

Preprotein import in chloroplast biogenesis – Identification and characterisation of a strong dimerisation mutant of atToc159

A dissertation submitted to the

Faculty of Sciences
Institute of Biology
University of Neuchâtel

For the degree of

Doctor es Sciences

Presented by

Meryll M. MARTIN

Laboratory of Plant Physiology, Institute of Biology,
Faculty of Sciences, University of Neuchâtel

Accepted after recommendation by the thesis committee

Pr. Felix KESSLER, Thesis Director
Pr. Sacha BAGINSKY
Pr. Jean-Marc NEUHAUS
Dr. Norbert ROLLAND

Presented on November 14th 2011

IMPRIMATUR POUR LA THESE

Preprotein import in chloroplast biogenesis – Identification and characterisation of a strong dimerisation mutant of atToc159

Meryll Michael MARTIN

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

Prof. Felix. Kessler (directeur de thèse),
Prof. Jean-Marc Neuhaus,
Dr Norbert Rolland (Université de Grenoble, F)
Prof. Sacha Baginsky (Martin-Luther-Universität, Halle-Wittenberg, D)

autorise l'impression de la présente thèse.

Neuchâtel, le 25 janvier 2012



Le doyen :
P. Kropf

Abstract

Abbreviations

1- Introduction	1
1.1- <i>The Plant kingdom</i>	1
1.1.1- Universal eukaryotic cell features	1
1.1.2- The theory of endosymbiosis and the origin of plastids	6
1.1.3- Three genomes and their coordination	8
1.2- <i>The process of protein import into the chloroplast</i>	12
1.2.1- The chloroplast transit peptide	12
1.2.2- Energy requirement	14
1.3- <i>The TOC core complex</i>	15
1.3.1- Toc GTPases	17
1.3.2- Toc75	21
1.4- <i>The TIC complex</i>	21
1.5- <i>Models of protein import by the TOC complex</i>	22
1.5.1- The “motor” model	23
1.5.2- The “targeting” model	23
1.5.3- Two pictures: spot the difference!	24
1.6- <i>Genetic of Toc33 and Toc159</i>	25
1.7- <i>Aims of the thesis</i>	27
2- Results	28
2.1- <i>Screening for a Toc159 mutant with strong binding for Toc33</i>	28
2.1.1- A single point mutation suppressing Toc33 R130A loss-of-interaction	30
2.1.2- Toc159G D919V suppresses Toc33 loss-of-interaction mutants in the D1 motif	30
2.2- <i>Expression and purification of recombinant proteins</i>	32
2.3- <i>Toc159G D919V interacts strongly with Toc33G in a Ni-NTA pull-down assay</i>	36
2.4- <i>Toc159G D919V is deficient in GTP binding</i>	38
2.5- <i>Toc159G D919V hydrolyses GTP like the WT</i>	38
2.6- <i>Complementation of Arabidopsis thaliana ppi2 by TAP-Toc159GM D919V</i>	40
2.7- <i>Insertion of Toc159 D919V mutant into the outer membrane of chloroplasts</i>	43
2.8- <i>Preprotein import into isolated Toc159GM D919V chloroplasts</i>	46
3-Discussion	48
3.1- <i>Synthesis</i>	48
3.1.1- Requirements for an optimal preprotein import	48
3.1.2- G-domain dimerisation is a key element of preprotein import	50
3.1.3- Toc159 D919V has GTPase properties distinct from the WT	51
3.2- <i>Outlook</i>	52
3.2.1- Crystallisation of Toc159G and its interacting partner	52
3.2.2- Deciphering the G-domain of Toc159	52

4- Materials and methods	56
4.1- Materials	56
4.1.1- Biological material	56
4.1.2- Oligonucleotides	56
4.1.3- Plasmids	56
4.1.4- Antibodies	57
4.1.5- Chemicals	57
4.2- Methods	57
4.2.1- Physiological methods	57
4.2.2- Molecular cloning	58
4.2.3- DNA constructs	59
4.2.4- Bacterial expression and purification of recombinant protein	61
4.2.5- Stable transformation of <i>Arabidopsis thaliana</i>	62
4.2.6- SDS-PAGE, Western blotting and immuno-detection	62
4.2.7- Yeast two-hybrid library	63
4.2.8- Pull-down assay	64
4.2.9- Guanosine Tri-Phosphate binding and hydrolysis	64
4.2.10- Insertion and import into the Chloroplasts	65
4.2.11- Electron microscopy	66
Bibliography	67

Annex

Acknowledgements

ABSTRACT

The chloroplast is the hallmark organelle of plant having evolved from the endosymbiotic event. Most chloroplast proteins are synthesized as preproteins in the cytosol. The import of these preproteins is mediated by molecular complexes located at the outer and inner membrane of the chloroplast. These complexes are called TOC (Translocon at the Outer envelope of the Chloroplast) and TIC (Translocon at the Inner envelope of the Chloroplast), respectively. In *Arabidopsis thaliana*, the TOC complex consists of three principle components: two homologous receptor GTPases, atToc159 and atToc33 and a protein-import channel, atToc75. During import, the two GTPases undergo complex interactions with precursor proteins and amongst themselves although precise mechanisms remain unknown. *In vitro* studies revealed that Toc159 and Toc33 interact with each other via the dimerisation of their GTP-binding domain (G-domain). According to the crystal structure of pea Toc34 homodimer and based on the G-domain homology of the TOC GTPases it is likely that the process of dimerisation is a key step for the process of import of preprotein into the chloroplast. This thesis intends to identify a mutant of Toc159 with an increased dimerisation interaction towards Toc33. Thus, Toc159 D919V was identified and revealed itself to bind strongly to Toc33, hydrolyse GTP and complement the TOC159 null mutant. This mutant is a promising candidate for crystallisation purposes and for the identification of interaction partners of TOC GTPases by TAP-tag purification.

Keywords: chloroplast biogenesis, TOC complex, Toc159, *Arabidopsis thaliana*, *Agrobacterium tumefaciens*, Yeast two-hybrid, protein translocation

ABBREVIATIONS

α -"protein"	antibodies recognizing the specified "protein"
A-domain	Acidic domain
Arabidopsis	<i>Arabidopsis thaliana</i>
at	<i>Arabidopsis thaliana</i>
atToc159G	GTP binding domain of atToc159
atToc33G	GTP binding domain of atToc33
atToc159A	acidic-domain of atToc159
BN-PAGE	Blue Native-PAGE
bp	base pairs
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
CBP	Calmodulin Binding Protein
cDNA	complementary DNA
Col	Columbia
D1	Dimerisation motif 1
DNA	DesoxyriboNucleic Acid
dNTP	desoxy Nucleotide TriPhosphate
DTT	1,4-Dithio-DL-ThreiTol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	EthyleneDiamine-N,N,N',N'-TetraAcetic acid
EGTA	Ethylene Glycol TetraAcetic acid
ER	Endoplasmic Reticulum
G-domain	GTP binding domain
GAD	G-proteins Activated by nucleotide-dependent Dimerisation
GAP	GTPase Activating Protein
GEF	GDP Exchange Factor

GST	Glutathione-S-Transferase
HEPES	4-(2-HydroxyEthyl)Piperazine-1-EthaneSulfonic acid
His6	hexahistidinyI-tag
IgG	Immunoglobulin G
IPTG	IsoPropyl-β-D-1-ThioGalactopyranoside
kbp	kilo base pairs
kDa	kilo Dalton
KOAc	Potassium acetate
LB	Luria Bertani
M-domain	Membrane anchoring domain
MeOH	Methanol
Mg(OAc) ₂	Magnesium Acetate
MS	Murashige and Skoog
Ni-NTA	Nickel-NitriloTriacetic Acid
ONPG	O-NitroPhenyl-beta-D-Galactopyranoside
<i>P. patens</i>	<i>Physcomitrella patens</i>
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEI-Cellulose	PolyEthyleneimine- Cellulose
ppi	plastid protein import
preSSU	preprotein of SSU
protA	protein A
ps	<i>Pisum sativum</i>
RER	Rough ER
rt	room temperature
RubisCO	Ribulose-1,5-bisphosphate Carboxylase Oxygenase
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	SDS-PolyAcrylamide Gel Electrophoresis
SER	Smooth ER
SR	SRP Receptor
SRP	Signal Recognition Particle
SSU	Small SubUnit of RubisCO
T0 lines	Arabidopsis plants that were transformed by floral dip
T1 lines	first generation of seeds after plant transformation by floral dip
Tn lines	n th generation of seeds after plant transformation by floral dip
TAP-tag	Tandem Affinity Purification tag
T-DNA	Transfer DNA
TEMED	N,N,N',N',-TetraMethyl-EthyleneDiamine
TIC	Translocon at the Inner membrane of the Chloroplast
TM	TransMembrane helix
TOC	Translocon at the Outer membrane of the Chloroplast
Tris	tris (hydroxymethyl) aminomethane
U	unit
UV	Ultra Violet
v/v	volume per volume
WT	Wild Type
w/v	weight per volume
atToc..., atTic...	indicates the Toc-, Tic-protein of <i>A. thaliana</i>
psToc..., psTic...	indicates the Toc-, Tic-protein of <i>P. sativum</i>
upper case	gene (e.g. TOC159)
lower case, italic	mutant allele (e.g. <i>ppi2</i>)
regular case	protein (e.g. atToc159)

1- Introduction

1.1- The Plant kingdom

This kingdom is characteristic of eukaryotic photosynthetic organisms containing plastids subsequently to the primary endosymbiosis (section 1.1.2) event and is divided into three lineages (Figure 1): Viridiplantae (land plants and green algae), Rhodophyte (red algae) and, Glaucophyte (Cavalier-Smith, 1981; Simpson & Roger, 2004).

1.1.1- Universal eukaryotic cell features

In 1838, Schleiden published the cellular theory. According to this theory the common point among all life forms, the functional unit, is the cell. Indeed, the study of plant physiology is, above all, the study of cell physiology. Therefore, it is of interest to have an overview of this functional unit of life. A plant cell is made of an aqueous solution, the protoplasm consisting of the cytoplasm, the nucleus and other organelles, surrounded by a plasma membrane. This ensemble is called a protoplast. The plasma membrane is of primary importance as it is the limit between the living and non-living worlds and regulates exchanges between the cell and its environment. The eukaryotic cell differs from the prokaryotic cell by its size and its intra-cellular organisation; the eukaryotic cell possesses a complex endomembrane system. The endomembrane system is a singular feature of eukaryotes, it defines sub-cellular compartments with specific structures and functions. These organelles take over and compartmentalise intra-cellular metabolic functions. It is important to note that both mitochondria and plastids are not under the strict definition of the endomembrane system; they are referred to as endosymbiont organelles (Alberts *et al*, 2002). A stereotypic eukaryotic plant cell is composed of a nucleus, a vacuole, the endoplasmic reticulum, the Golgi apparatus, the chondriome, microbodies and, last but not least, plastids (Figure 2).

1.1.1.1- The nucleus

The nucleus contains the DNA encoding for genes and regulates its transcription into RNA that is exported to the cytosol in order to be translated into a protein (Brenner *et al*, 1961). This sequence of events, from the DNA to the protein, defines the paradigm of the modern biological science; it opened a vast field of investigation along with the era of

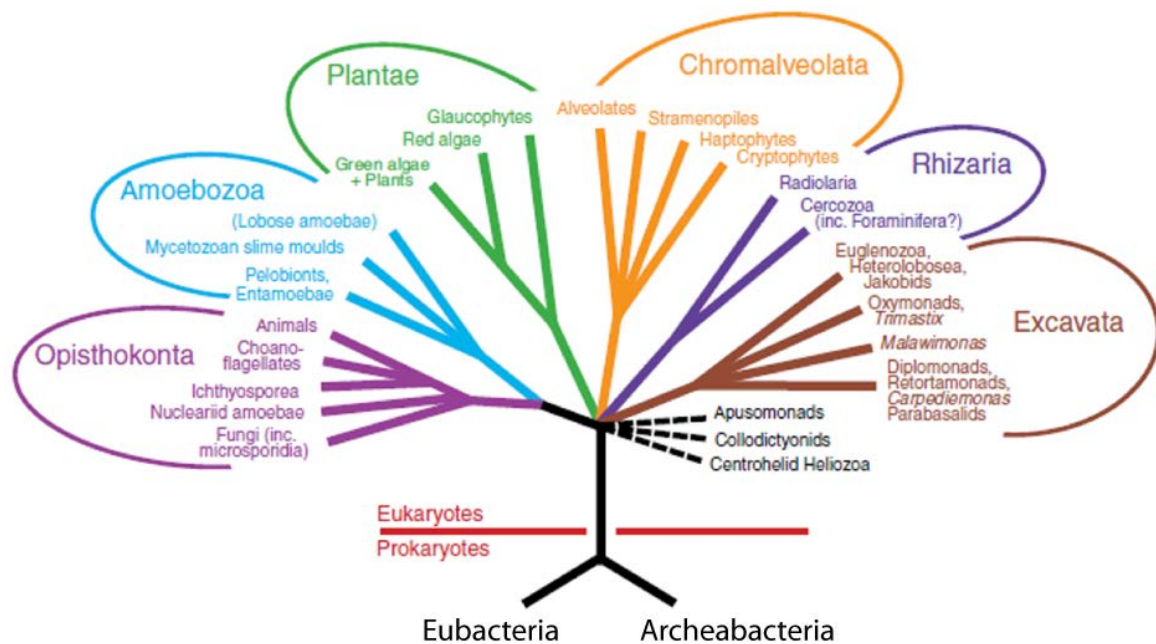


Figure 1: The subdivision of lifeforms. Schematic phylogenetic tree of lifeforms depicting the organisation of eukaryotes in six major clades. The Plantae kingdom (in green colour) covers the three lineages of Glaucophytae, Rhodophytae (red algae) and Viridiplantae (green algae and land plants). Adapted from Simpson & Roger(2004).

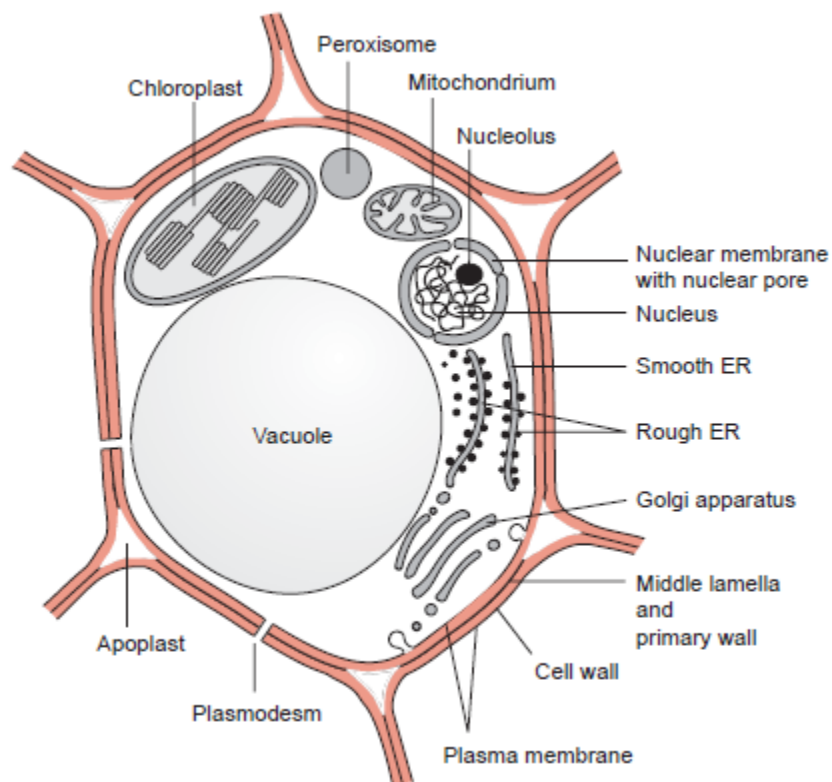


Figure 2: The universal eukaryotic cell. Schematic representation of the cross-section of a plant cell and its organelles; surrounded by six neighbouring cells. The cell-wall is coloured in orange and defines the cell boundary. Adapted from Heldt & Piechulla (2004).

scientific reductionism. The dogma describing the road from DNA to RNA to protein is not so straight though and protein is not the sole functional agent of cell mechanisms. For instance, small RNAs are involved in several processes of DNA and gene products regulation. Several reports have been published on small RNA implication in plant suppression of invading viruses, silencing of aberrant transcripts, transposons, repetitive elements and maintenance of the epigenetically silent state of genes (Lam *et al*, 2001; Ratcliff *et al*, 1997); for a review see Obbard *et al*. (2008). In a non-dividing cell the DNA is spread in the nucleus and associated with proteins, forming the chromatin. During cell division, DNA is condensed into short compact structures, the chromosomes. The non-dividing cell also contains subnuclear bodies called the nucleolus known to be the ribosome synthesizing site. The nucleus is surrounded by the nuclear envelope, composed of a double membrane exhibiting fusion points defining nuclear pores responsible for exchanges between the nucleus and the cytosol by gated transport (for a review see Lamond & Earnshaw, 1998; Park & De Boni, 1999).

1.1.1.2- The endoplasmic reticulum and the Golgi apparatus

The endoplasmic reticulum (ER) and the Golgi apparatus constitute a highly complex membrane structure responsible for the synthesis of lipids, protein neo-synthesis and the secretory pathway.

The ER forms a continuum with the nuclear external membrane and is largely associated with ribosomes on its cytoplasmic face ("Rough ER"). Neo-synthesized proteins migrate to the Smooth ER, a region of the ER lacking association with ribosomes, where post-translational modifications take place. Subsequently, the newly synthesized proteins are carried to the Golgi apparatus by transport vesicles budding out from the ER. The Smooth ER is also the site of lipid biosynthesis for membrane formation and storage. The ER is a very polyvalent organelle taking over a large variety of processes required by the cell, from protein neo-synthesis, phospholipid synthesis to calcium homeostasis (for review see Groenendyk *et al*, 2010; Papp *et al*, 2003).

The Golgi apparatus is made of a stack of cisternae and is physically distinct from the ER. It is responsible for further post-translational modifications of proteins carried by transport vesicles fusing with the Golgi membranes. The subsequently modified proteins exit the Golgi apparatus via secretory vesicles to be targeted to their destination site (for review see Klumperman, 2000; Lippincott-Schwartz, 1993). An important function of the Golgi apparatus in plants is the biosynthesis of complex polysaccharides involved in cell wall formation, e.g. during cell division (Faso *et al*, 2009; Lycett, 2008; Sandhu *et al*, 2009).

1.1.1.3- The vacuole

Plant cells contain a fluid filled compartment called the vacuole delimited by its membrane, the tonoplast. This compartment usually occupies most of the cell's volume and relegates other organelles to the periphery of the cell. The vacuole is filled with a variety of compounds e.g. ions, carbohydrates, enzymes. It is therefore related to a wide variety of cell physiological functions and can operate as a degradation compartment as well as a storage site and drive cell expansion by its turgor pressure (Frigerio *et al*, 2008). Vacuoles often store products of interest for human consumption, e.g. latex, pigments responsible for flower coloration in order to attract pollinators or noxious molecules released when the plant is damaged by a herbivore (Alberts *et al*, 2002; Hopkins, 2003).

1.1.1.4- Mitochondria and chloroplasts

Mitochondria, present in all eukaryotic cells and, chloroplasts, characteristic for plants, are two particular organelles because of their structure and function. They both exhibit a complex membrane system responsible for their energy converting function: the aerobic respiration of mitochondria and the photosynthesis of chloroplasts. These two chemiosmotic coupling phenomena, crucial for the cell, are performed by protein complexes embedded in their membranes.

Performing photosynthesis, chloroplasts are the site of CO₂, nitrite and sulphate reduction and assimilation into carbohydrates, amino acids, fatty acids, and terpenoid compounds. The chloroplast belongs to a family of organelles, referred to as plastids (see section 1.1.3.3). The chloroplast is the hallmark organelle of plants and is characterised by its high content of the pigment chlorophyll responsible for the green colour of plants, along with other pigments e.g. carotenes and xanthophylls. Chloroplasts are discoid structures, about 5 to 10 µm in diameter, present in each cell at a number of 10 to more than 100, depending on the cell type and the species (as reviewed in Block *et al*, 2007; Lopez-Juez & Pyke, 2005). They are delimited by their envelope constituted by an outer and an inner membrane. The internal compartment, the stroma, is an aqueous solution rich in soluble proteins, ribosomes, starch and lipoprotein particles called plastoglobuli (Brehelin *et al*, 2007). It also contains the thylakoids, rich in galactolipids while other plant membranes are phospholipid rich (Douce & Joyard, 1990; Jarvis *et al*, 2000), a highly organised membrane system appearing as stacked cisternae, the grana, interconnected by single flat vesicles exposed to the stroma, the stromal lamellae. This membrane system creates an internal compartment, the thylakoid

lumen. The thylakoid is the place of interrelated photochemical and redox reactions and its organisation allow for spatial compartmentalisation of supramolecular assemblies of proteins, pigments and electron carriers, e.g. photosystem I is present in the stromal thylakoids whereas photosystem II is located to grana stacks (Anderson & Melis, 1983).

