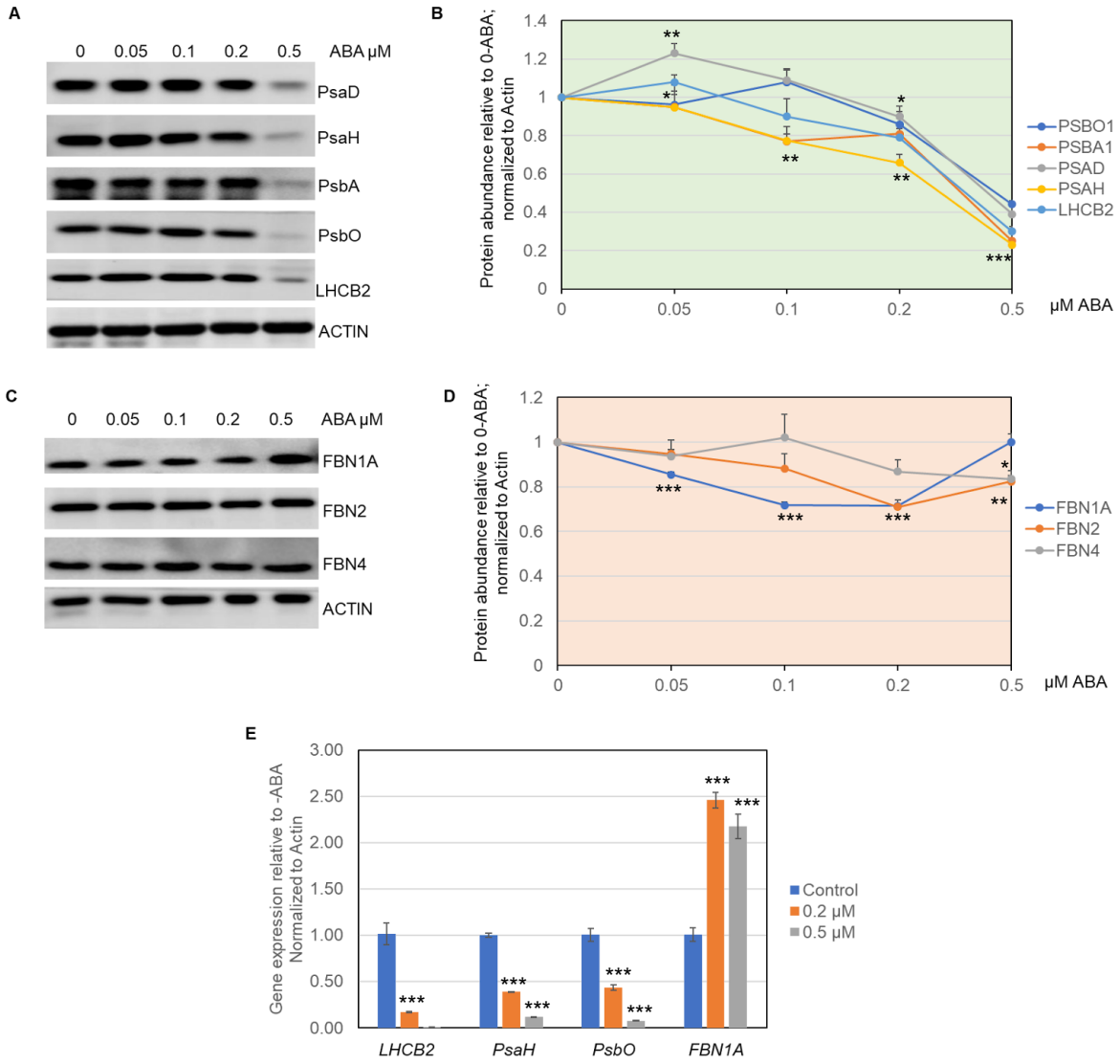

Figure 8: ABA regulation of photosynthetic and PG proteins dependent on transcriptional control