Interestingly, the chloroplasts, as well as mitochondria, possess their own genomic DNA and show similarities, e.g. size and shape, with bacteria suggesting an endosymbiotic origin.

1.1.2- The theory of endosymbiosis and the origin of plastids

This paragraph will deal with primary endosymbiosis only as the model plant used in this thesis is a land plant, *Arabidopsis thaliana*; a descendant of the green algae, emerging after their last common ancestor engulfed a cyanobacterium.

According to the theory of endosymbiosis, formulated by Margulis (1971), both mitochondria and plastids are of prokaryotic origin; α -proteobacteria are the ancestor of mitochondria (Andersson *et al*, 1998) and cyanobacteria are the ancestor of chloroplasts (Gould *et al*, 2008; Reyes-Prieto *et al*, 2007). A potential scenario for plastid origin is that cyanobacteria were engulfed through phagocytosis as a prey item countless times by the Plantae ancestor, and in some of these cells, the cyanobacterium was not digested but maintained as an endosymbiont. Over time, the prokaryote was reduced to a double membrane-bound plastid and vertically transmitted to subsequent generations (Figure 3).

As reviewed in Cavalier-Smith (2000), the phagotrophic membrane surrounding the endosymbiont was lost resulting in a plastid with a double-membrane envelope and the outer membrane seems to be a chimera as it has been altered by the host cell to suit nuclear-encoded preprotein import.

According to several lines of evidence, the Plantae clades are considered monophyletic (McFadden & van Dooren, 2004). The evidence for Plantae monophyly comes from molecular phylogenetic and comparative analyses of plastid and nuclear genes involved in plastid functions such as members of the TIC-TOC supertranslocon (section 1.3 and 1.4) responsible for plastid protein import. Indeed, primary endosymbiont plastids are characterised by a double membrane envelope, derived from the plasma membrane and the outer membrane of the Gram-negative cyanobacterium (Vesteg *et al*, 2009). Comparison of membrane's lipid (Nakamura *et al*, 2007) and protein composition of both plastids and cyanobacteria shows similarities supporting homology between them, like the presence of β -barrel proteins in their membrane (Schleiff *et al*, 2003a) or enzymes involved in the chemiosmotic coupling and other biochemical reactions localised in the plastid (Reyes-Prieto & Bhattacharya, 2007).

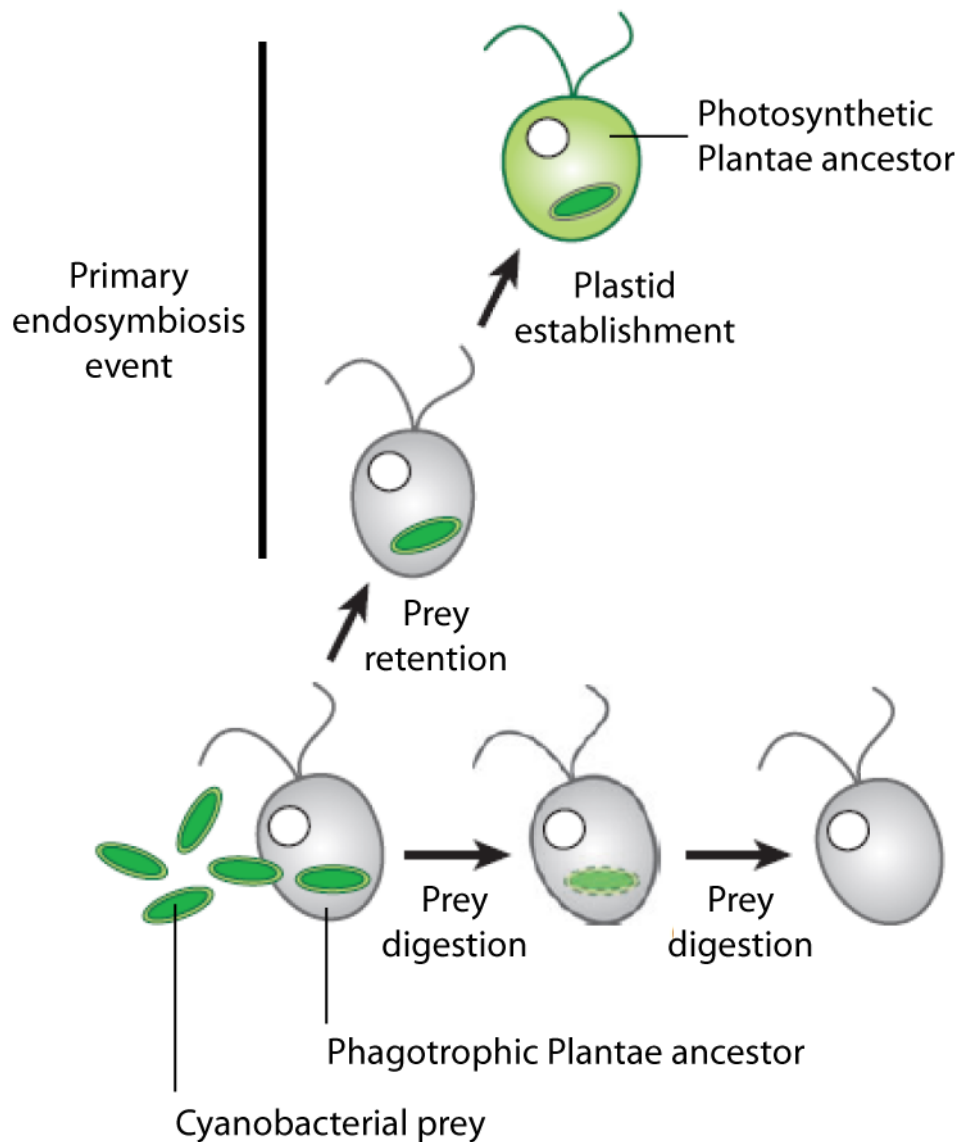


Figure 3: Model of the primary endosymbiotic origin of the plastid in the common Plantae ancestor. During the course of evolution, the phagotrophic Plantae ancestor usually digests the cyanobacterial prey. During the course of evolution, the event of phagotrophy led to the retention of the cyanobacterial prey and subsequently, establishing the lineage of photosynthetic Plantae. Reproduced and modified after Reyes-Prieto *et al* (2007).

Nevertheless, an alternative hypothesis has been proposed suggesting that the apparent monophyly of the Plantae clades is due to a convergent evolution (Stiller *et al*, 2003).

1.1.3- Three genomes and their coordination

As mentioned before, all eukaryotic cells harbour at least two genomes: the nuclear and mitochondrial ones. Plants harbour a third one: the chloroplastic genome (section 1.1.1.4). These three genomes do not function independently; they underwent evolution together towards a complex coordination of gene expression. Additionally, based on tissue specificity, plastids assume different structures and functions as a reflection of their genome expression.

1.1.3.1- Gene transfer from the plastid to the nucleus

During endosymbiosis, gene transfer from the plastid to the host cell's nucleus took place. The cyanobacterial ancestor of plastid counts approximately 3000 genes (Kaneko *et al*, 1996). There is a relic of 10% of these genes coming from the cyanobacterial ancestor still present in the plastid DNA. The remaining 90% were transferred from the endosymbiont to the nucleus. Nevertheless, it has been published that of these 90%, a fifth is derived from the cyanobacterial ancestor whereas the large remaining part of this pool of genes is derived from the host (Martin *et al*, 2002). The replacement or addition of components was frequently observed for most of the plastid functions except for the light reaction of photosynthesis and the translation/degradation of proteins in the plastid. A considerable amount of bacterial genomic material other than cyanobacteria, as well as host's and endosymbiont's genomic material, has contributed to the establishment of the plastid before the separation of rhodophyte and glaucophyte (Suzuki & Miyagishima, 2009).

The cause for gene transfer has not been elucidated but it seems that the endosymbiosis is mainly a host driven process. The nucleus controls most cellular activities and the synthesis of proteins destined for the plastid is regulated in a tissue specific fashion (Egea *et al*, 2010; Padmanabhan & Dinesh-Kumar, 2010). This observation is an element that supports gene transfer from the plastid to the nucleus. A complementary observation proposes that the gene transfer is still an ongoing process that is not yet finished or supports evolutionary flexibility for further adaptation (Matsuo *et al*, 2005).

1.1.3.2- The cross-talk between the nucleus and plastids

One reason for massive gene transfer from the plastid to the nucleus is to ensure the anterograde (from nucleus to plastid) control of the organelle. Indeed the nucleus controls most cellular activities. The regulation of the plastid also includes sensing the plastid's state which is achieved by retrograde (from plastid to nucleus) signalling (for review see Leister, 2005). It is not clear to date which molecules are responsible for acting as signalling factors regulating the nuclear gene expression. It is possible that the chloroplast controls nuclear gene expression indirectly by metabolic signalling with ROS and redox state modification (for review see Kleine *et al*, 2009). In addition, it has been demonstrated that intermediates of the plastidial tetrapyrrole pathway, may control nuclear gene expression (Rintamaki *et al.*, 2009). This cross-talk between the nucleus and the plastid implicates mitochondria as well, making the overall picture even more complex (Figure 4).

1.1.3.3- Chloroplast biogenesis and the different plastid types

Early, in the course of plant growth and development, plastids differentiate from a proplastid (Figure 5). All plastid types can therefore be considered as interrelated and interconvertible. In leaves though, most chloroplasts arise through plastid division and not directly from a proplastid. Depending on the tissue specificity and environmental cues, plastids will differentiate into a specific plastid type classified as either photosynthetic or non-photosynthetic plastid (Bowsher & Tobin, 2001). The photosynthetic plastid in green tissues is the chloroplast. Other non-photosynthetic plastids can be distinguished by their structure, pigment composition, and function (Thomson, 1980); e.g. eoplast, a basal-state plastid resulting from the de-differentiation of a chloroplast during the late stages of embryo maturation (Ruppel *et al*, 2011), chromoplast, a pigment accumulating plastid present in flowers or ripening fruit and differentiated from a chloroplast (Kahlau & Bock, 2008), amyloplast, present in many root cells for starch storage, elaioplast, for the storage of lipids, leucoplast, for the storage of aromatic oils, xeroplast, a desiccation tolerant proplastid present in *Xerophyta humilis* (Ingle *et al*, 2008) (for a review see Lopez-Juez & Pyke, 2005). When a seedling grows in the dark, its proplastids differentiate into etioplasts, subsequently the plant develops the etiolation process characterised by an elongated hypocotyl, an apical hook and closed cotyledons. Etioplast have pro-lamellar bodies, precursors of the thylakoid membranes, and represent primed organelles ready to undergo a rapid differentiation upon light exposure (Waters & Langdale, 2009). Indeed the etioplast proteome shows drastic and

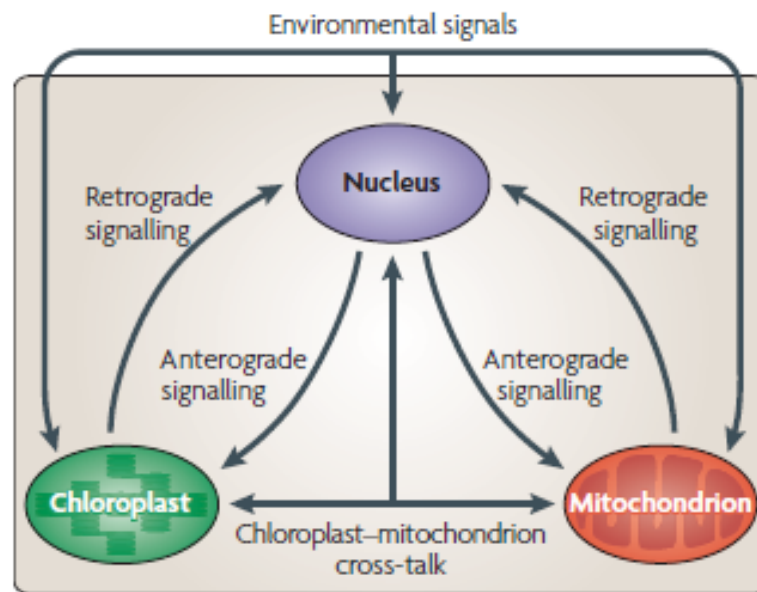


Figure 4: An overview of genome co-ordination between the nucleus and intracellular organelles. The diagram depicts communication between the nucleus, chloroplast and mitochondrion. Environmental signals such as stress, oxygen, nutrient availability, light intensity or quality, and hormones affect the expression of nuclear genes that encode organellar proteins. This process affects organelle functions and gene expression through anterograde mechanisms. Chloroplasts and mitochondria are also able to sense certain environmental conditions and stimuli that can affect their functional activities, for example, light intensity or quality (chloroplasts) and O_2 availability (mitochondria). Using retrograde signals, organelles communicate these received stimuli and their functional status to the nucleus, which leads to nuclear gene regulation. Adapted from Woodson & Chory (2008).

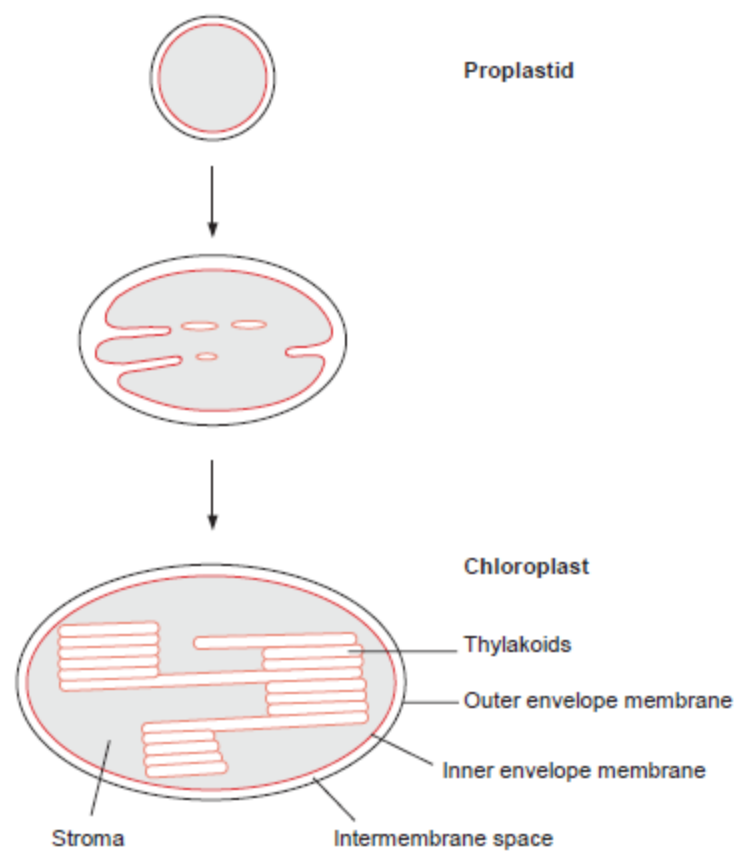


Figure 5: Differentiation of a proplastid into a chloroplast. This scheme depicts the transition from an undifferentiated proplastid to a complex organelle, the chloroplast with its specialised membrane system. Adapted from Heldt & Piechulla (2004).

rapid changes as early as two hours after illumination, reflecting a shift from a heterotrophic towards an autotrophic metabolism (Kleffmann *et al*, 2007).

1.2- The process of protein import into the chloroplast

Most of the proteins required for plastid functions are encoded in the nucleus and translated by cytoplasmic ribosomes. These nuclear-encoded proteins need to be addressed to their final destination in the plastid and pass through the two envelope membranes (Figure 6). They are post-translationally targeted to the plastid's envelope which is one of the main sites for the coordination of plastids with other compartments of the cell (Block *et al*, 2007). The chloroplast outer envelope membrane is permeable to molecules up to 10 kDa while the inner envelope membrane is selective and contains many specific transporters (Lopez-Juez & Pyke, 2005). The transmembrane transport of neo-synthesised proteins en route to the chloroplast occurs in an energy-dependent manner through a multi-protein import machinery, the TOC-TIC supertranslocon (section 1.3 and 1.4) spanning the outer and inner membranes of the chloroplast envelope. At the N-terminal end of the neo-synthesised protein there is a targeting sequence, the transit peptide, which will help to deliver the protein to its final destination compartment. The transit peptide is a requisite for protein import through the plastid's envelope and is cleaved after transport by a soluble peptidase (Grossman *et al*, 1980), the Stromal Processing Peptidase (Richter & Lamppa, 1999). For instance, the small subunit (SSU) of Rubisco is synthesised in the cytosol as a preprotein with a cleavable N-terminal transit peptide and imported post-translationally into the chloroplasts and is widely used as a model import substrate. Further targeting of plastid nuclear-encoded proteins inside the plastid, to the thylakoid membranes and lumen occurs through four independent pathways: the spontaneous insertion pathway, the Signal Recognition Particle (SRP) system, the Secretory pathway (Sec) and the Twin-Arginine Translocase (Tat) system, (for review see Aldrige *et al*, 2009).

1.2.1- The chloroplast transit peptide

Transit peptides are N-terminal extensions of nuclear encoded precursor proteins (termed preprotein in this thesis) en route to the chloroplast. The transit peptide is necessary and sufficient for the targeting and translocation into plastids via a post-translational mechanism. Transit peptides are highly divergent in length (from 20 to 80 amino-acids), composition, and organisation (as reviewed in Bruce, 2001); however, they are rich in Serine

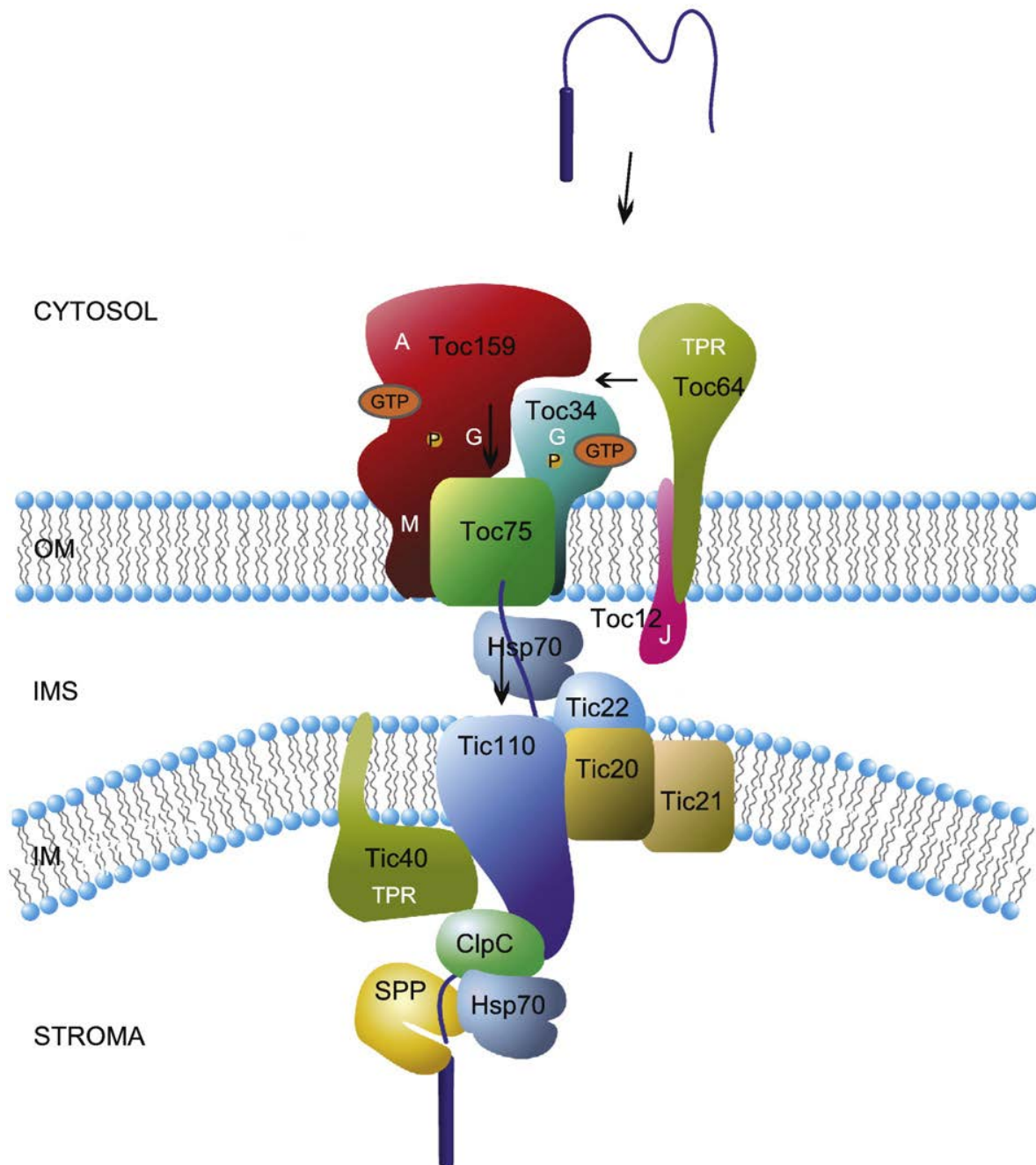


Figure 6: Import of a preprotein across the chloroplast envelope is mediated by the TOC-TIC supertranslocon. The nuclear-encoded preprotein bearing an N-terminal transit peptide is targeted towards the outer membrane of the chloroplast where it is recognised by the Toc GTPases, Toc159 and Toc33. Subsequently is formed the early import intermediate which corresponds to the GTP- and ATP-dependant association of the preprotein with the import machinery, the preprotein spans the outer membrane. Finally, during the ATP-dependant late translocation intermediate, the preprotein spans both the outer and inner membranes. After its complete translocation from the cytosol to the stroma, the transit peptide of the preprotein is cleaved and degraded. Adapted from Andres *et al* (2010).

and Leucine (Bhushan *et al*, 2006) and exhibit a high proportion of hydroxylated, hydrophobic and positively charged amino-acids (Zhang & Glaser, 2002). Transit peptides have multiple semi-conserved subdomains with complex relationships that seem to be involved at different stages of the targeting and translocation process (Lee *et al*, 2006; Lee *et al*, 2008; Rensink *et al*, 1998). A direct interaction with outer envelope lipids (Pinnaduwaage & Bruce, 1996), as well as association with a guidance complex favoured by the phosphorylated state of the preprotein (May & Soll, 2000), recognition by envelope receptors, insertion into the TOC-TIC supertranslocon, interaction with molecular motors (Becker *et al*, 2004) and finally, recognition/cleavage by the stromal processing peptidase (Gavel & von Heijne, 1990) have been shown. Despite the large body of experimental evidence, it still remains difficult to draw a clear picture of the events involving the transit peptide in the translocation process.

Upon entering the chloroplast, the transit peptide is cleaved by the stromal processing peptidase followed by folding and assembly of the mature form (Richter & Lamppa, 1998); any defect in this process may result in the loss of biochemical function of that protein (Zhong *et al*, 2003). However, the canonical view of the role of the transit peptide is completed by experimental evidence highlighting the existence of non-cleavable transit peptides (Armbruster *et al*, 2009).

1.2.2- Energy requirement

The transport across the chloroplastic envelope can be divided into three stages according to its energy requirement.

At the first stage, there is no need for energy and the preprotein associates with the chloroplastic envelope reversibly (Ma *et al*, 1996; Perry & Keegstra, 1994).

In the second stage of import, the preprotein at the chloroplast surface forms an early import intermediate with the import machinery. The envelope associated preprotein spans the outer chloroplast membrane en route to the stroma. This binding step is irreversible and is promoted by hydrolysis of low concentrations of GTP and ATP (100 μ M), the ATP being hydrolysed in the intermembrane space (Kouranov & Schnell, 1997; Olsen & Keegstra, 1992; Pain & Blobel, 1987; Schnell & Blobel, 1993). The requirement for ATP has been attributed to the activity of molecular chaperones that are postulated to bind and stabilise preproteins during their transport across the outer membrane (Chen & Schnell, 1999; Schnell *et al*, 1994).

The ultimate stage is the complete translocation of the preprotein through both the outer and the inner envelope membrane and, when arrested, results in the late translocation

intermediate. This step requires high concentrations of ATP within the stroma, ranging from 1 to 3 mM (Pain & Blobel, 1987; Theg *et al*, 1989). Its hydrolysis is presumably mediated by stromal molecular chaperones such as Hsp60, Hsp70 or Hsp100 (Kessler & Blobel, 1996; Nielsen *et al*, 1997; Shi & Theg, 2010; Su & Li, 2010). One of these is Hsp93 (a member of the Hsp100 family of molecular chaperones) that functions in close association with the TIC complex and has been proposed to bind the preprotein as it penetrates into the stromal compartment and provide part of the driving force (Akita *et al*, 1997; Kovacheva *et al*, 2007).

1.3- The TOC core complex

The initial plant model that was used to study chloroplast protein import was *Pisum sativum*. In this plant, the protein import machinery of the outer envelope membrane, the TOC complex is composed of psToc159, psToc75 and psToc34.

The TOC complex of *A. thaliana* includes three core components, namely atToc159, atToc75 and atToc33 (Kessler *et al*, 1994; Schnell *et al*, 1994; Tranel *et al*, 1995); all homologues of the Toc proteins found in *P. sativum*. The number of the Toc proteins corresponds to their molecular mass in kDa. Both atToc159 and atToc33 are GTPases; Toc159 belongs to the family of large acidic Toc GTPases (Toc90, Toc120, Toc132 and Toc159) and atToc33 to the family of small Toc GTPases (Toc33 and Toc34) as classified by Reddick *et al*. (2007). As shown in Figure 7 the protein Toc75 is the translocation channel found in any TOC complex. In Arabidopsis, Toc GTPases are encoded by more than one gene and appear to associate into distinct but homologous TOC-TIC complexes reflecting different import pathways essential for the differentiation and specific functions of distinct plastid types during the course of plant growth and development (Kessler & Schnell, 2006; Smith, 2006). It is possible to find different types of TOC complex with specificity for different types of preprotein. In Arabidopsis, the photosynthetic-related preproteins are translocated preferentially by a TOC complex made of Toc159 and Toc33 whereas other types of preproteins will preferentially be translocated by a complex composed of Toc132 or Toc120 and Toc34. Another complex involving Toc90 seems to be partially redundant with the complex involving Toc159 and Toc33. Electron microscopy and molecular analysis reveal a calculated mass of the TOC core complex of approximately 550 kDa with a stoichiometry of 4:4-5:1 for psToc75:psToc34:psToc159, respectively (Schleiff *et al*, 2003c). However, slightly different stoichiometries have also been reported (Kikuchi *et al*, 2006; Vojta *et al*, 2004). The three proteins that form the trimeric TOC core complex will be described below.

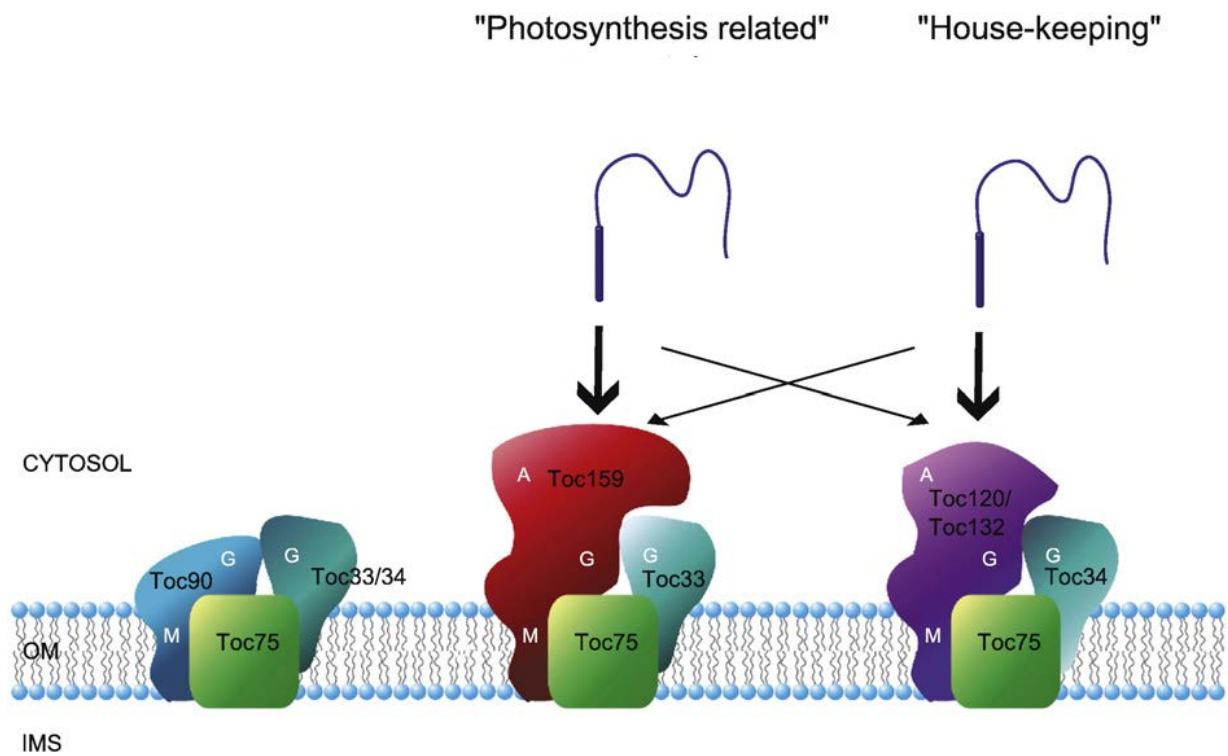


Figure 7: Alternative chloroplast protein import pathways in *Arabidopsis*. atToc159, atToc33, and atToc75 cooperate to import photosynthetic related proteins during chloroplast biogenesis. Varying combinations of atToc132/120/90, atToc33/34, and atToc75 support the import of non-photosynthetic plastid housekeeping proteins. The functional overlap of the different type of TOC complex is represented by arrow cross-over. Numbers indicate the molecular mass of the Toc proteins. Adapted from Andres *et al* (2010).

1.3.1- Toc GTPases

atToc159 and atToc33 are the two GTPases of the TOC core complex and share a highly conserved GTP-binding domain (G-domain). They are both of eukaryotic origin (Reumann & Keegstra, 1999) and belong to a distinct family of plant GTPases (Sun *et al*, 2002), the septin family of GTPases within the TRAFAC class (Aronsson & Jarvis, 2011). Yet, there has been a long debate to which of the two GTPases acts as the primary receptor (Chen *et al*, 2000; Schleiff *et al*, 2003b). Now, it seems appropriate to consider them as mediating the recognition of the chloroplast targeting peptide in concert (Kouranov & Schnell, 1997).

In Arabidopsis, two genes encode proteins homologous to pea Toc34: *atTOC33* and *atTOC34* (Hiltbrunner *et al*, 2001a; Jarvis *et al*, 1998). Large Toc GTPases, the Toc159 family, are encoded by four genes: *atTOC159*, *atTOC132*, *atTOC120* and *atTOC90*. The homologues of Toc159 share a similar domain structure, only their N-terminal acidic domains (A-domain) differ in length and sequence (Bauer *et al*, 2000; Hiltbrunner *et al*, 2001a) (Figure 8).

Toc159 is a tripartite protein composed of an N-terminal acidic domain (A-domain), a central GTP binding domain (G-domain) and a C-terminal membrane anchoring domain of 52 kDa (M-domain) (Chen *et al*, 2000; Hiltbrunner *et al*, 2001a). Both the A- and G-domain are exposed to the cytosol whereas the M-domain is anchored in the outer membrane of the chloroplast envelope (Hirsch *et al*, 1994). atToc33 is mostly constituted of its G-domain and is anchored in the outer membrane by a short C-terminal part which contains a stretch of hydrophobic amino-acids (Chen & Schnell, 1997).

The G-domain of Toc GTPases exhibits classical motifs of guanine nucleotide binding and hydrolysis characteristic of many GTPases (Bourne *et al*, 1991; Bourne *et al*, 1990) as well as a dimerisation motif. The crystal structure of the G-domain of psToc34 and *in vitro* studies suggest that the homodimerisation of psToc34 as well as heterodimerisation with the G-domain of Toc159 occurs in its GDP-bound state (Sun *et al*, 2002; Weibel *et al*, 2003).

Phosphorylation of the G-domain of small Toc GTPases has been reported previously although the phosphorylation pattern is not the same when comparing Pea and Arabidopsis. Phosphorylation leads to a negative regulation of both preprotein (Sveshnikova *et al*, 2000) and GTP binding (Jelic *et al*, 2003; Jelic *et al*, 2002). The phosphorylation of the G-domain regulates the assembly of the chloroplast import apparatus, more specifically in the case of Toc33 (Oreb *et al*, 2008). Nevertheless, the role of post-translational phosphorylation of small Toc GTPases is controversial and the importance of this process *in vivo* has been challenged (Aronsson *et al*, 2006; Oreb *et al*, 2007).

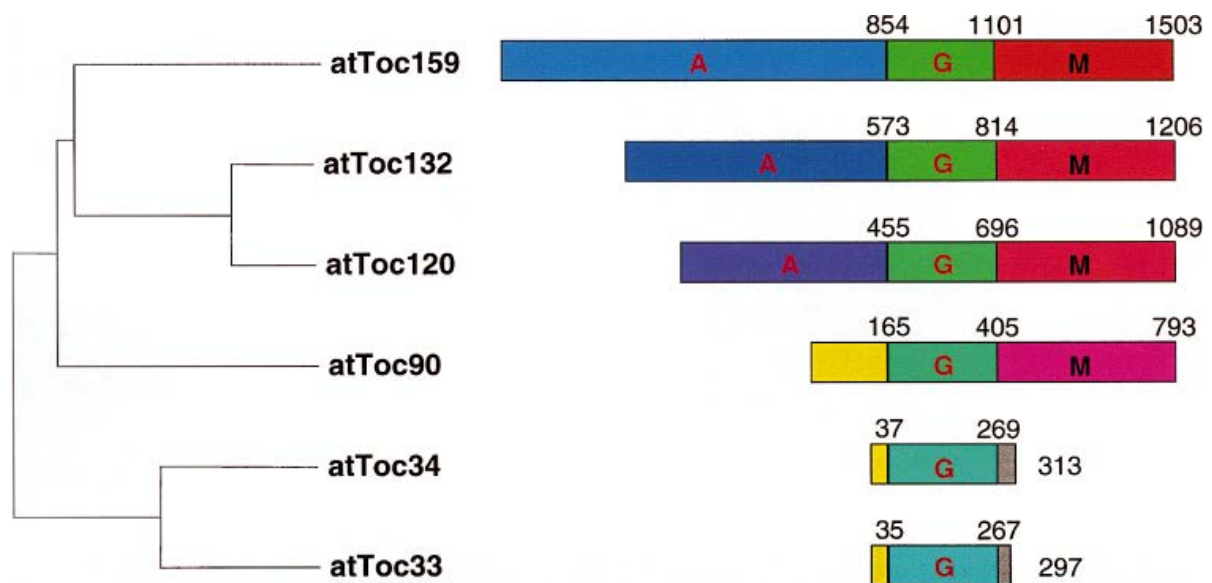


Figure 8: Phylogenetic tree and structure of the GTP-binding components of the TOC complex in *Arabidopsis*. The members of the Toc GTPase family fall into two subgroups, the large Toc GTPases homologues of Toc159 (atToc159, atToc132, atToc120, and atToc90) and the small Toc GTPases atToc33 and atToc34. AtToc90 lacks the characteristic A-domain of atToc159, atToc132, and atToc120. M, membrane anchor domain (given in shades of red); G, GTP-binding domain (given in shades of green); A, acidic domains (given in shades of blue); regions other than the A, G, and M domains are given in yellow; transmembrane helices of atToc33 and atToc34 are given in grey. Numbers above the Toc protein structure indicate the amino acid number. Adapted from Hiltbrunner *et al* (2001a).

1.3.1.1- Large Toc GTPases: Toc159 and its homologues

Toc159 was initially identified as an 86 kDa fragment (Toc86 or Toc159GM) because of its high sensitivity to proteolysis (Bolter *et al*, 1998; Kessler *et al*, 1994; Waegemann & Soll, 1991). Toc159 has been proposed to be involved in initial preprotein binding (Chen *et al*, 2000).

The initial docking and insertion of Toc159 receptor at the TOC complex requires its intrinsic GTPase activity along with the presence of Toc33. The association of the two G-domains stimulates membrane anchoring of Toc159 to the translocon (Bauer *et al*, 2002; Smith *et al*, 2002). Additionally, the M-domain of Toc159 interacts with both the G-domain of Toc33 and Toc75 which are the two necessary components for the proper integration of Toc159 into the chloroplast outer envelope (Wallas *et al*, 2003). The M-domain, the function of which is to anchor the protein in the outer membrane, was shown to partially complement the preprotein import defect in the *ppi2* mutant (Lee *et al*, 2003).

Characterisation of the *ppi2* mutant (plastid protein import), an atToc159 T-DNA insertion mutant, showed that the differentiation of proplastids into chloroplasts is blocked, resulting in an albino phenotype (Bauer *et al*, 2000): the plant cannot develop photo-autotrophically. Both the accumulation of photosynthesis-related proteins was decreased along with their level of expression. This did not appear to be the case for non-photosynthetic plastid proteins. It was proposed that these are imported by other members of the large Toc GTPases family, namely Toc132 and Toc120 and suggest the existence of functionally distinct photosynthetic and non-photosynthetic plastid protein import pathways (Bauer *et al*, 2000; Kubis *et al*, 2004) (Figure 7).

Single knock-out mutants of atToc132, atToc120 or atToc90 show no specific phenotypes, indicating that their function is not essential (Hiltbrunner *et al*, 2004; Kubis *et al*, 2004). However, the double mutant atToc132/atToc120 is lethal (Ivanova *et al*, 2004), or has a phenotype similar to *ppi2* (Kubis *et al*, 2004), which confirms that these two proteins are redundant and constitute a structural and functional subclass of protein import receptors. The double knock-out mutants atToc90/atToc132 and atToc90/atToc120 have no phenotype even though *atTOC90* expression level is high throughout development of WT plants (Kubis *et al*, 2004). These results show that there is no functional redundancy between atToc90 and atToc132/atToc120 subgroup. Therefore the role of Toc90 still has to be unravelled. Although the role of Toc90 is not clearly understood, it was able to partially complement *A. thaliana ppi2* mutant (Infanger *et al*, 2011). Toc90 was shown to increase the accumulation of photosynthetic proteins in the *ppi2* genetic background and therefore proposed to contribute to the same import pathway (Hiltbrunner *et al*, 2004).

A soluble form of Toc159, shuttling between the cytosol and the outer membrane of the chloroplast, has been proposed but was under debate (Becker *et al*, 2004; Hiltbrunner *et al*, 2001b). Recently, however, it was discovered that the A-domain of Toc159 exists as a separate soluble phospho-protein. It now appears likely that the A-domain was responsible for the observation of the soluble form of Toc159. The physiological role of the separated A-domain remains elusive (Agne *et al*. 2010). The function of the A-domain of Toc159 is still under investigation though it has been demonstrated to be non-essential for chloroplast biogenesis. Indeed, proteins lacking this domain can complement the *ppi2* mutant (Lee *et al*, 2003). The A-domains of the Toc159 homologues are proposed to be major determinants of distinct pathways for protein import into chloroplasts as demonstrated by observations made after A-domain swapping experiments (Inoue *et al*, 2010). Also, Toc132GM, lacking the A-domain, was able to partially complement the *ppi2* mutant (Inoue *et al*, 2010). Furthermore, the isolated A-domain behaves as an intrinsically disordered protein (Richardson *et al*, 2009). Evidence for the presence of the separated A-domain as a stable and abundant cytosolic phosphoprotein and also extrinsically associated with the outer membrane of chloroplast envelope has been demonstrated by Agne *et al* (2010). Together, these data strongly suggest a regulatory role for the A-domain during import of preprotein into the chloroplast.

The detailed analysis of the G-domain will be discussed in a later chapter (section 1.6).

1.3.1.2- Small Toc GTPases, Toc33 and Toc34

psToc34 is synthesized without a cleavable transit peptide. It belongs to the class of TA (Tail Anchored proteins) requiring an AKR2A (Bae *et al.*, 2008) for membrane insertion into the outer membrane lipid bilayer of the chloroplast in its GTP-bound form (Qbadou *et al*, 2003). In *Arabidopsis* the knock-out mutant for atToc33, the *ppi1* mutant, has a pale green phenotype disappearing after the first weeks of development. This indicates that atToc33 is involved during the early stages of plant development. atToc34 can complement the loss of atToc33 expression (Jarvis *et al*, 1998). The *ppi1* mutant also exhibits a defect in expression and import of photosynthetic related proteins suggesting that atToc33 is mainly involved in import of photosynthetic proteins (Kubis *et al*, 2003). The *ppi3* mutant plant, knock-out for atToc34, has no visible phenotype apart from delayed root growth. These observations suggest that atToc34 has a more important role for plastid biogenesis in roots and that its function overlaps with that of atToc33 (Constan *et al*, 2004). Additionally, the double mutant *ppi1/ppi3* is embryo lethal, indicating that the combined function of these two proteins is

essential for protein import into plastids in different tissues during early development (Constan *et al*, 2004).