(A) Total protein extracts from WT seedlings, grown in the absence or presence (0.05 to 0.5 µM) or without ABA treatment, were subjected to immunoblot analysis. Antibodies specific to PsaD, PsaH, PsbA, PsbO, LHCB2, and actin (used as a loading control) were used for protein detection. (B) Quantification of specific bands in western blotting and normalization to actin. Data represent means. Error bars indicate ± SEM (n =3). Student's t-tests (two-sided, paired); ***p < 0.005. (C) Total protein extracts from WT seedlings, grown in the absence or presence (0.05 to 0.5 µM) or without ABA treatment, were subjected to immunoblot analysis. Antibodies specific to FBN1A, FBN2, FBN4, and actin (used as a loading control) were used for protein detection. (D) Quantifying specific bands in western blotting and normalizing actin. Data represent means. Error bars indicate ± SEM (n =3). Student's t-test (two-sided, paired); ***p < 0.005. (E) Transcript levels of LHCB2, PsaH, PsbO, and FBN1A were measured from WT seedlings grown in the absence or presence of ABA (0.2, 0.5, and 2 µM). Data represent means ± SD (n=3) of TOCs mRNA level relative to actin. Student's t-test (two-sided, paired); ***p < 0.005

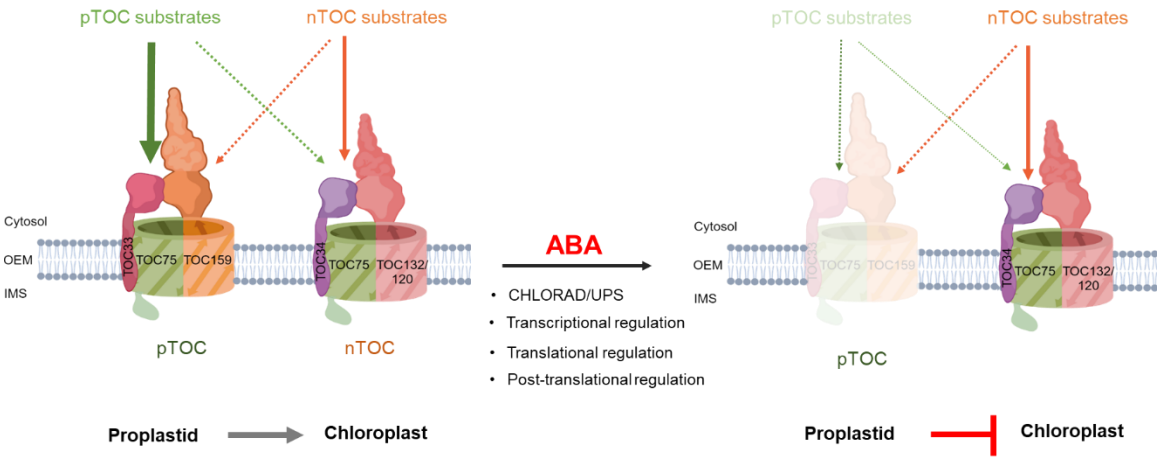

Figure 9: Hypothetical model for the ABA regulation of chloroplast biogenesis at seed to seedling stage Environmental cues influence the concentrations of ABA that accumulate upon seed to seedling stage. When ABA levels are increased by stress (+ABA) (right-hand panel), the photosynthesis-associated genes are downregulated, and non-photosynthetic genes are upregulated via transcriptional regulation. The associated translocon of photosynthetic protein is downregulated via SP1-dependent UPS and may be via translation regulation. The associated translocon of non-photosynthetic protein are upregulated at the protein level may via translation regulation. Afterward, non-photosynthetic stress-related proteins are imported mostly via an associated translocon of non-photosynthetic protein. When ABA concentrations decrease (-ABA) (left-hand panel), transcriptional and translational regulation are attenuated, allowing photosynthetic protein import via photosynthetic translocon to promote chloroplast biogenesis.