1.3.2- Toc75

Toc75 is a protein of the chloroplast outer envelope membrane of prokaryotic origin (Reumann *et al*, 1999). It has been identified as a component of the transport apparatus and shown to be a channel protein (Perry & Keegstra, 1994; Schnell *et al*, 1994; Tranel *et al*, 1995; Hinnah *et al*, 2002; Schleiff *et al*, 2003a). The model plant *A. thaliana* has three genes encoding for homologues of pea Toc75 (psToc75): *atTOC75-I*, *atTOC75-III*, and *atTOC75-IV*. *atTOC75-III* seems to code for the functional homologue of psToc75 and is likely to be the major isoform of Toc75 in *Arabidopsis* (Jackson-Constan & Keegstra, 2001). Toc75 is synthesised as a preprotein with a bipartite targeting peptide containing chloroplastic and intraorganellar targeting information for its proper insertion into the chloroplast outer membrane (Tranel & Keegstra, 1996). The N-terminal part of its targeting peptide consists of a transit peptide and targets the protein to the stroma and is cleaved by the stromal processing peptidase whereas the C-terminal part prevents import of the remainder of the protein to the stroma and mediates outer envelope insertion. Toc75 is a β -barrel protein bearing a POTRA (POLypeptide TRansport Associated) domain (Sanchez-Pulido *et al*, 2003). This protein has been predicted to be a cation selective conducting channel (Hinnah *et al*, 1997) and is a major component of the TOC apparatus making contact with regions of preproteins that are inserted across the outer membrane (Ma *et al*, 1996).

1.4- The TIC complex

The TIC complex is in charge of the late stages of preprotein import into the stromal compartment. The transient association of TOC and TIC complexes allow for the precursor protein to be transferred through the chloroplastic double membrane from the cytosol to the stroma. As reviewed in Dutta *et al* (2009), the TIC complex is composed of several components consisting of Tic110 (Kessler & Blobel, 1996; Lubeck *et al*, 1996), Tic62 (Kuchler *et al*, 2002), Tic55 (Caliebe *et al*, 1997), Tic40 (Stahl *et al*, 1999), Tic32 (Hormann *et al*, 2004), Tic22 (Kouranov & Schnell, 1997), Tic21 (Ma *et al*, 1996), Tic20 (Chen *et al*, 2002). The number of the different Tic proteins corresponds to their molecular mass in kDa.

The *in vitro* analysis of Tic110 has revealed potential roles in several key events of protein import into plastids such as TIC complex assembly, preprotein binding and the recruitment of molecular chaperones to translocon sites. Tic110 is essential for plastid biogenesis in Arabidopsis, indeed T-DNA insertion lines are embryo-lethal (Inaba *et al*, 2003). The central role of Tic110 in the TIC complex makes it an essential and general component of the protein import apparatus. Tic110 appears to participate in the import of all transit peptide mediated import pathways of plastids as it associates in TOC-TIC supercomplexes with any component of the Toc159 GTPase family (Kovacs-Bogdan *et al*, 2010). Molecular studies and computational analysis predict Tic110, Tic21 and Tic20 to be the putative components of the inner membrane preprotein conducting channel (Heins *et al*, 2002; Reumann & Keegstra, 1999; Teng *et al*, 2006).

During the early import intermediate phase, Tic22 is postulated to interact with the preprotein at the intermembrane space and probably facilitates its movement from TOC to TIC, as evidenced by cross-linking experiments (Kouranov & Schnell, 1997).

Biochemical analysis and tertiary structure analysis showed that Tic40 has a large hydrophilic domain projecting in the stroma and it has been proposed to function as a co-chaperone facilitating preprotein translocation across the inner membrane of the chloroplast through the interaction with ClpC (Bedard *et al*, 2007; Chou *et al*, 2003).

The other TIC components, Tic62, Tic55, and Tic32, have been proposed to act as redox regulators for import of preproteins (Caliebe *et al*, 1997; Hormann *et al*, 2004; Kuchler *et al*, 2002). Recently, interaction of the stromal Hsp70 with TIC components has emerged as an important process in preprotein import into chloroplasts of *A. thaliana*, *P. sativum* and *P. patens* (Shi & Theg, 2010; Su & Li, 2010).

1.5- Models of protein import by the TOC complex

Based on almost 20 years of molecular and cellular studies, two models have been drafted in order to depict the mechanistic events happening during the process of preprotein translocation across the envelope of chloroplasts from the cytosol to the stroma. In summary, the available data suggest the central role of both Toc159 and Toc33 GTPase families which dimerise in a nucleotide-dependant mechanism.

1.5.1- The “motor” model

The guidance complex is a multi-protein complex composed of the preprotein, a 14-3-3 protein and the chaperone Hsp70. The formation of the guidance complex is favoured by the phosphorylated state of the preprotein and renders it import competent (May & Soll, 2000). The motor model states that the preprotein, through interaction with the guidance complex (May & Soll, 2000), is targeted to the GTP-bound form of psToc34 (Schleiff *et al*, 2002). Subsequently, the TOC core complex associates, psToc34 forms a heterodimer with Toc159 (Smith *et al*, 2002; Sun *et al*, 2002). The C-terminal part of the transit peptide, in the phosphorylated state, is recognised by psToc34, whereas Toc159 recognises only the unphosphorylated transit peptide (Becker *et al*, 2004). The N-terminal part of the transit peptide binds to the GTP-bound form of Toc159. The transit peptide stays bound to psToc34 long enough for the phosphate to be removed, in order to be completely passed to the motor and the channel proteins, Toc159 and Toc75, respectively (Becker *et al*, 2004). The interaction with the transit peptide stimulates GTP activity of psToc34 (Jelic *et al*, 2002) and the small Toc GTPase dissociates from the preprotein. Toc159 acting as a GTP-dependent motor, drives the preprotein across the Toc75 channel in the outer envelope membrane. After dephosphorylation of the transit peptide, it induces GTP hydrolysis at Toc159 receptor which pushes the preprotein, through the channel Toc75, by performing several cycles of GTP hydrolysis, until the preprotein is completely transferred to the stromal compartment (Becker *et al*, 2004).

According to the stoichiometry of the TOC core complex (Schleiff *et al*, 2003c), psToc34 has been proposed to function as a receptor and Toc159 as a central catalytic motor (Schleiff *et al*, 2003b) for the translocation of preproteins. Furthermore, psToc34 was predominantly found in association with cross-linked preprotein at the early import stage, whereas Toc159 interaction was more pronounced in later stages (Kouranov & Schnell, 1997).

1.5.2- The “targeting” model

According to the original targeting model (Kessler & Schnell, 2004), Toc159 is the primary soluble receptor of preproteins, conferring specificity to the import pathway. A soluble pool of Toc159 was detected (Hiltbrunner *et al*, 2001b), supporting a shuttling mechanism of Toc159 between a soluble cytosolic form and a membrane-bound form, similar to both that of the Signal Recognition Particle (SRP) and the Sec systems. The former involving a targeting mechanism based on two homotypic GTP- binding proteins (Keenan *et al*, 2001) and the latter involving a mobile subunit of an integral membrane receptor

consuming ATP for its insertion/de-insertion while guiding preproteins across the membrane (Economou & Wickner, 1994). The soluble GTP-bound Toc159 would bind the preprotein then targets and integrates to the chloroplast envelope by a process involving the GTP-bound atToc33 as a docking receptor. The preprotein stimulates the GTP hydrolysis of both GTPases (Chen *et al*, 2000; Jelic *et al*, 2002) thereby favouring their heterodimerisation (Sun *et al*, 2002) and a conformational modification allowing the preprotein translocation through the protein conducting channel, Toc75, followed by the engagement of the TIC complex. Recently, however, it was discovered that the A-domain alone, separated from the rest of the protein makes up the majority of the soluble Toc159 (Agne *et al*, 2010). It now appears that Toc159 is only soluble immediately before its targeting to the outer membrane and the TOC complex. This model of the TOC complex agrees well with the available data: the GTPase activity of both Toc159 and Toc33 is required for the proper targeting and insertion of Toc159 into the membrane (Wallas *et al*, 2003) and the GDP-bound state conformation stabilises the association of Toc159 and Toc33 *in vitro* (Smith *et al*, 2002).

1.5.3- Two pictures: spot the difference!

Both of the models presented above have been contradicted to certain extents by published experimental evidences. Yet, the author of this manuscript thinks the “targeting” model, but excluding Toc159 as a soluble preprotein receptor, gathers more convincing elements.

On one hand, challenging the “motor” model, it has been shown that phosphorylation of the transit peptide of preprotein is non-essential for import into the chloroplast (Nakrieko *et al*, 2004). Also, the fact that the M-domain alone can partially complement the *ppi2* mutant (Lee *et al*, 2003) and that GTP hydrolysis influences events leading to the formation of early-import intermediates but not subsequent steps, such as the preprotein translocation (Young *et al*, 1999), suggest that the revolving GTP motor of Toc159 is unlikely. Furthermore, the ability of defective GTPase defective hydrolysis Toc159 mutant to support preprotein import into chloroplasts has been demonstrated (Wang *et al*, 2008; Agne *et al*, 2009). On the other hand, the “targeting” model has been questioned by the fact that the soluble population of Toc159 derives from partial membrane disruption due to the experimental set-up and that the vast majority of the soluble Toc159 consists of the A-domain alone (Agne *et al*, 2010). This indicates that all preprotein interactions with the TOC complex occur on the membrane (Becker *et al*, 2004).

Although "motor" and "targeting" models diverge on which is the primary preprotein receptor and the import mechanism, the most important point is what they share. First and foremost,

both psToc34 (Sveshnikova *et al*, 2000) and Toc159 (Perry & Keegstra, 1994) have been shown to recognise and bind to the preprotein. Second, the importance of the interaction between atToc33 and Toc159, forming a heterodimer in their GDP-bound state. As revealed by the crystal structure of psToc34 (Sun *et al*, 2002), the GDP-bound dimer suggests a central role for the nucleotide-dependent dimerisation of the Toc GTPases in the mechanism of the translocon. Binding studies also confirmed the involvement of the G-domains for the direct interaction of Toc159 and psToc34 (Weibel *et al*, 2003) and their preferential interaction in the GDP-bound state. However, it has been shown *in vitro* that homodimerisation of Toc34 or Toc33 is not strictly dependent on the GDP- or GTP-bound state (Koenig *et al*, 2008). Thus, it is likely that the TOC complex activity is regulated to some extent by the Toc GTPases interaction through GTP-binding and GTP-hydrolysis.

1.6- Genetic analysis of the Toc33 and Toc159 GTPases

psToc34 and psToc159 first identified in *Pisum sativum* (Schnell *et al.*, 1994) and share common motifs with the canonical Ras related GTPases (Kessler *et al*, 1994; Sun *et al*, 2002). This indicates that they may function according to the principles established for the superfamily of small GTPases (for review see Bourne *et al.*, 1990, 1991; Colicelli, 2004). The crystal structure of psToc34 suggested that homodimerisation of the G-domain of the Toc GTPases may play a role during the process of preprotein import into chloroplasts and may be regulated by GTP hydrolysis and GDP exchange (Sun *et al.*, 2002). Heterodimerisation of atToc33 with atToc159 was also reported (Hiltbrunner *et al.*, 2001). According to the characterisation of *ppi1* (the pale mutant lacking Toc33) and *ppi2* (the albino mutant lacking Toc159) plants, the functional redundancy within the family of small Toc GTPases is greater in comparison to that within the family of large acidic Toc GTPases, (Jarvis *et al.*, 1998; Bauer *et al.*, 2000). Indeed, homologues of Toc159 appear to have much more stringent specificity for their preprotein substrates (Kessler & Schnell, 2006).

Early studies demonstrated that the G-domain of Toc159 is essential for its targeting and insertion into the outer membrane of chloroplast, its assembly into the TOC complex as well as binding of preprotein (Smith *et al.*, 2004). For instance, transient expression of strong mutants such as Toc159mGTP (A864R/K868R/S869R) (Bauer *et al.*, 2002), Toc159 S869N and Toc159 D909L (Lee *et al.*, 2003) did not support preprotein import in isolated Arabidopsis chloroplasts. These observations suggested that the G-domain along with its GTPase cycle are key elements of Toc159 (Sun *et al.*, 2002; Bauer *et al.*, 2002).

Due to inability to complement the *ppi2* mutant in the case of Toc159 mGTP, Toc159 S869R and Toc159 D909L, it was not possible to perform biochemical characterisations such as

import experiments or purification of mutant TOC complexes (Bauer *et al.*, 2002; Lee *et al.*, 2003). Single point mutations of the G-domain of Toc159 that are non-lethal, A864R (Wang *et al.*, 2008) and K868R (Agne *et al.*, 2009), were recently identified. Hence, it was possible to dissect the implication of the GTPase function in the process of preprotein import. Both A864R and K868R were able to reinstate the green phenotype in homozygous *ppi2* plants although their GTPase activity was strongly reduced if not measurable. Only Toc159 A864R was able to bind GTP with strong affinity and was therefore loaded in the GTP-bound state. Its import activity was higher than that of the WT. In the case of Toc159 K868R the GTP-binding is hardly detectable. These observations lead to the conclusion that the import efficiency is stimulated by GTP-binding (Agne *et al.*, 2009).

Mutations in the G-domain of Toc33, such as Toc33 F67A and Toc33 R130A (Weibel *et al.*, 2003; Lee *et al.*, 2009) deficient for dimerisation but not for the GTPase cycle, were also able to complement *ppi1* plants albeit reduced chloroplast preprotein import efficiency. The binding of preprotein to the TOC complex was unaltered (Lee *et al.*, 2009; Aronsson *et al.*, 2010). The fact that it is possible to disrupt the GTPase cycle at Toc33 and yet retain *in vivo* functionality has also been puzzling. Actually, plants can survive easily without Toc33 (*ppi1* mutant has a pale phenotype) due to its large functional redundancy with Toc34 (Jarvis *et al.*, 1998). Experiments with a large diversity of point mutations within the G-domain of atToc33 showed that these mutants were all able to complement *ppi1* plants (Aronsson *et al.*, 2010). This observation is even more striking for atToc33 G45R/K49N/S50R, which is the equivalent of atToc159mGTP mutant (Bauer *et al.*, 2002). Indeed, it is likely that the strong GTPase deficiency of Toc159mGTP lead to a larger structural defect and prevented the successful complementation of *ppi2* plants. Residues 869 and 909 are predicted to be involved in Mg²⁺ chelation which may be important for the structural integrity of the GTPase Toc159 (Farnsworth & Feig, 1991; Lee *et al.*, 2003; Agne *et al.*, 2009; Aronsson *et al.*, 2010). The hypothesis is that the greater structural complexity of atToc159, compared to atToc33, may account for the failure of these mutants to complement *ppi2* plants (Aronsson *et al.*, 2010).

Overall, these data suggest that alone neither the functions of GTP binding and hydrolysis nor the dimerisation of the G-domains of the Toc GTPases are strictly required. In fact, they are both necessary in concert for an optimal preprotein import process and an optimal chloroplast biogenesis. The GTPase cycle and the G-domain dimerisation promote preprotein import into chloroplasts (Agne *et al.*, 2009; Aronsson *et al.*, 2010). The cycle of Toc GTPases appears to promote more specifically preprotein targeting and docking to the TOC complex (Wang *et al.*, 2008; Agne *et al.*, 2009; Aronsson *et al.*, 2010) when the

homo/heterotypic interaction of the G-domain act in order to support preprotein translocation across the channel of the outer membrane (Lee *et al.*, 2009; Aronsson *et al.*, 2010).

1.7- Aims of the thesis

As presented above (section 1.3.1.1, section 1.3.1.2 and section 1.5.3), one key element in preprotein import into the chloroplast is the targeting and docking of the preprotein at the outer envelope membrane. This involves the interaction of the two Toc GTPases, at the TOC core complex, engaged in a process of homo/hetero-dimerisation coupled with mechanisms of GTP binding and hydrolysis (section 1.6).

The set of experimental data available to date is focused on the disruption of the GTPase domain's properties of Toc159. Therefore, the starting hypothesis is to study this component of the TOC complex from an opposite approach, i.e. to reinforce the interaction of Toc159 with its dimerising partner Toc33. The author wanted to identify a mutant of Toc159 for which the GTPase activity, in terms of GTP-binding and GTP-hydrolysis, is not altered whereas the dimerisation would be strengthened.

To study the importance of dimerisation of the TOC GTPases components, in the process of preprotein import into the chloroplast, this thesis presents experimental research that identified a mutant of Toc159 for which the dimerisation property is increased but the GTPase function remains unaffected. To reach this goal, a random mutant library of the Toc159 G-domain was generated and screened using the yeast Two-Hybrid system for a phenotype restoring the loss-of-interaction of Toc33G R130A; this mutant of Toc33 is impaired in the dimerisation but still binds and hydrolyses GTP like the WT protein. The next goal was to confirm whether the mutant of Toc159G identified in the screen is deficient or not in its GTPase functions, binding and hydrolysis of GTP, and to determine its ability to dimerise *in vitro* with Toc33. The third and last point was to show whether such a mutant is able to support preprotein import process both *in vitro* and *in vivo*.

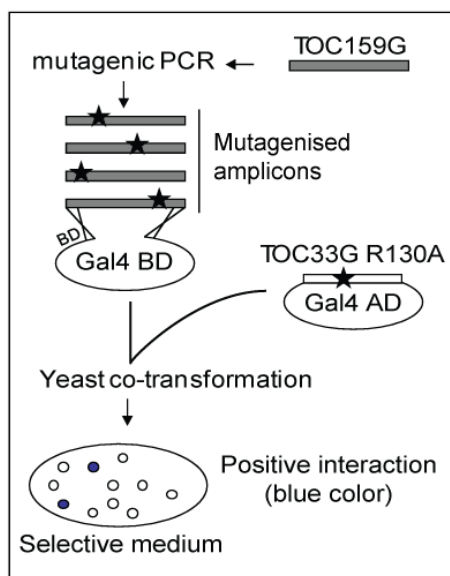
2- Results

2.1- Screening for a Toc159 mutant with strong binding for Toc33

To isolate a Toc159 mutant with a high binding affinity for Toc33, the yeast Two-Hybrid system (Rahim *et al.*, 2009) and a well characterised loss-of-interaction mutant of Toc33, Toc33 R130A, were used. Rahim *et al.* (2009) demonstrated that the G-domains of Toc33 and Toc159 interact in the yeast Two-Hybrid system. Weibel *et al.*, (2003) demonstrated that the Toc33 R130A mutant binds and hydrolyses GTP like the wild type but reduces both, the homo- and heterotypic interaction of Toc33 with the GTP-binding domains of Toc33 (Toc33G) or Toc159 (Toc159G).

For screening purpose, the Toc33 R130A mutation was introduced into the Gal-AD-Toc33G construct and co-transformed with Gal-BD-Toc159G WT. Figure 9B, upper panel, shows the loss of interaction of Toc33G R130A in a yeast Two-Hybrid β -galactosidase colony-lift filter assay when compared to the WT Toc33G construct. Thus, Toc159G WT, in fusion with Gal4-BD, did not interact with Gal4-AD-Toc33G R130A. Based on this observation, a strategy for screening mutants of Toc159G that would suppress the non-dimerising phenotype of Toc33G R130A was set up in a yeast Two-Hybrid system (Figure 9A). A random mutagenic PCR was performed on the coding sequence of the G-domain of Toc159 (TOC159G) to generate a library of mutant alleles. The mutagenized amplicons were subsequently cloned by homologous recombination to a binding domain Two-Hybrid vector (pGBKT7) in yeast cells, co-transformed with the activating domain vector coding for the G-domain of Toc33 carrying the R130A mutation (pGADT7-Toc33G R130A). Co-transformed yeast cells were first grown on selective medium and then, subjected to a β -galactosidase colony-lift filter assay to screen for mutants that restore the interaction and therefore result in a blue colour of the yeast cells in the filter assay. Several blue colonies were obtained but only two, designated Toc159G Sup1 and Toc159G Sup2 showed, after isolation of the mutagenized plasmid and retransformation into yeast, the desired suppressor phenotype (Figure 9B, middle and lower panel). Next, it was tested if Toc159G Sup1 and Toc159G Sup2, beyond restoring interaction with Toc33G R130A, bind more strongly to wild-type Toc33G. Indeed, the interaction of the two suppressor mutants with both Toc33G and Toc33 R130A appeared to be stronger when compared to the interaction of wild-type Toc159G with Toc33G as indicated by a darker blue coloration in the β -galactosidase colony-lift filter assay (Figure 9B).

A



B

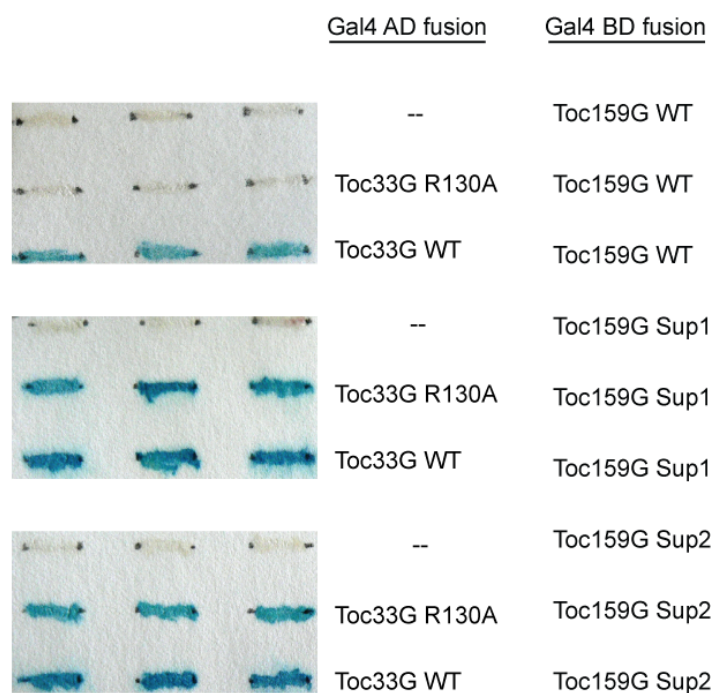


Figure 9: Screening mutant of Toc159G suppressing Toc33G R130A loss of interaction by yeast two hybrid. (A) Scheme describing the strategy for generating and screening the yeast library. (B) Yeast two hybrid analysis of heterodimerisation by X-Gal filter assay. Yeast carrying the Gal4 Activation Domain (AD) and the Gal4 DNA Binding Domain (BD) constructs, either atToc33G or atToc33G R130A in fusion with AD in pGADT7 and either atToc159G WT or mutant in fusion with BD in pGBKT7, respectively, were subjected to X-Gal filter assay. Development of a blue coloration due to LacZ reporter gene activation indicates a positive interaction result.

2.1.1- A single point mutation suppressing Toc33 R130A loss-of-interaction

Sequencing revealed that several residues were mutated in both suppressor mutant alleles of Toc159G. As shown in the protein sequence alignment in Figure 10A, mutations resulted in two amino-acid changes in Toc159G Sup1 (D919V and N1052D) and 7 amino-acid changes in Toc159G Sup2 (K784E, I812T, V903A, D919V, S997G, Q1054R, F1089L). Interestingly, Toc159G Sup1 and Toc159G Sup2 have a common point mutation in position 919 of the amino-acid sequence of Toc159, underscored in Figure 10A, where the aspartic acid has been changed to a valine.

To test whether the suppressor phenotype in both Toc159G Sup1 and Toc159G Sup2 could be attributed to the D919V mutation, the corresponding nucleic acid change was introduced into the yeast Two-Hybrid vector pGBKT7-Toc159G by QuikChange site directed mutagenesis (Stratagene). As shown in Figure 10B, Toc159G D919V, like Toc159G Sup1 and Toc159G Sup2, is able to interact with both Toc33 R130A and Toc33 WT. It also appears, in the β -galactosidase colony-lift filter assay, that the mutant D919V binds stronger to Toc33 WT, compared to Toc33 R130A, as indicated by the darker blue coloration. This hypothesis was tested by performing a quantitative yeast Two-Hybrid liquid culture assay using O-NitroPhenyl β -d-Galactopyranoside (ONPG) as substrate. According to this quantitative assay, which is based on the correlation between binding strength and the optical density, Toc159G D919V binds 3.6 times stronger to Toc33G than unmutated Toc159G. However, the enzymatic activity in yeast cells transformed with Toc159G Sup1 or Toc159G Sup2 and Toc33G was 4.4 and 7 times higher, respectively, when compared to the interaction of Toc159G WT and Toc33G WT. In average 7.7 β -galactosidase units were measured for the Toc159G Sup2-Toc33G interaction, 4.9 units for the Toc159G Sup1-Toc33G interaction and 4 units for the Toc159G D919V-Toc33G interaction.

Thus, D919V appeared to be a promising binding mutation that justified to be characterised with regard to both GTP-binding and GTP-hydrolysis.

2.1.2- Toc159G D919V suppresses Toc33 loss-of-interaction mutants in the D1 motif

The mutated residue D919V is located in a putative Switch II region of the G-domain of Toc159 (Figure 11). Switch regions in GTPases undergo important conformational changes upon GTP binding (active state of the GTPase) and hydrolysis (inactive state). Furthermore this residue is conserved in the Toc159 homologues Toc132 and Toc120 but not conserved in the Toc33 GTPase family (Figure 11).

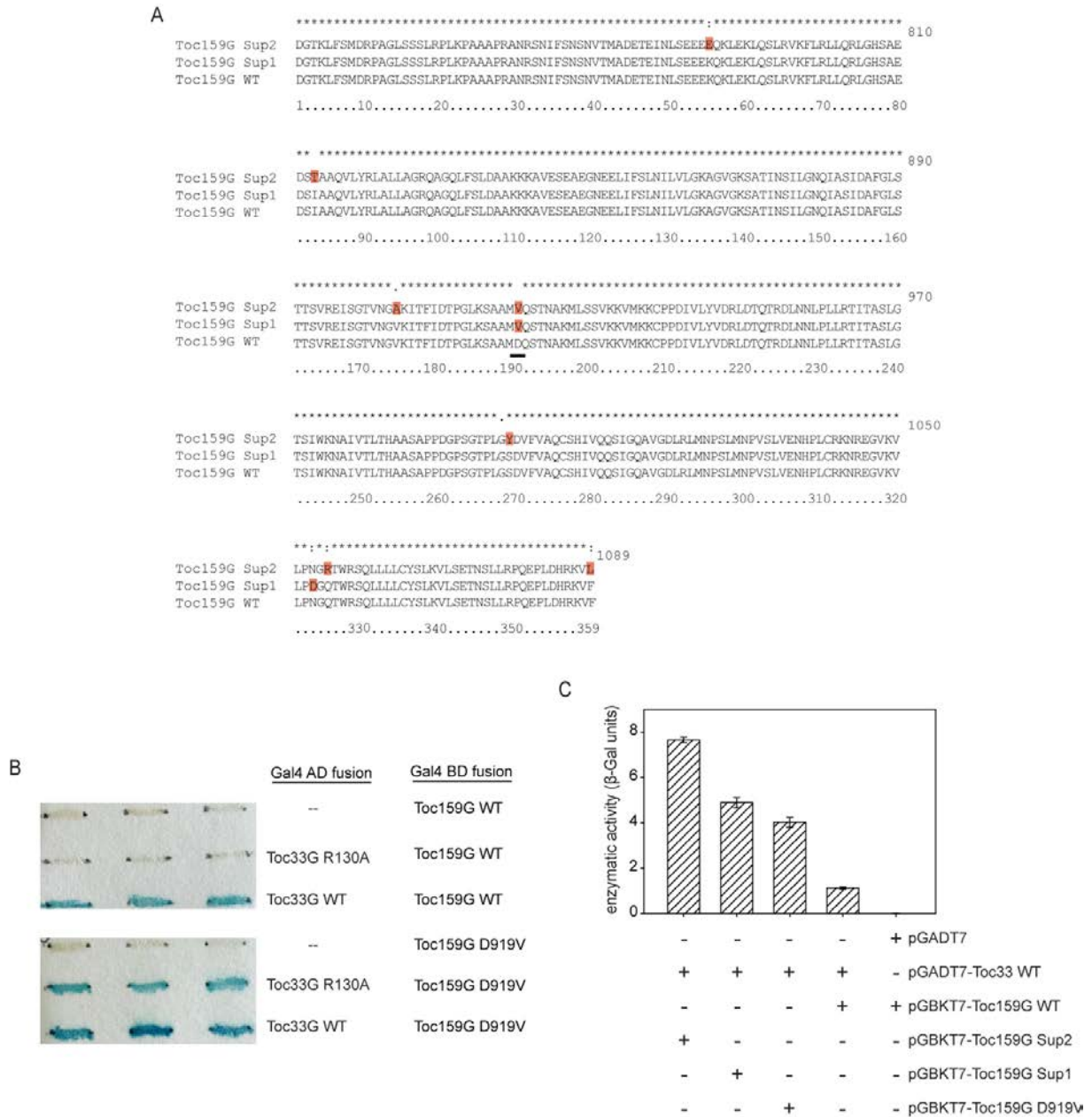


Figure 10: Isolation of single point mutation responsible for the heterodimerisation phenotype. (A) Amino acid sequence partial alignment of atToc159G WT, atToc159G Sup1 and atToc159G Sup2. Amino acid position 919 is underscored to emphasize the common point mutation of atToc159G Sup1 and atToc159G Sup2. (B) Yeast two hybrid analysis of heterodimerisation by X-Gal filter assay. Yeast carrying the Gal4- activation domain (AD) and the Gal4 DNA binding domain (BD) constructs, corresponding to atToc33G in fusion with AD in pGADT7 and either atToc159G WT or mutants in fusion with BD in pGBKT7, respectively, were subjected to X-Gal filter assay. Development of a blue coloration due to LacZ reporter gene activation indicates a positive interaction result. Either atToc33G or atToc33G R130A were cloned in fusion with AD in pGADT7. Either atToc159G WT or atToc159G D919V was cloned in fusion with BD in pGBKT7. (C) Yeast two hybrid analysis of heterodimerisation by liquid culture assay using o-nitrophenyl-beta-D-galactopyranoside (ONPG). Yeast carrying the Gal4- activation domain (AD) and the Gal4 DNA binding domain (BD) constructs, pGADT7 and pGBKT7, respectively, were subjected to ONPG liquid assay. Quantification of the interaction between AD and BD is done by measurement of the beta-galactosidase activity.

Two sequence motifs are important for both homo and hetero-dimerisation of Toc GTPases. One is the so-called D1 (dimerisation) motif conserved in all Toc GTPases (Figure 11). The other is the G1 phosphate-binding loop (P-loop) characteristic of many nucleotide binding proteins. It is known that Toc GTPase dimerisation involves residues in the D1 motif and that it is regulated by the GTPase activity (Sun *et al*, 2002). Several point mutations in Toc33G have been described that disrupt heterodimerisation with Toc159G (Aronsson *et al*, 2010; Lee *et al*, 2009). For example the critical arginine residue R130, positioned in the D1 motif (Weibel *et al*, 2003) first described as psToc34 R133A (Sun *et al*, 2002), but also mutations in the G1 motif, involved in the coordination of the nucleotide, disrupt the interaction. For the screening of the Toc159G D919V suppressor mutant a Toc33G mutant in the D1 motif (Toc33G R130A) was used. Therefore, it was of interest to know whether or not the mutation D919V suppresses other Toc33G loss-of-interaction mutants. For this purpose, heterodimerisation of either Toc159G WT or Toc159G D919V with loss-of-interaction mutants of Toc33G were tested with the yeast Two-Hybrid system (Figure 12). Two additional D1 mutants (Toc33 R125A and Toc33 D127A) and two G1 mutants (Toc33 S50N, Toc33 G45R K49N S50R) were also tested.

It turned out that Toc159G D919V is able to restore dimerisation with all three mutants of Toc33G in the D1 motif but not with the two mutants in the G1 motif. It is likely that an impairment of the nucleotide coordination by the G1 motif of Toc33G prevents the process of dimerisation with the interacting partner. This issue will be addressed later on in section 3.

2.2- Expression and purification of recombinant proteins

To confirm the strong interaction phenotype of Toc159G D919V by another method and to check if its GTPase activity is affected by the mutation, the G-domains of Toc159 WT and Toc159 D919V were expressed as N-terminally GST-tagged proteins and purified (Figure 13, lanes 2/5 and lanes 7/10, respectively). For this purpose, the point mutation for D919V was introduced into the plasmid pGEX4T1-Toc159G by QuikChange site directed mutagenesis. Over-expression of the recombinant proteins GST-Toc159G WT and GST-Toc159G D919V was achieved by transformation of *E. coli* BL21 with pGEX4T1-Toc159G, previously described in Rahim *et al*. (2009), and pGEX4T1-Toc159G D919V. Both proteins were expressed upon IPTG induction (Figure 13, lanes 2 and 7). After over-expression of the recombinant proteins in liquid growth medium, bacteria were harvested by centrifugation, resuspended and incubated in lysis buffer containing lysozyme for 30 minutes under constant agitation at 4°C. This suspension was subsequently passed through a French press. The bacterial lysate was centrifuged in order to separate insoluble (P, Figure 13, lanes

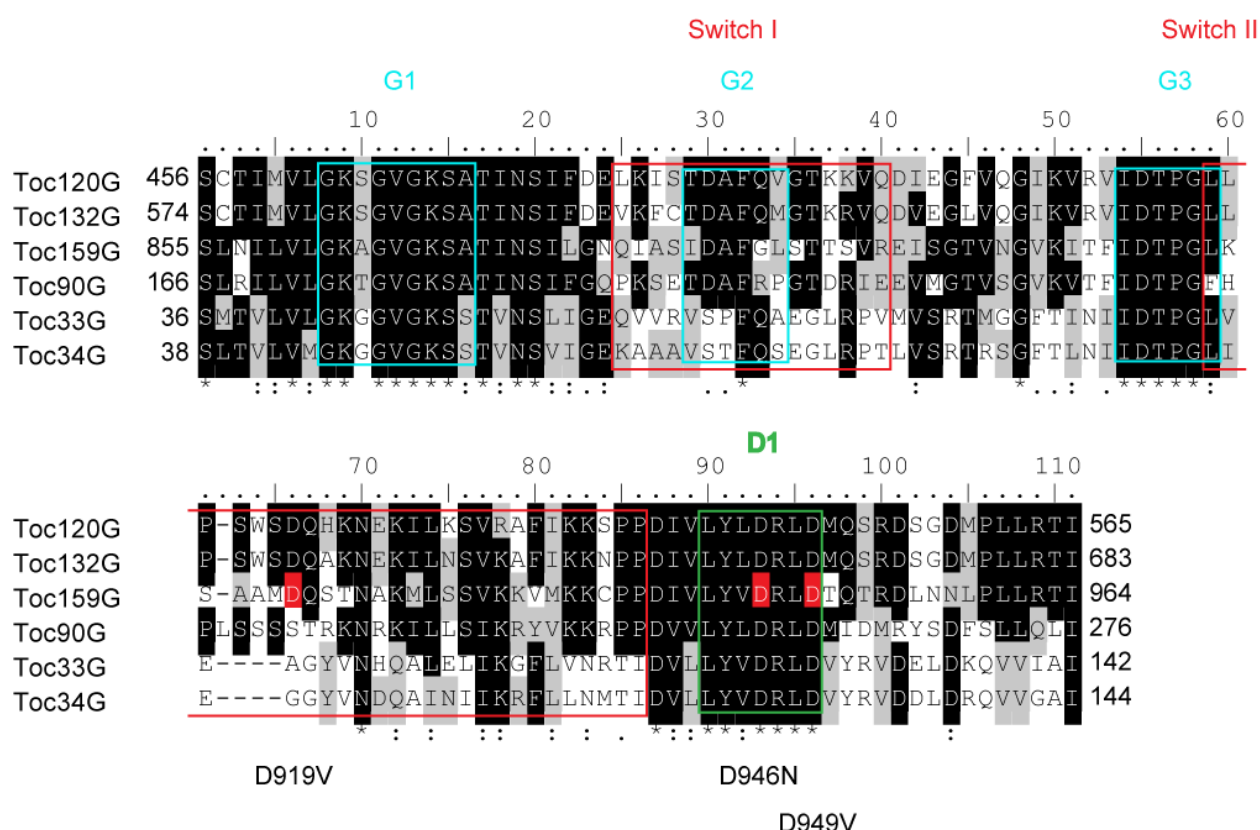


Figure 11: Partial sequence alignment of the G-domain of Toc33/Toc159 subfamily in *A. thaliana*. Blue boxes are enclosing G1 to G3 motifs when red boxes enclose Switches I and II and the green box encloses the D1 motif. Numbers at the start and end of sequences indicate positions within the corresponding full-length proteins. Residues identical in at least four sequences are shaded black whereas, similar residues are shaded grey. Residues in Toc159 sequence that are relevant point mutations for this study are shaded red, namely mutations D919V, D946N and D949V. The point mutations D946N and D949V will be addressed to later in the discussion. The features present in the alignment are made after (Sun *et al*, 2002), the alignment was generated using ClustalW within BioEdit (Hall, 1999; Thompson *et al*, 1994).

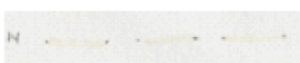
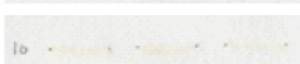
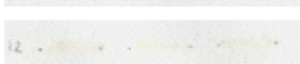
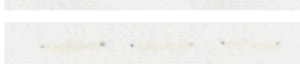
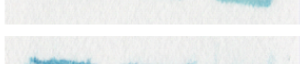
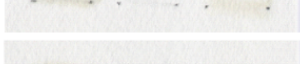
		<u>Gal4 AD fusion</u>	<u>Gal4 BD fusion</u>
Mutation in D1 motif		Toc33G WT	Toc159G WT
		Toc33G R125A	Toc159G WT
		Toc33G D127A	Toc159G WT
		Toc33G R130A	Toc159G WT
Mutation in G1 motif		Toc33G S50N	Toc159G WT
		Toc33G G45R K49N S50R	Toc159G WT
Mutation in D1 motif		Toc33G WT	Toc159G D919V
		Toc33G R125A	Toc159G D919V
		Toc33G D127A	Toc159G D919V
		Toc33G R130A	Toc159G D919V
Mutation in G1 motif		Toc33G S50N	Toc159G D919V
		Toc33G G45R K49N S50R	Toc159G D919V

Figure 12: Mutation D919V suppresses Toc33G loss of interaction mutants in the D1 dimerisation motif. Yeast two hybrid analysis of heterodimerisation by X-Gal filter assay. Yeast carrying the Gal4-activation domain (AD) and the Gal4 DNA binding domain (BD) constructs, either atToc33G WT or mutants in fusion with AD in pGADT7 and either atToc159G WT or atToc159G D919V in fusion with BD in pGBKT7, respectively, were subjected to X-Gal filter assay. Development of a blue coloration due to LacZ reporter gene activation indicates a positive interaction result.

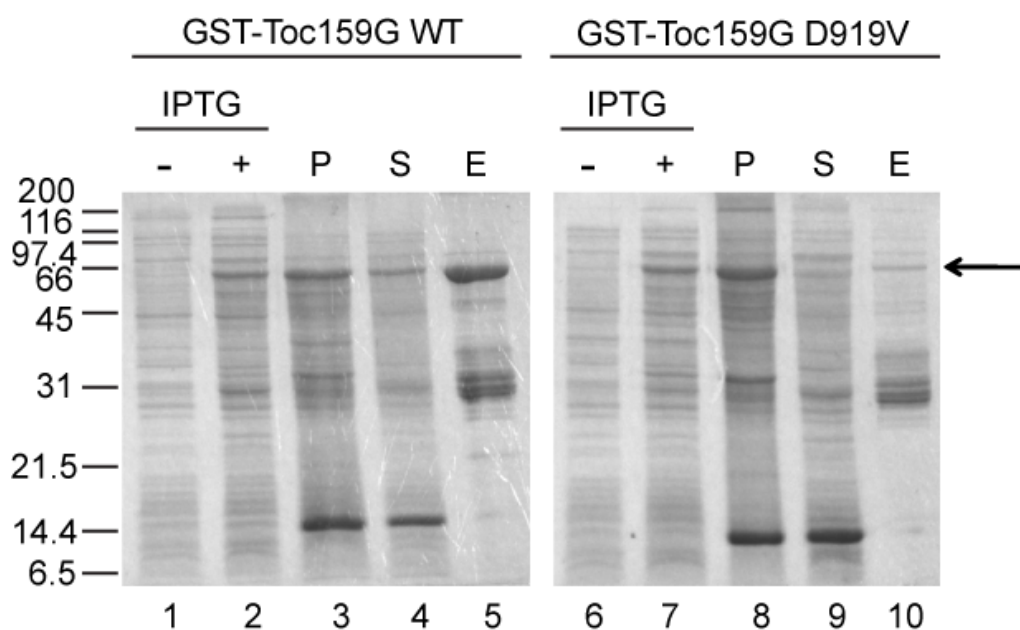


Figure 13: Over-expression and purification of GST tagged Toc159G WT and Toc159G D919V. The 2 GST-tagged proteins were expressed in *E. coli* BL21 (DE3) cells. The non-denaturing purification of recombinant proteins was carried out using a GSTrap HP chromatography column on an AKTA Prime Plus purification system. Bound proteins were eluted using 10 mM reduced glutathione in 50 mM Tris/HCl pH 8. Aliquots corresponding to 100 μ l of non-induced culture (-IPTG, lane 1 and lane 6), 50 μ l of induced culture (+IPTG, lane 2 and lane 7), 0.01% of pellet (P, lane 3 and 8), 0.01% of soluble fraction (S, lane 4 and 9) and 0.12% of eluate (E, lanes 5 and 10) were used for SDS-PAGE analysis followed by Coomassie blue staining. The black arrow on the right indicates the position of the recombinant GST-tagged protein.