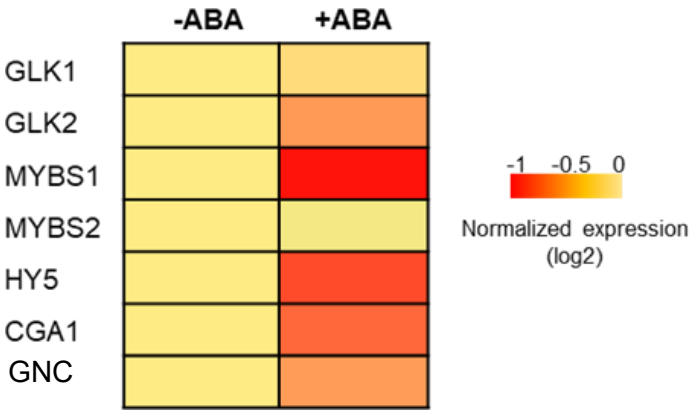

Figure S1: Heat map illustrating differentially expressed chloroplast biogenesis transcription factors HY5, GNC, CGA1, GLK1/2, and MYB, respectively, with (+) and without (-) ABA.

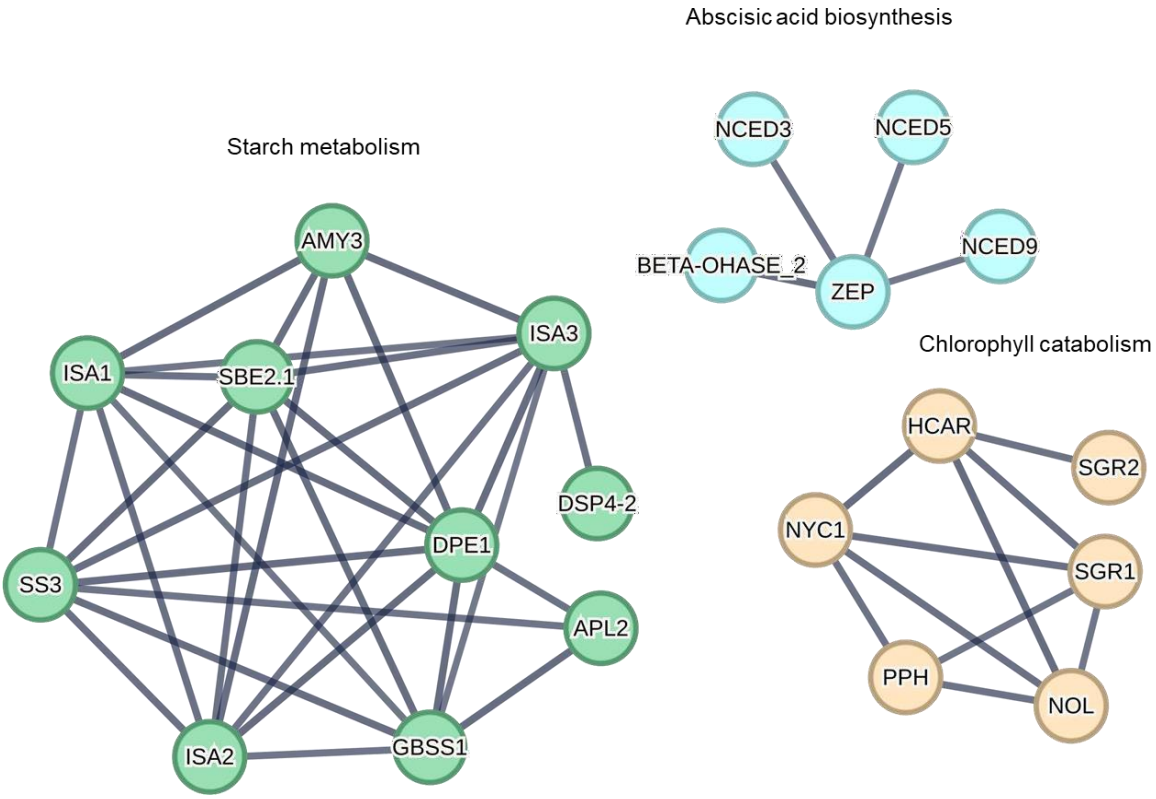

Figure S2: Interaction network of proteins upregulated under ABA. The upregulated proteins analysis using the STRING software identified three clusters. Green (cluster 1) enriched in starch metabolic enzymes; Blue (cluster 2) enriched in abscisic biosynthesis enzymes; Yellow (cluster 3) enriched in chlorophyll catabolism enzymes.