3 and 8) and soluble (S, Figure 13, lanes 4 and 9) fractions. The supernatant (S) was subsequently used for purification of the GST-tagged recombinant proteins under native conditions by affinity chromatography using GSTrap HP column on an AKTAPrime Plus (GE Healthcare). After the purification, aliquots were analysed by SDS-PAGE (12%) followed by Coomassie blue staining. The over-expressed fusion proteins had the expected relative mass of 66 kDa. In the case of GST-Toc159G D919V there was a high degree of degradation products (fragments around 31 kDa), especially after a multi-step purification protocol (data not shown). Therefore it was decided to continue with a single purification step. In the case of GST-Toc159 WT recombinant protein, a sufficient quantity was present as soluble protein allowing for its purification under native conditions, resulting in purified GST-Toc159G WT to a final concentration of approximately 22 μ M, average of several purification experiments (not pooled). It was more challenging to purify the fusion protein GST-Toc159G D919V as less protein was soluble (compare 66 kDa bands in Figure 13, lanes 8 and 9) and most of the mutant fusion protein remained in the pellet as insoluble material. Purified GST-Toc159G D919V had a final concentration of approximately 7 μ M, average of several purification experiments (not pooled).

2.3- Toc159G D919V interacts strongly with Toc33G in a Ni-NTA pull-down assay

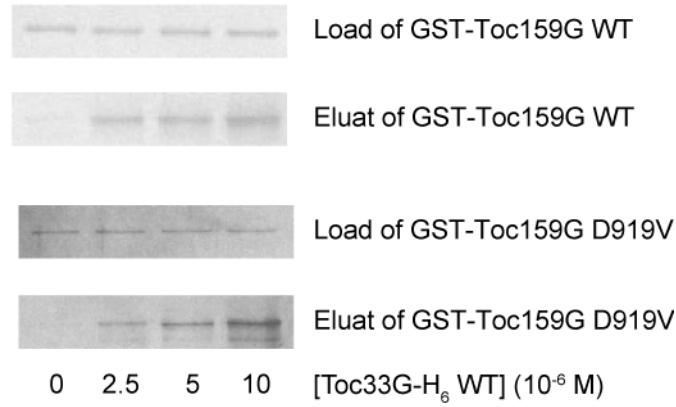
In order to confirm the stronger affinity of Toc159G D919V for Toc33G (section 2.1), a pull-down assay was performed.

Either GST-Toc159G WT or D919V, at a concentration of 2.5 μ M, were incubated with increasing concentrations (from 0 to 10 μ M) of His₆-tagged Toc33G. After the incubation of binding partners, Ni-NTA agarose was added to the reaction tube in order to isolate the His₆-tagged proteins bound together with the interaction partner.

Subsequently, the Ni-NTA agarose was extensively washed and eluted with imidazole. The eluates were analysed by SDS-PAGE followed by Coomassie blue staining (Figure 14A). The bands corresponding to the GST fusion protein and His₆-tagged protein were quantified (Figure 14B).

To calculate the amount of bound material, an aliquot (5% of the starting volume) was removed before the addition of the Ni-NTA agarose beads (Load, Figure 14A). The entire protein eluate was precipitated and dissolved in Sample buffer (Eluate, Figure 14A). After separation of the proteins by SDS-PAGE and quantification with Biorad QuantityOne software, the percentage of binding of GST-Toc159G D919V to Toc33G-His₆ was calculated relatively to the binding of Toc33G-His₆ to GST-Toc159G WT, at a concentration of 10 and

A



B

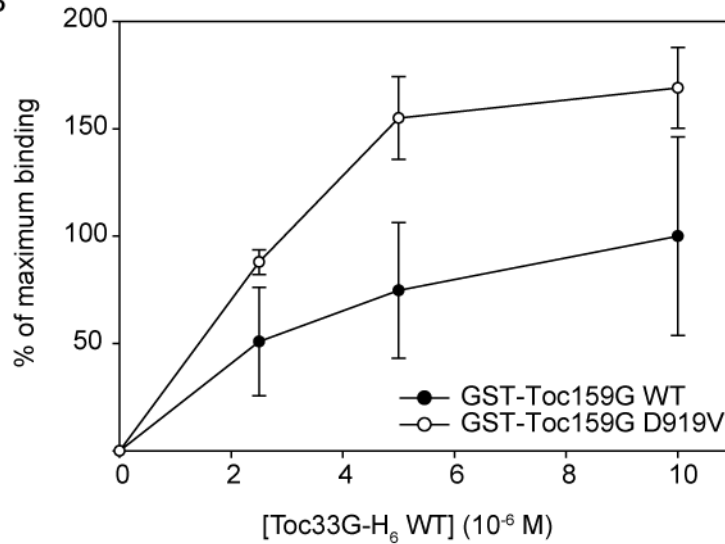


Figure 14: Hetero-dimerisation of GST-Toc159G WT and GST-Toc159G D919V with Toc33G-H₆ WT. (A), GST-Toc159G WT/D919V (2.5 μ M) was incubated with increasing concentration (0 to 10 μ M) of Toc33G-H₆ WT in binding buffer (25 mM HEPES-KOH pH 7.5, 40 mM KOAc, 2 mM Mg(OAc)₂, 20 μ M DTT, 0.1% (v/v) Triton X-100). After 10 min of pre-incubation at 4°C, Toc33G-H₆ was re-isolated by affinity chromatography with Ni-NTA beads during 1 h at 4°C, along with bound Toc159G WT or Toc159G D919V. After extensive washing with binding buffer complemented with 0.4 mM GTP, proteins were eluted with 200 mM imidazole in binding buffer. Aliquots of 5% of loaded material and 100% of eluted material were used for SDS-PAGE analysis followed by Coomassie blue staining. (B), Quantification of (A) using the QuantityOne software (Biorad). Ratio of eluted to loaded Toc159G recombinant protein was calculated; the amount of GST-Toc159G WT bound to Toc33G-H₆ WT was defined as 100% binding. GST-Toc159G binding to Ni-NTA in absence of His-tagged protein was deduced as background and adjusted to 0% binding. The experiment was performed in triplicate.

2.5 μ M respectively, set as 100% binding. Binding was set to 0% when protein concentration of Toc33G-His₆ was 0 μ M.

Figure 14B clearly shows that Toc33G WT pulls down more of the interacting partner in the case of Toc159G D919V than for Toc159G WT. At a concentration of 10 μ M of WT Toc33G, relative binding of Toc159G D919V is 155%.

These data indicate that the Toc159 mutant D919V binds more strongly to Toc33 than the wild type, thereby confirming the observations made above with the yeast Two-Hybrid experiments (section 2.1).

2.4- Toc159G D919V is deficient in GTP binding

In order to determine whether Toc159G D919V is able to bind GTP and to what extent, the purified recombinant proteins GST (negative control), GST-Toc159G WT and GST-Toc159G D919V were incubated in the presence of radioactive [α^{33} P]-GTP. One μ M of recombinant protein was incubated with 100 nM of [α^{33} P]-GTP and 0,4 mM cold GTP in Binding buffer. After 1h, aliquots were spotted on Nitrocellulose membrane, which was thoroughly washed to remove any unbound radioactive GTP. The membrane was air-dried and spots detected and quantified by PhosphorImaging (Figure 15A and 14B, respectively). The binding of [α^{33} P]-GTP to recombinant Toc33G-His₆ WT was used as a positive control and set to 100% binding (data not shown). Relatively to the positive control, it was possible to calculate the relative [α^{33} P]-GTP binding to the recombinant GST-Toc159G D919V. As can be seen in Figure 15, GTP binding to D919V mutant is strongly reduced (25.1 % of relative binding) compared to WT GST-Toc159G (69.1 % of relative binding) and identical to that of the negative control. It therefore appears that GST6Toc159 D919V is unable to stably bind GTP at the given concentration.

2.5- Toc159G D919V hydrolyses GTP like the WT

To further examine the effects of the D919V mutation on the G-domain of Toc159 it was of interest to determine the rate of GTP hydrolysis of the mutated protein and to calculate enzymatic parameters such as the maximum velocity (V_m) and the Michaelis constant (K_m). For this purpose, the recombinant proteins GST, GST-Toc159G or GST-Toc159G D919V at 2.5 μ M were incubated with 50 nM of [α^{33} P]-GTP, as a tracer, in Hydrolysis buffer at 25°C for 2h, allowing for GTP hydrolysis with increasing concentrations of un-labelled GTP (from 0 to 10 mM). At different time-points, reactions were sampled,

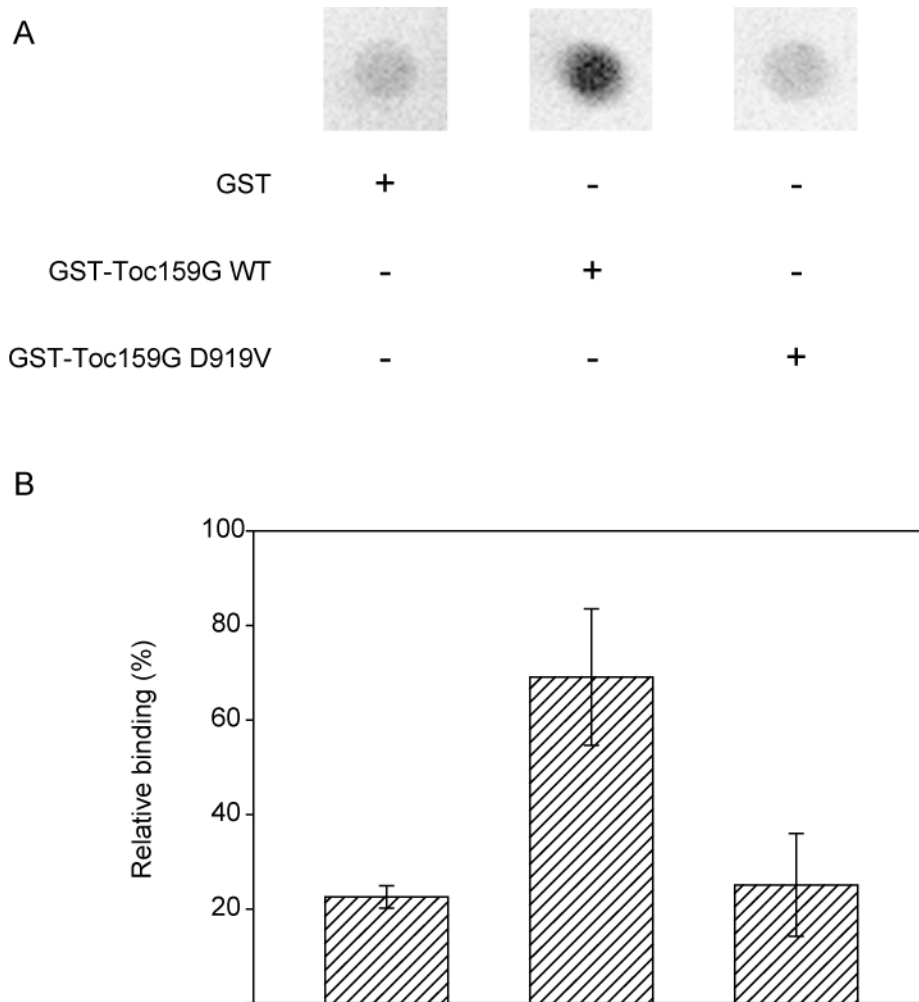


Figure 15: GTP binding of atToc159G D919V. (A), Purified recombinant protein (1 μ M), either GST (negative control), GST-Toc159G WT or, GST-Toc159G D919V, was incubated with 0.1 μ M [α^{33} P]-GTP for 1h at 4°C in binding buffer (20 mM Tris-HCl pH8, 50 μ M MgCl₂, 0.3% [v/v] Tween 20 and 0.4 mM GTP). Aliquot of 2 μ l were spotted on nitrocellulose membrane which was subsequently washed, dried and exposed on PhosphorImager plate (Biorad) for detection of radiolabeled bound [α^{33} P]-GTP. (B), Quantification of data shown in (A) using the QuantityOne software (Biorad). [α^{33} P]-GTP binding to Toc33 was used as a positive control (data not shown), this interaction was defined as a 100% GTP binding reference. The experiment was performed in triplicate.

stopped and guanine nucleotides were separated by thin layer chromatography on PEI-cellulose. By PhosphorImaging, spots corresponding to GTP and GDP were quantified allowing for calculation of the GTP hydrolysis rate. Figure 16 represents the rate of GTP hydrolysis as a function of GTP concentration. GST was used in this assay as a negative control. Unfortunately, GTPase activity was detected with the negative control and gives a high background. Therefore, fitting a non-linear regression curve (SigmaPlot), according to the model of Michaelis-Menten, was not successful and prevented the calculation of the enzymatic parameters (V_m and K_m). It is possible to conclude from Figure 16 that both GST-Toc159G WT and GST-Toc159G D919V hydrolyse GTP at similar rates. It appears that Toc159G-D919V is able to stably bind GTP, otherwise it would be unable to hydrolyse GTP (like Toc159 K868R).

2.6- Complementation of *Arabidopsis thaliana ppi2* by TAP-Toc159GM D919V

To determine whether the Toc159 mutant D919V can rescue the *ppi2* phenotype *in vivo*, a T-DNA construct encoding the G and M-domains of Toc159 containing the D919V mutation was engineered. An additional PstI restriction site was introduced along with the D919V mutation in the transgene allowing for identification of the specific PCR product after enzymatic restriction of PCR products. Indeed, the A-domain has been reported to be non-essential (Lee *et al*, 2003; Agne *et al*, 2009). The D919V GM construct was fused to a N-terminal TAP-tag consisting of two IgG binding domains of Protein A and a Calmodulin Binding Protein (CBP). The resulting construct was TAP-Toc159GM D919V. Subsequently, this construct was introduced into heterozygous *ppi2* plants by *Agrobacterium* mediated transformation and yielded eight individual plants resistant to BASTA. Out of these eight transgenic lines, two were wild-type, three were not able to produce seeds. Two out of the three remaining heterozygous *WT/ppi2* lines were used to perform a segregation experiment in order to isolate independent homozygous lines (line #3-5/7 and line #7-5/5). The genotypes were confirmed using primers pairs specific for the *WT* or *ppi2* alleles. A specific primer set was used to confirm the presence of the T-DNA construct (Figure 17B). The transformation with the TAP-Toc159GM D919V construct resulted in a green phenotype in the *ppi2* homozygous background, similar to *WT* plants, suggesting functional complementation (Figure 17A). The expression of the transgene, in both line #3-5/7 and line #7-5/5, was confirmed by Western Blot analysis (Figure 17C). Identical amounts of total protein extract from these two lines were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies raised against the A-domain of Toc159 (α Toc159A) as well as purified rabbit IgG binding directly to the protein A, part of the TAP-

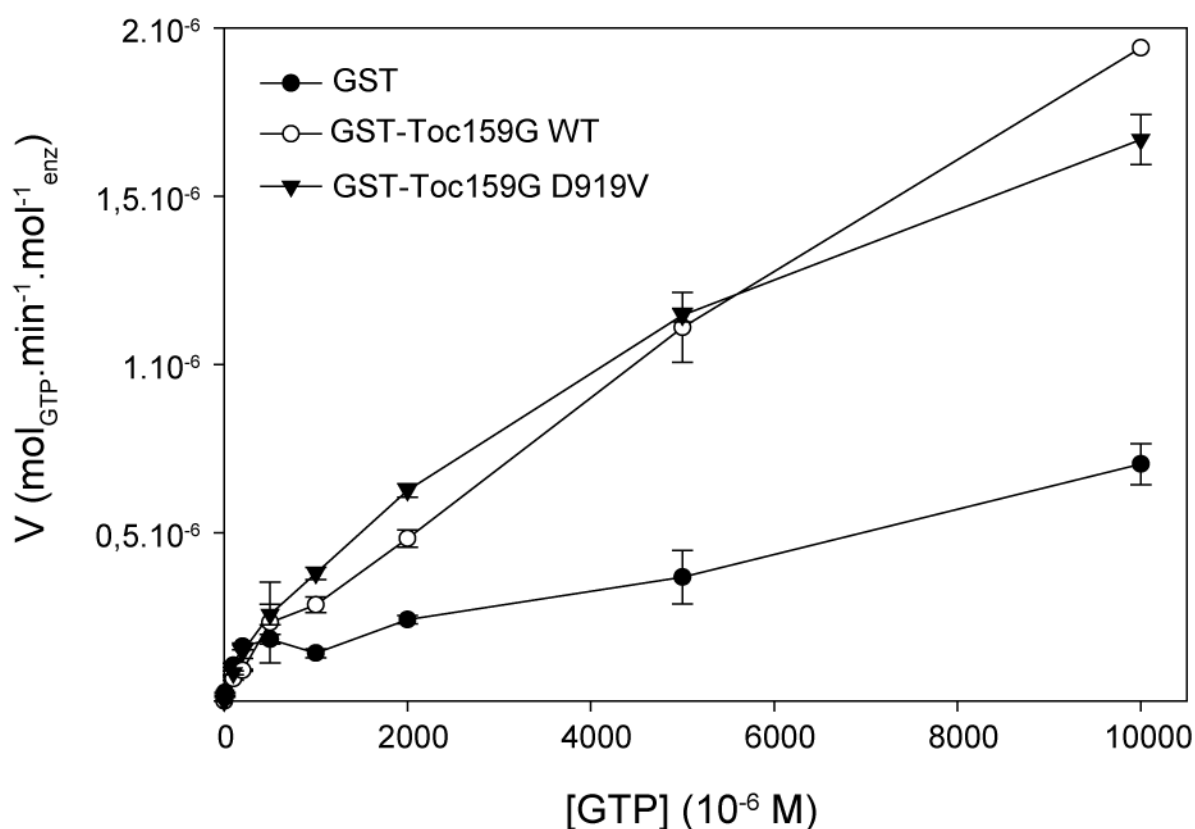


Figure 16: GTP hydrolysis by GST-Toc159G WT and GST-Toc159G D919V. Purified recombinant proteins, either GST (negative control), GST-Toc159G WT or D919V, were incubated at a concentration of 2.5 μ M at 25°C with 50 nM [α^{33} P]-GTP and increasing concentrations of non-radioactive GTP (from 0 to 10 mM) in hydrolysis buffer (20 mM Tris/Cl pH 8, 25 mM KOAc, 2 mM MgCl₂, 0.1 g/l BSA). After 0, 30, 60 and 120 min, 10 μ l of the reaction was aliquoted in stop buffer and spotted on PEI-cellulose plates for chromatographic separation of GTP and GDP with 0.75 M KH₂PO₄ pH 3.5 as solvent. Spots corresponding to GTP and GDP were detected and quantified using a PhosphorImager (Biorad). The rate of GTP hydrolysis was subsequently calculated and plotted against GTP concentration. The experiment was performed in triplicate.

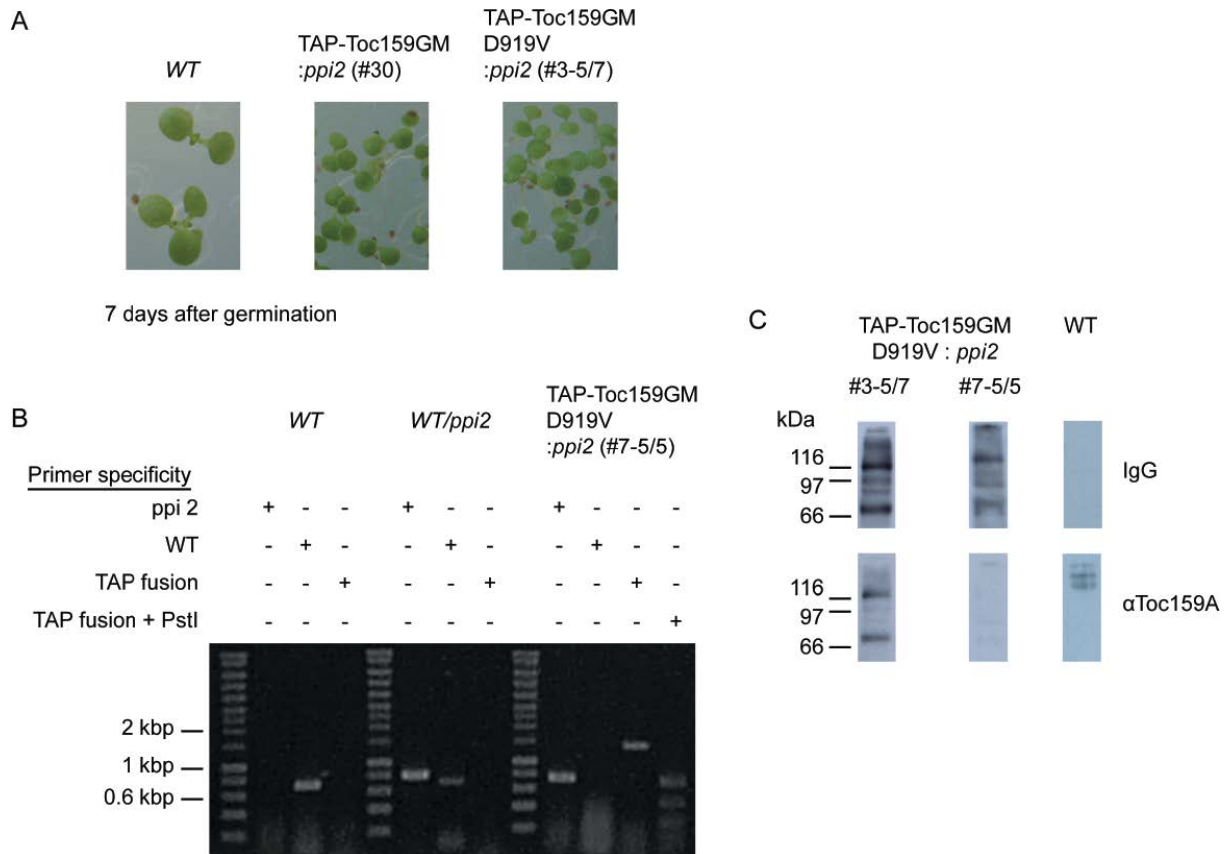


Figure 17: Complementation of *ppi2* mutant by Toc159GM D919V. (A), phenotypic comparison of homozygous *ppi2* plants transformed with TAP-Toc159GM or TAP-Toc159GM D919V with untransformed *A. thaliana* (WT). (B), confirmation of genotype of transformed homozygous *ppi2* with TAP-Toc159GM D919V (line 7-5/5) and presence of the single point mutation D919V. PCR analysis of genomic DNA with primer set specific to *ppi2* alleles (*ppi2*), non-disrupted WT gene TOC159 (WT) and the transgene (TAP fusion). The digestion of the transgene specific PCR product by PstI restriction enzyme is indicative of the presence of the D919V mutation that was introduced along with a PstI restriction site. *Arabidopsis* WT (Ws) and heterozygous *ppi2* were used as negative control. (C), expression of the TAP-tagged protein. Identical amounts of total protein extracts were used for Western Blotting with rabbit IgG, for the detection of TAP-tagged protein, and anti-Toc159 A-domain, for the detection of endogenous full-length atToc159.

tag. The WT plants were used as a control in order to identify the profile of WT Toc159 (Figure 17C). This Western Blot indicates that, in both transformed lines homozygous for *ppi2*, the WT gene TOC159 is not expressed and the transgenic protein TAP-Toc159GM D919V is detected. The bands visible on the blot of line 3-5/7 immuno-hybridised with anti-Toc159A antibody (Figure 17C – α Toc159A) corresponds to a false positive due to the previous immuno-hybridisation with rabbit IgG.

The electron micrograph images (Figure 18), showing the ultra-structure of chloroplast thin-sections of *ppi2* homozygous plants complemented with the TAP-Toc159GM D919V construct, confirm that the plants are fully complemented. Indeed, there is no significant difference between chloroplasts of *A. thaliana* expressing WT TAP-Toc159GM (WT L30 – Figure 18, upper right micrograph) or TAP-Toc159GM D919V (D919V#3-5/7 and D919V#7-5/5 – Figure 18, lower left and lower right micrograph, respectively). *A. thaliana* homozygous for *ppi2* expressing TAP-Toc159GM WT (WT L30 – Figure 18, upper right micrograph) (Agne *et al*, 2009) and untransformed *A. thaliana* chloroplasts (Ws – Figure 18, upper left micrograph) were used as controls were used as negative control.

2.7- Insertion of Toc159 D919V mutant into the outer membrane of chloroplasts

We wanted to test whether or not the D919V mutant of Toc159 is impaired in insertion into the outer membrane of chloroplasts. To address this question, pET21-Toc159 WT and pET21-Toc159 D919V were *in vitro* translated using [³⁵S]-methionine in order to express radio-labelled recombinant proteins [³⁵S]-Toc159 WT and [³⁵S]-Toc159 D919V. The [³⁵S]-recombinant proteins were incubated with isolated chloroplasts *in vitro* and subsequently incubated in absence (-TL – Figure 19A) or presence (+TL – Figure 19A) of thermolysine. In the TL+ treatment, the part of Toc159 which is inserted into the chloroplast membrane is protected from the thermolysine protease, resulting in a 52 kDa M-domain fragment (159M – Figure 19A) if correctly inserted (Chen *et al*, 2000). A 52 KDa band was detected both in the case of Toc159 WT and Toc159 D919V. This corresponds, in Figure 19A, to lane 3 for Toc159 WT and lane 7 for Toc159 D919V. For the band quantification, the number of methionine residues was considered. These bands were quantified relatively to the amount of Toc159 *in vitro* translate (IVT – Figure 19A) bound to the chloroplasts (lane 2 and 6 for Toc159 WT and Toc159 D919V, respectively, Figure 19A) and represented in Figure 19B. It appears that 63.7% of bound Toc159 WT was inserted correctly into the outer membrane of chloroplasts while 73% was inserted in the case of D919V mutant. Therefore, it is possible to conclude that the mutation D919V does not affect the insertion of Toc159 into the outer membrane of chloroplasts.

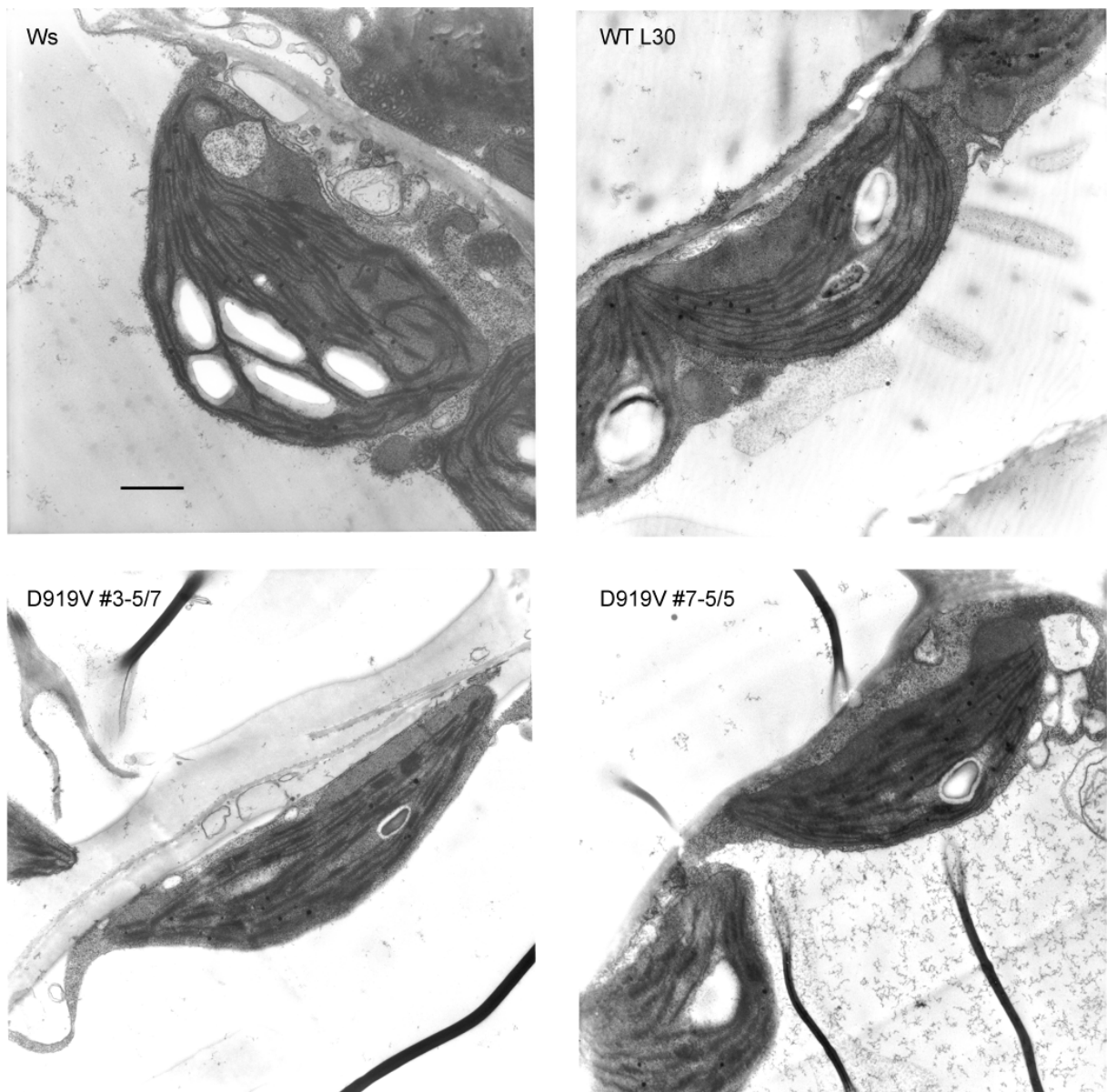


Figure 18: Ultra-structural analysis of chloroplasts in *ppi2* plants complemented with TAP-Toc159GM D919V. Transmission electron microscopy of leaf tissue thin sections from 21 day-old *in vitro* grown plants. Genotype is indicated on the upper left corner of each photograph. Ws corresponds to ecotype Wassilewskija of *A. thaliana* WT. WT L30 corresponds to homozygous *ppi2* complemented with TAP-Toc159GM WT (Agne *et al*, 2009). D919V #3-5/7 and #7-5/5 are two independent lines corresponding to homozygous *ppi2* complemented with TAP-Toc159GM D919V. Scale bar, in the upper left panel, is 1 μ m.

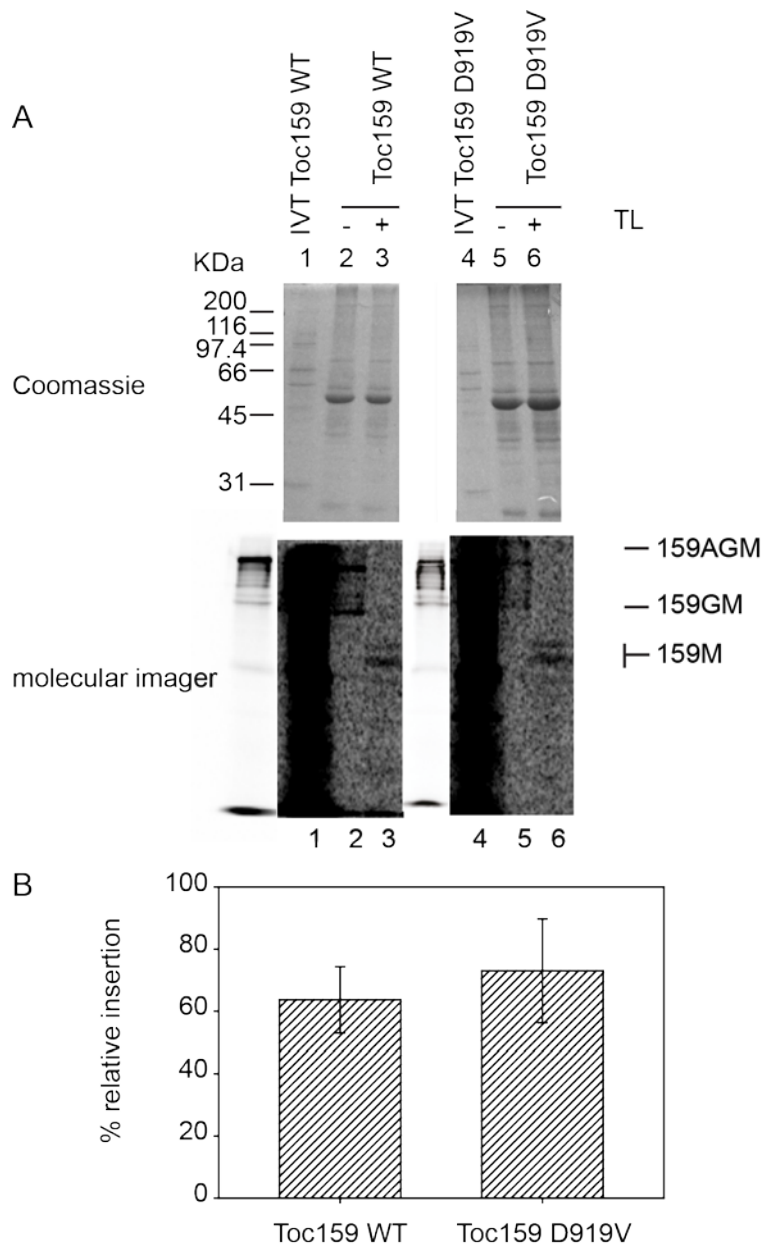


Figure 19: Effect of D919V mutation on the *in vitro* insertion of Toc159 into the outer membrane of chloroplasts. (A), *in vitro* translated [35 S]-methionine labelled (IVT) Toc159 WT or Toc159 D919V were incubated with isolated *Arabidopsis thaliana* chloroplasts at 25°C during 15 min. A normal contrast image of the IVT is represented on the left of lane 1 and 4 for the WT and D919V, respectively. Chloroplasts were re-isolated and incubated in presence (+TL, lane 3 and 6) or absence of thermolysine (-TL, lane 2 and 5) at 4°C during 15 min. Chloroplasts were re-isolated for precipitation of total proteins by chloroform/methanol and analysed by SDS-PAGE followed by Coomassie blue staining and PhosphorImager (Biorad) detection and quantification. (B), quantitative analysis of data shown in (A) using the Quantity One software (Biorad). The quantification was calibrated to the amount of methionine present in Toc159, Toc159GM and Toc159M. Percentage of inserted Toc159 (Toc159M, lane 3 and 6) was calculated relatively to Toc159 bound to chloroplast (-TL, lane 2 and 5). The experiment was performed in triplicate.

2.8- Preprotein import into isolated Toc159GM D919V chloroplasts

To test the capacity of Toc159GM D919V plants to import proteins, chloroplasts of two distinct lines of complemented plants (#3-5/7 and #7-5/5) were isolated *in vitro* and incubated in the presence of radio-labelled small sub-unit of the RubisCO (pSSU). The import experiment was allowed to proceed for 0, 5, 10 and 15 minutes (Figure 20A). Time points at 0 and 15 minutes were taken into account for the signal quantification in Figure 20B. This analysis, although performed once due to the difficulty of isolating sufficient chloroplasts, shows that the mutation D919V results in an increased import of preprotein into chloroplasts. Complemented plants #3-5/7 and #7-5/5 import 44% and 73% more, respectively, relatively to the WT chloroplasts.

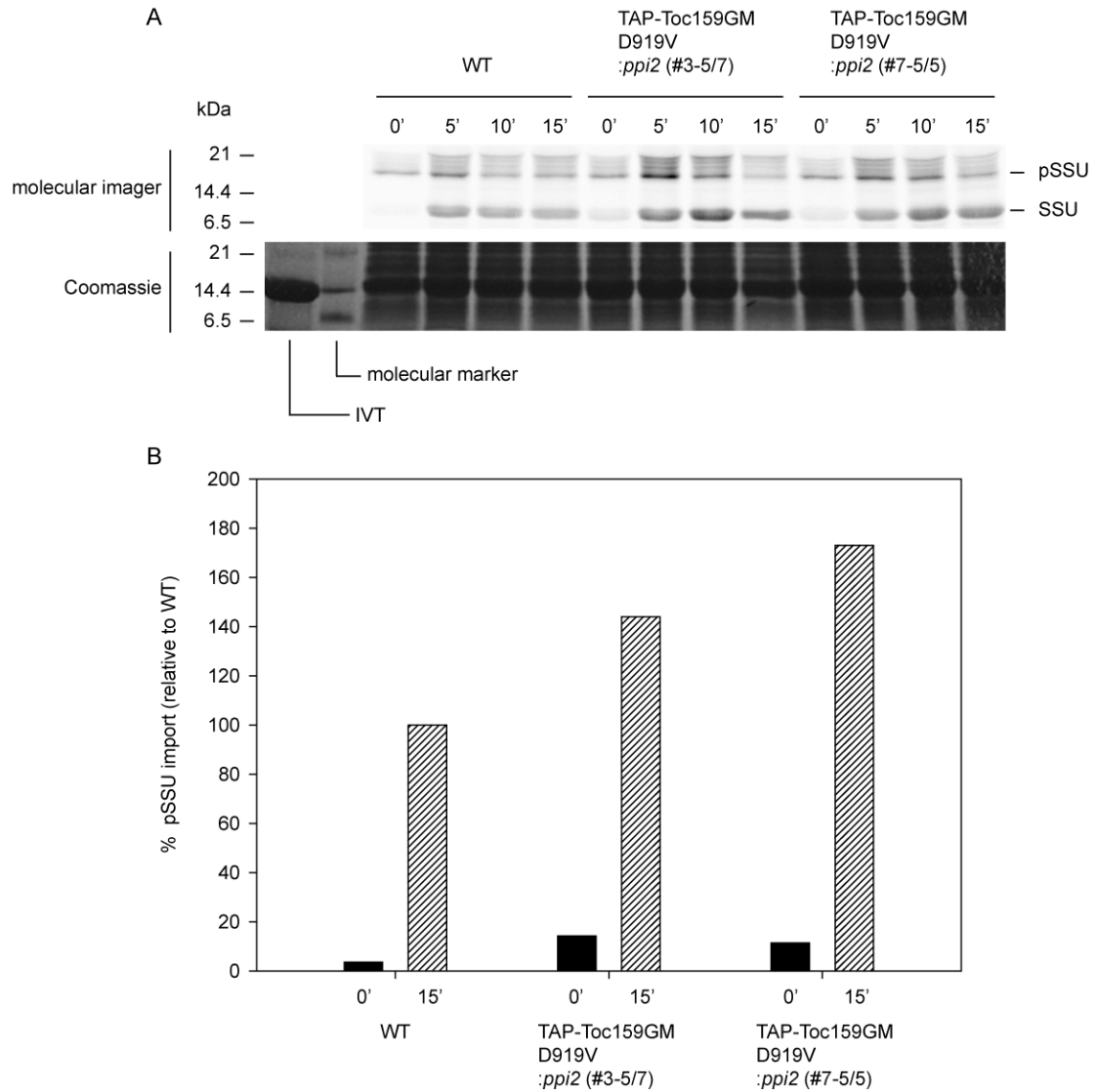


Figure 20: *in vitro* import of the precursor of the small subunit of the RubisCO (pSSU) into TAP-Toc159GM D919V chloroplasts. (A), *in vitro* translated [35 S]-methionine labelled pSSU was incubated with chloroplasts isolated from two distinct lines expressing TAP-Toc159GM D919V in the homozygous *ppi2* genetic background. Import was allowed to proceed for 0, 5, 10 and 15 min. Chloroplast were subsequently re-isolated and total protein was precipitated for SDS-PAGE analysis followed by Coomassie blue staining and PhosphorImager detection. (B), Quantification of data shown in (A) with the QuantityOne software (Biorad) at 0 and 15 min of import of pSSU. The amount of imported SSU after 15 min of import into WT isolated chloroplast was set to 100%.