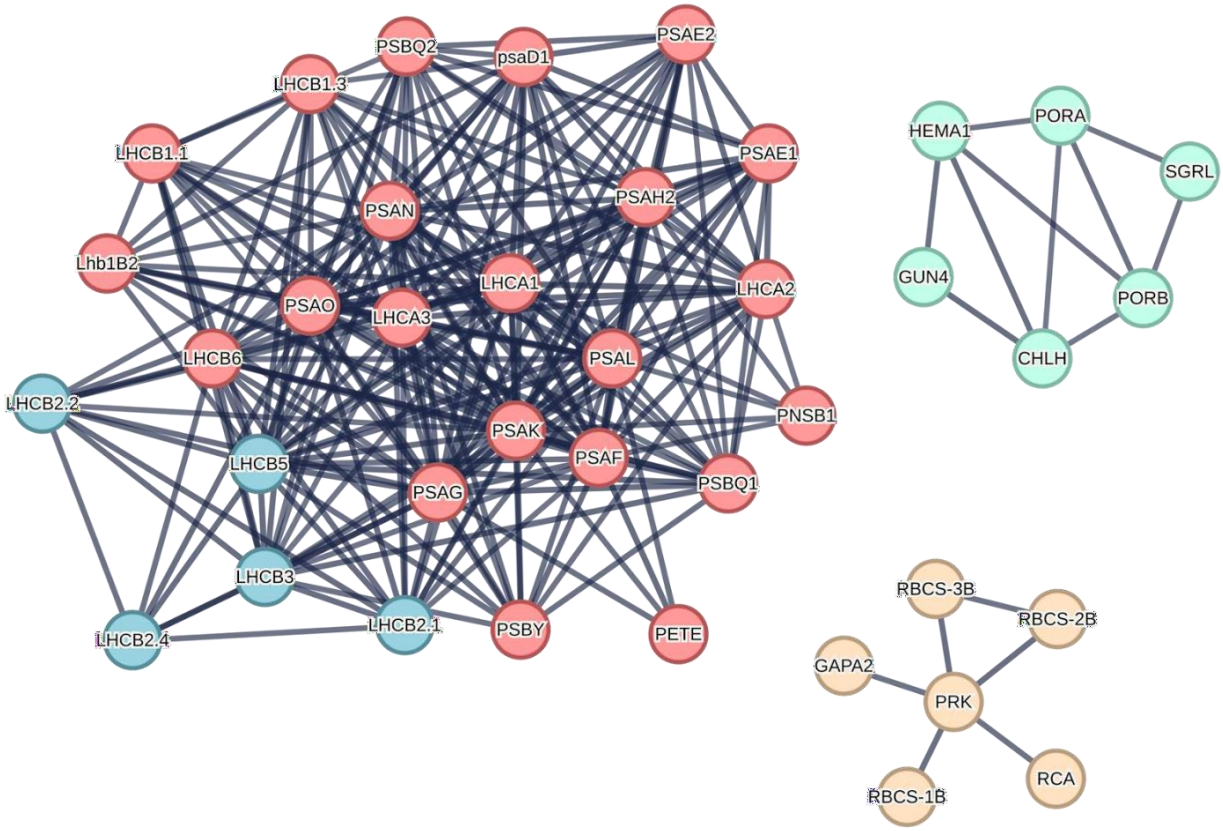

Figure S3: Interaction network of proteins downregulated under ABA. The downregulated proteins analysis using the STRING software identified three clusters. Red and blue (cluster 1) enriched photosynthetic proteins; Yellow (cluster 2) enriched in Calvin Benson Bassham (CBB) cycle enzymes; Green (cluster 3) enriched in chlorophyll biosynthetic enzymes.

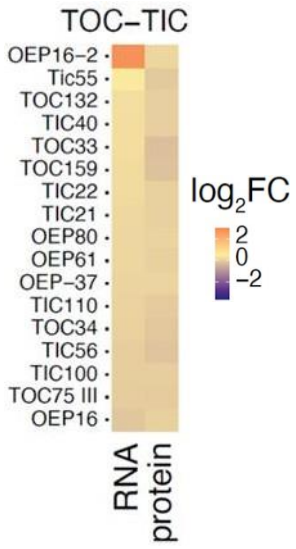

Figure S4: Heat map illustrating relative comparison between RNA (genome-wide RNAseq) and protein (proteome-wide analyses) from selected TOC-TIC complex genes.

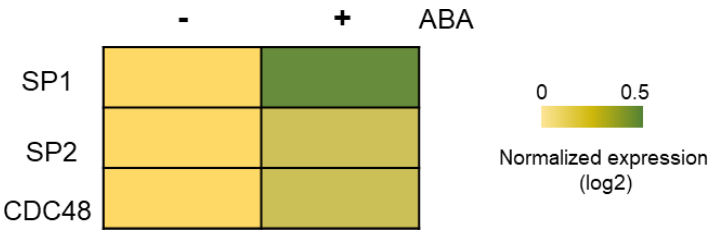

Figure S5: Heat map illustrating differentially expressed CHLORAD components SP1, SP2 and CDC48, with (+) and without (-) ABA.

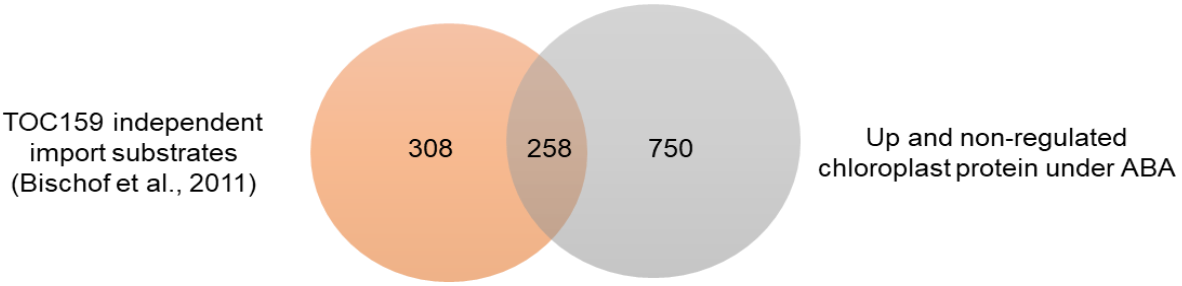

Figure S6: Venn diagram illustrating comparison between TOC159 independent import substrates and up, non-regulated chloroplast protein under ABA

Conclusion

Chloroplast biogenesis is a crucial process for establishing photoautotrophic growth in plants. During the seed-to-seedling transition, the rapid differentiation of undifferentiated proplastids into fully functional chloroplasts is indispensable for harnessing light energy and sustaining plant development. This process involves the coordinated import of thousands of nucleus-encoded proteins from the cytoplasm to the chloroplast through TOC (Translocon at the Outer envelope of the Chloroplast) and TIC (Translocon at the Inner envelope of the Chloroplast), leading to the establishment of photosynthetic complexes. ABA hormone known as a stress hormone negatively regulates seed germination. Furthermore, it has been shown that long-term ABA treatment impairs chloroplast metabolism and increases chlorophyll degradation, leading to leaf yellowing. However, there is still a lack of understanding of how ABA impacts chloroplast biogenesis and coordinates it with overall plant development. In my PhD thesis, I investigated how ABA-induced remodeling of the plastid proteome and transcriptome influences chloroplast development and how ABA regulates protein translocation via nTOC and pTOC during the transition from seed to seedling.

The first chapter of my thesis, is a review article "The journey of preproteins across the chloroplast membrane systems" (Frontiers in Physiology, 2023), which I co-authored, I contributed significantly to the writing and conceptual development of the sections related to the mechanisms by which nuclear-encoded preproteins are imported into chloroplasts and subsequently into thylakoids. Chloroplasts have an endosymbiotic origin, and for this reason, most of their proteins are encoded in the nucleus and synthesized as preproteins in the cytosol. These preproteins possess N-terminal transit peptides that function as targeting signals, directing them to the chloroplast. The import process involves recognition by cytosolic factors and subsequent translocation through the TOC and TIC complexes, with the assistance of GTP and ATP. Once delivered inside the stroma, the transit peptide is cleaved by the stromal processing peptidase. For proteins destined for the thylakoid lumen or membrane, additional targeting sequences guide them via specific pathways namely the Twin Arginine Transport (Tat), Secretion pathway (Sec), or Signal Recognition Particle (SRP) pathways. This intricate import system is essential for chloroplast biogenesis and function, underpinning the photosynthetic capacity and overall

development of plants.

The second chapter of the present thesis is devoted to the discovery of the effect of the plant hormone ABA on chloroplast biogenesis with regard to chloroplast import machinery in the transition from seed to seedling in Arabidopsis. In this study, I examined the impact of ABA on two distinct seedling stages: 36- and 72-hours post-imbibition. By integrating transcriptome-wide RNA sequencing (RNA-seq) and proteome-wide mass spectrometry, I provide a comprehensive dissection of ABA's influence on plastid development in seedlings. The whole transcriptome analysis revealed that approximately half of all Arabidopsis genes were differentially expressed under ABA treatment. While GO terms associated with general chloroplast, mitochondria, and peroxisomes were only moderately downregulated, a strikingly stronger repression was observed for photosynthesis-specific categories, including photosystem I and II assembly, the Calvin–Benson–Bassham (CBB) cycle, and chlorophyll biosynthesis. These patterns were mirrored at the proteome level, with the accumulation of photosystem components, Calvin cycle enzymes, and chlorophyll biosynthetic enzymes significantly reduced under ABA. This selectivity suggests that ABA does not exert a broad, indiscriminate suppression of chloroplast functions, but instead targets a subset of processes specifically linked to light harvesting, electron transport and carbon fixation. Such a targeted repression aligns with earlier studies showing that ABA downregulates *LHCB* and *RBCS* expression through ABI4- and ABI5-mediated promoter binding (Koussevitzky et al., 2007; Hernández et al., 2005). However, the narrow scope of the repression observed here, compared to the modest downregulation of the general "chloroplast" GO term, points toward a finely tuned developmental checkpoint rather than whole plastid shutdown. In parallel with the suppression of photosynthesis-associated pathways, ABA strongly induced transcripts and proteins linked to stress adaptation. Notably, plastoglobule (PG)-associated proteins such as fibrillins (FBN1A, FBN2), ABC1K kinases, and VTE1 were robustly upregulated. These proteins are implicated in lipid remodeling, antioxidant biosynthesis (tocopherols, plastoquinone), and stabilization of thylakoid structures under stress (Bréhélin et al., 2007; Shanmugabalaji et al., 2013). ABA biosynthesis genes were also induced, suggesting a feed-forward regulatory loop, while chlorophyll catabolism genes (*SGR1*, *NYC1*) known ABI5 targets (Skubacz et al., 2016) were activated, promoting pigment breakdown. Collectively, these changes indicate a hormonal reprogramming from energy capture to oxidative stress mitigation, a shift

that is likely adaptive under environmental or developmental stress scenarios where photosynthetic ROS production could be damaging.

A major mechanistic insight from my study is the ABA-induced reconfiguration of the TOC translocon complex. Under ABA, pTOC (TCOC159 and 33) transcripts were elevated, however their protein levels declined strongly suggesting post-transcriptional regulation. This transcript–protein uncoupling may involve translational repression, proteasomal degradation, or both. Indeed, previous work has shown that SP1 E3 ligase ubiquitinates TOC33 and TOC75 for CHLORAD-mediated degradation under certain stress conditions (Ling et al., 2012; Shanmugabalaji et al., 2018). While our data indicate that SP1 contributes to ABA-induced pTOC degradation, the effect is only partial, implicating additional E3 ligases or other regulatory mechanisms. In contrast, nTOC components (TOC120 and -132) exhibited coordinated transcript and protein upregulation under ABA, maintaining or enhancing the import of stress-protective proteins such as FBN1A. This inverse regulation (downregulation of pTOC and upregulation of nTOC) marks a significant change in the capacity of plastids to import proteins, shifting the balance from photosynthetic development to stress resilience. To explore potential cis-regulatory contributions, we analyzed TOC33 and TOC159 transgenes lacking native 5′ and 3′ untranslated regions. These constructs exhibited ABA responses the same as wild type, indicating that their UTRs are not involved in ABA-induced downregulation. This finding suggests that other trans-acting regulatory factors such as RNA-binding proteins or non-coding RNAs may play a role. Integrating these findings, I propose that ABA functions as a master regulator of plastid development during the seed-to-seedling transition through a three-pronged mechanism:

1. Transcriptional repression of photosynthesis-associated nuclear genes.
2. Post-transcriptional suppression of pTOC translocons accumulation, via translational inhibition and selective proteasomal degradation, partially mediated by SP1.
3. Preferential upregulation of nTOC components, sustaining import of stress-protective proteins.

From this study several questions have emerged. First, the exact post-transcriptional mechanisms repressing pTOC accumulation remain unknown. Future research could build on this work by using ribosome profiling (Ribo-seq) to test whether ABA actively suppresses translation of pTOC mRNAs, which encode components of the chloroplast

outer envelope translocon. By pairing Ribo-seq with RNA-seq across defined ABA treatment time courses in Arabidopsis, translation efficiency changes could be quantified genome-wide and compared between pTOC transcripts and matched controls. Analyses of ribosome occupancy patterns, 5′UTR features, and uORFs could reveal cis-elements underlying translational repression. Orthogonal approaches such as polysome profiling, reporter assays, and targeted proteomics would validate and extend these findings, ultimately providing a mechanistic framework for understanding how ABA modulates chloroplast protein import capacity through selective control of cytosolic translation. In addition, to investigate whether small RNAs, particularly microRNAs, contribute to the regulation of pTOC mRNAs under ABA. Parallel small-RNA sequencing (sRNA-seq) alongside RNA-seq and Ribo-seq across defined ABA treatment time courses could identify ABA-responsive miRNAs with potential binding sites in pTOC transcripts. Candidate interactions could then be validated using reporter assays with native and mutated target sites, and tested functionally through gain- and loss-of-function approaches such as overexpression or knock down constructs. Linking changes in miRNA abundance to corresponding reductions in pTOC mRNA levels, translation efficiency, and protein abundance would establish a mechanistic model in which ABA-responsive miRNAs fine-tune chloroplast protein import by selectively targeting pTOC transcripts.

Another promising direction would be to use electron microscopy to investigate how ABA influences the abundance, organization, or ultrastructure of TOC complexes at the chloroplast outer envelope. Correlating ultrastructural observations with molecular data from Ribo-seq, RNA-seq, and proteomics would provide a multi-scale understanding of how ABA affects chloroplast biogenesis from transcript regulation to protein complex architecture.

In conclusion, this work establishes a comprehensive framework for understanding ABA-mediated remodeling of chloroplast protein import during early plant development. By integrating transcriptomic, proteomic, and mechanistic evidence, I demonstrated that ABA selectively inhibits pTOC-mediated photosynthetic protein import while maintaining nTOC-mediated stress-protective import. This adaptive rebalancing underscores the sophistication of hormonal control over organelle biogenesis and opens new avenues for dissecting plastid protein trafficking under stress.

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