3- Discussion

3.1- Synthesis

3.1.1- Requirements for an optimal preprotein import

Earlier research from our lab indicated that weakening the interactions between Toc-GTPases by single point mutants in the D1 motif reduced import capacity but was non-lethal. We therefore attempted to identify Toc159 mutants with an increased affinity for Toc33 to determine what their effects on a variety of chloroplast development and protein import parameters would be. We used a yeast Two-Hybrid based screening strategy to identify Toc159G mutants able to suppress the Toc33G R130A loss-of-interaction mutant. We identified two mutants of Toc159G able to rescue the loss-of-interaction phenotype of Toc33G R130A. Both Toc159G mutants had several amino-acids changes and, strikingly, one of these point mutations, D919V, was common to both mutants. This point mutation was further characterised. The D919V mutation alone was able to restore the loss-of-interaction with Toc33G R130A and resulted in a stronger binding of Toc159G to Toc33G WT. Interestingly, D919V restored interaction with Toc33G variants mutated in the D1 motif but not in the G1 motif. The D1 motif corresponds to the second half of the β 4-sheet of Toc33 and the G1 motif is positioned between the β 1-sheet and the α 1-helix (Figure 21B). Based on the homology of the G-domains it is possible to extrapolate the structure of Toc159G based on the crystal structure of psToc34 and atToc33 (Sun *et al*, 2002; Koenig *et al*, 2008). Thereby, the homodimeric crystal of psToc34 (Figure 21A) resembles that of the Toc159G/Toc33G heterodimer. The dimer appears to have a central symmetry and the Switch II regions of the two monomers interact with each other. The D919V mutation of Toc159 is positioned in the Switch II region. So apparently there is no direct link between the mutation D919V, the D1 motif and the G1 motif. The replacement of an aspartic acid residue, which is the most acidic amino-acid, by a valine, an apolar residue, might account for the strengthened Switch II / Switch II interaction between Toc159 and Toc33. This local increase in the strength of interaction seems to be sufficient to compensate for the loss-of-interaction of dimerisation mutants of Toc33G such as R130A. The fact that Toc159G D919V could restore loss-of-interaction mutants of the D1 motif but couldn't restore interaction with mutants of the G1 motif suggests that the GTP cycle and the dimerisation are two independent co-acting processes important for the optimal preprotein import into chloroplasts.

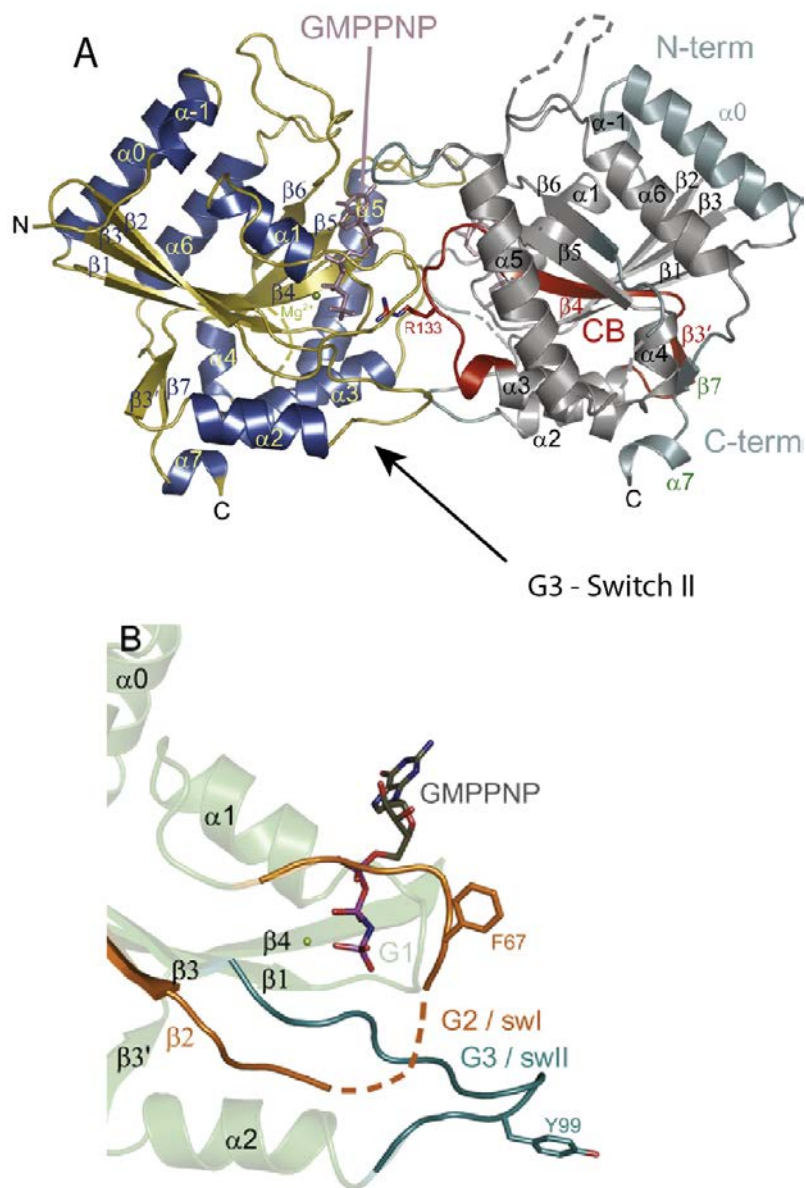


Figure 21: Crystal structure analysis of psToc34/atToc33. (A), The protein psToc34 is shown as a homodimer as ribbon diagram with GMP-PNP in stick representation. The monomer on the left-hand side is coloured in blue and the monomer on the right-hand side is coloured in grey. The conserved box (CB) characteristic for the paraseptin family is highlighted in red on the right-and side monomer. The arrow points out the G3/Switch II region of the left-hand monomer. (B), Zoom in of the G2/Switch I and G3/Switch II regions in monomeric atToc33 with GMP-PNP shown as ribbon diagram. Only selected secondary structure elements are shown for clarity, GMP-PNP is shown in stick representation. Adapted from Koenig *et al.* (2008).

3.1.2- G-domain dimerisation of the Toc GTPases is a key element of preprotein import

The use of the recombinant over-expressed protein GST-Toc159G D919V in a pull-down assay (Figure 14) confirmed the dimerisation data observed by means of the yeast Two-Hybrid assay (Figure 9 and Figure 10). Although the over-expression of the recombinant protein Toc159G D919V with a GST tag in N-terminal position was successful, its yield was lower than that of GST-Toc159G WT (Figure 13).

This may be due to the fact that the recombinant protein GST-Toc159G D919V is less soluble than the WT recombinant protein. To address this issue it may be useful to use a C-terminal GST-tag instead of an N-terminal tag to Toc159G. Two arguments are in favour of cloning the G-domain of Toc159 with a tag in C-terminal position likely to support the over-expression of a more soluble protein and its purification to homogeneity. (1) both Toc159GM and Toc159 full-length exist *in vivo*; the G-domain alone doesn't. So, it is possible that, rather than removing the M-domain of Toc159, replacing it by the GST-tag may reinforce the stability and the solubility of the recombinant protein, especially in the case of the Toc159G D919V. (2), HCA (Hydrophobic Cluster Analysis) established that the A, G and M domains of proteins belonging to the Toc159 family originate from tandem duplications of the GTPase G-domain, evolving in an unstructured spatial conformation in the case of the A-domain (Hernandez Torres *et al*, 2007). The A-domain has been shown to belong to the class of IDPs (Intrinsically Disordered Proteins) that are able to undergo conformational changes upon various environmental stimuli (Richardson *et al*, 2009). While the A-domain is not essential for viability its function is unresolved and the fact that the GST-tag in N-terminal position of Toc159G replaces the A-domain can induce artefacts responsible for the lower solubility and stability of the GST-Toc159G D919V recombinant protein.

According to data presented in Figure 14, the D919V mutation increased the heterotypic interaction of Toc159 with Toc33. Given the high structural identity of the G-domain of Toc33 and Toc159 (Figure 11), it is likely that the homotypic Toc159-Toc159 interactions would also be increased by the D919V mutation. For this purpose, it will be interesting to perform a pull-down assay in order to confirm that binding of Toc159 D919V with Toc159 WT is indeed increased.

Furthermore, the mutation D919V can rescue the *ppi2* phenotype of *A. thaliana in vivo* (Figure 17), suggesting that a stronger dimerisation (Figure 14), either in the case of homo or heterodimerisation, is not detrimental to the growth and development of the plant. Also, the rate of preprotein import into *ppi2*:TAP-Toc159GM D919V chloroplasts is higher than that of WT chloroplasts (Figure 20). Taken together, these data are consistent with observations made by Lee *et al.*, 2009, Agne *et al.*, 2009 and Aronsson *et al.*, 2010: A reduced ability to perform dimerisation resulted in lower preprotein import efficiency in the case of *ppi1* plants

complemented with Toc33 R130A. The present study shows that reciprocally a higher ability for dimerisation supports higher rate of preprotein import into chloroplasts of *ppi2* plants complemented with Toc159 D919V (Figure 20).

It is noteworthy that complementation experiments on *ppi2* were done utilising a 35S promoter. Hence, it would be interesting to see if the same results appear when the mutant recombinant Toc159 protein is expressed under the control of the endogenous promoter. In order to complete the overall picture, it would be interesting (1) to test if the greening kinetic, the acquisition of the photosynthetic capacity, is faster in the case of *ppi2*:TAP-Toc159GM D919V when compared to WT plants by means of a de-etiolation experiment and (2) to measure the chlorophyll content of *ppi2*:TAP-Toc159GM D919V plants in comparison with WT plants.

3.1.3- Toc159 D919V has GTPase properties distinct from the WT

The GTP hydrolysis data (Figure 14) seem to contradict the GTP binding data (Figure 15): despite a GTP hydrolysis rate similar to WT, Toc159 D919V exhibits strongly reduced GTP binding in our blot-based binding assay. The GTP-binding assay was only carried out at one concentration of GTP. It is possible that at the given GTP concentration, the steady-state binding of GTP was low. Also, if the affinity for GTP in the D919V were reduced the GTP may have been removed during the washing steps in our GTP-binding assay. So, the absence of GTP-binding in the case of GST-Toc159 D919V does not exclude that GTP-binding still takes place and might explain why the hydrolysis of GTP is still active.

According to recent studies, preprotein translocation efficiency is regulated by the GTPase cycle at Toc159 (Wang *et al.*, 2008; Agne *et al.*, 2009) Data demonstrating that mutants with strongly reduced GTP-binding or hydrolysis rates argue against a model implicating multiple rounds of GTP-hydrolysis per preprotein and suggest that the "motor model" hypothesis is not robust (Smith *et al.*, 2002; Wang *et al.*, 2008; Agne *et al.*, 2009). In fact, it appears that the translocation event may be dissociated from GTPase activity at Toc159 (as well as Toc33). Here, we demonstrate that the Toc159 D919V which binds to Toc33 more strongly than the wild type also retains at least partial functionality.

In order to go deeper in the characterisation of the GTPase cycle of Toc159G, it would be of interest to assess the nucleotide binding properties of Toc159 D919V in more detail firstly by testing its ability to bind different nucleotides such as GDP and GMP-PNP by means of a nucleotide binding assay similar to the one presented in Figure 15. In the case of Toc159 D919V it is possible that the steady state binding is too unstable to allow for the measurements of nucleotide binding by the method presented in this thesis. The surface

plasmon resonance would offer an alternative way to monitor the nucleotide binding properties of GST-Toc159 D919V by measuring not only the equilibrium binding constants but also association and dissociation rates. In Reddick *et al*, (2007) the authors were not surprised by the variation of kinetic parameters between the different homologues of TOC GTPases. Nevertheless they were troubled by the huge variation reported for kinetic properties between different groups and, on occasion, within a given group for the same protein. For this reason they proposed the use of a systematic and robust method enabling a direct comparison of the GTPases enzymatic and dimerisation properties.

3.2- Outlook

3.2.1- Crystallisation of Toc159G and its interaction partner

Owing to a number of crystallography studies, a large body of data has become available on the 3D structure of Toc33G. Based on the sequence similarity of Toc159G with Toc33G, it is possible to extrapolate the structure of Toc159G. However, it would be important to determine the actual crystal structure of Toc159G. But to date, no crystal structure of Toc159G has been published and this has been attributed to relatively low expression rates as well as protein stability (Sun *et al*, 2002; Weibel *et al*, 2003). In order to achieve crystallization it may be useful to employ new expression strategies such as co-expression (using for example the pETDuet plasmid system) to achieve dimerisation of the partners Toc33G and Toc159G *in vivo*. Using this strategy it may be possible to isolate sufficient quantities of Toc159G in the form of a stable heterodimer *in vivo*, rather than by separate expression followed by *in vitro* dimer reconstitution. Such a strategy may yield both the crystal structure of Toc159G as well as that of the heterodimer and could be used in combination with a set of mutants of Toc159G. In particular, it is possible that the Toc159 D919V mutation may prove useful because of its increase ability to interact with Toc33G. This approach is currently underway.

3.2.2- Deciphering the G-domain of Toc159

The data presented in this thesis have led to the identification and characterisation of an interesting mutant of the G-domain of Toc159 where an Asp residue was changed to a Val residue in position 919 of the amino acid sequence of Toc159. This mutant has allowed

important achievements regarding the functional characterisation of Toc159G. Strikingly, the dimerisation property of Toc159G D919V was altered independently of the GTP-hydrolysis activity but reduced GTP-binding. The complementation of *ppi2* with TAP-Toc159GM D919V indicated its functionality. Toc159GM D919V supported a higher preprotein import rate in isolated chloroplasts. Nevertheless, many questions regarding the mechanistic details of preprotein import at the TOC complex are pending.

For instance, and along the same lines of what has been done in this study, it would be interesting to perform a similar set of experiments to isolate and characterise with mutants of Toc159G that exhibit a weak dimerising phenotype but unaltered GTPase functions. The strategy used for screening mutants able to rescue the loss-of-interaction of Toc33G R130A has been adapted in order to identify mutations in Toc159G with a decreased binding to Toc33G WT. This strategy allowed the identification of two point mutations: D946N and D949V (Figure 11). Indeed, these two mutants are promising in the way that their binding properties, yet to be analysed, reflect the exact opposite of mutant D919V. The comparison of data obtained from these dimerisation mutants would certainly help unravel more precisely the mechanism and implication of the dimerisation of Toc GTPases during the process of preprotein import into the chloroplast. The characterisation of these mutants is underway.

Now we know that dimerisation and GTPase functions in Toc159 are at least partially separable as we showed that Toc159G D919V can restore interaction with Toc33G mutated in the D1 motif but not in the G1 motif. It would be of interest to generate Toc159 mutants bearing different combinations of G-domain double mutants such as, A864R/D919V, A864R/D946N or D949V, K868R/D919V and K868R/D946N or D949V simultaneously affecting dimerisation and GTP-binding and/or -hydrolysis. Enzymatic characterisation and complementation experiments carried on such Toc159 G-domain double mutants would allow new insight as they affect the functionality of Toc159G at two different levels which, based on our results, cannot be mutually compensated.

Finally, another important question addresses the interrelation of the A- and G-domains of Toc159. Recently, phosphorylation of the A-domain has been demonstrated. The phosphorylation state of the A-domain may be related to the maturation of the chloroplast. Indeed, the need for photosynthesis-related proteins is greater during the earlier stages of growth and development of the plant and decreases with time according to the profile of expression of Toc159 (Figure 22 – red curve). It is therefore conceivable that phosphorylation of the A-domain may have a regulatory effect during the course of chloroplast development. Likely, the A-domain plays a role in the regulation of preprotein import into plastids. An interesting hypothesis to verify is that the A-domain somehow primes the TOC complex in order to foster its selectivity for subset of preproteins e.g. photosynthetic-related proteins in the case of chloroplasts. The A-domain, detached from

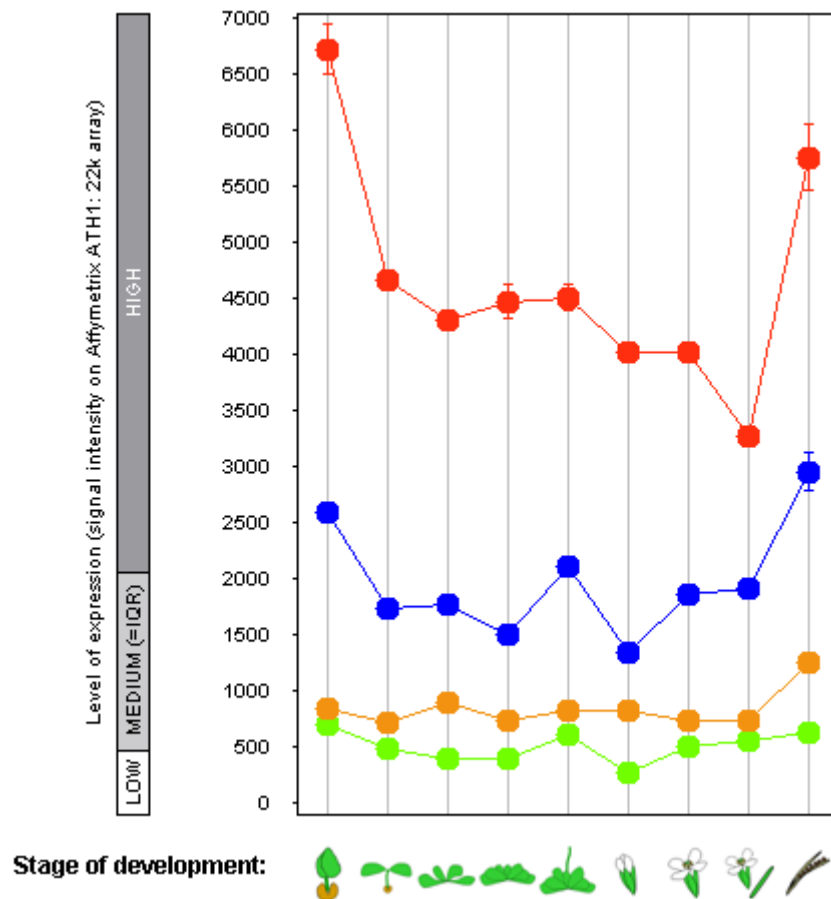


Figure 22: Level of expression of Toc159 family members in the course of plant development. The red expression profile corresponds to Toc159. The blue expression profile corresponds to Toc132. The orange expression profile corresponds to Toc120. The green expression profile corresponds to Toc90. The data were obtained from Genevestigator (Hruz *et al*, 2008).

Toc159, senses the physiological state of the chloroplast and adapts the quantity of photosynthetic-related pathways according to the needs of the plastid. It has been demonstrated that the A-domain of atToc159 and atToc132 have properties of intrinsically disordered proteins (Hernandez Torres *et al*, 2007; Richardson *et al*, 2009), suggesting an involvement of the A-domain in transient and multiple protein-protein interactions, possibly with the transit peptides of preproteins or other unidentified partners (Agne *et al*, 2010). Overall, the implication of Toc159A during preprotein import constitutes a new field of investigation in close relation to that of Toc159G. Given the proposed regulatory role it appears likely that the A-domain directly influences the activity of the G-domain.

As it can easily be seen, many Toc GTPases G-domain mysteries still have to be unveiled in order to understand its precise functions and implications during the course of chloroplasts biogenesis.

4- Materials and methods

4.1- Materials

4.1.1- Biological material

4.1.1.1- Plants

Wild type (WT) *Arabidopsis* plant, if not specified otherwise, refers to *A. thaliana* (L.) Heynh. var. Columbia 2. The *Arabidopsis* line CS11072 (*ppi2*) is in the Wassilewskija background and was obtained from the Arabidopsis Biological Resource Center (ABRC), The Ohio State University, Columbus OH, USA (Bauer *et al*, 2000).

4.1.1.2- Micro-organisms

Yeast strain pCY2 was obtained from Clontech laboratories AG, Basel, Switzerland. *Escherichia coli* strain DH5 α was obtained from Invitrogen AG, Basel, Switzerland and *E. coli* strain BL21-DE3 was obtained from Novagen, Inc., Madison WI, USA. *Agrobacterium tumefaciens* strain C58 was kindly provided by Dr. R. Kuhn, Institute of Plant Sciences, ETH Zurich, Switzerland.

4.1.2- Oligonucleotides

Oligonucleotides were synthesized at Microsynth GmbH, Balgach, Switzerland.

4.1.3- Plasmids

pGADT7 and pGBKT7 were obtained from Clontech laboratories AG, Basel, Switzerland. pET21d was purchased from Novagen, Inc., Madison WI, USA. pGEX4T-1 was obtained from Amersham Biosciences AB, Uppsala, Sweden. pGEM-T Easy Vector Systems was purchased from Promega AG, Dubendorf, Switzerland. The binary vector pCHF7 (Dr.

Christian Fankhauser, Center for Integrative Genomics, University of Lausanne, CH) contains two CaMV35S eukaryotic promoters, one of them having a duplicated enhancer region, an rbcS terminator and the phosphinotricin acetyltransferase (pat) gene for transgene selection.

4.1.4- Antibodies

Polyclonal antibodies specific to atToc33 (Agne *et al.*, 2009) and atToc159A (Bauer *et al.*, 2000) have been described. Rabbit IgGs are from ICN Immunobiological.

4.1.5- Chemicals

Unless noted otherwise, the chemicals were from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland. Radionuclides ^{35}S and ^{33}P chemicals were from Hartmann Analytic GmbH, Braunschweig, Germany.

4.2- Methods

4.2.1- Physiological methods

4.2.1.1- Growing *Arabidopsis thaliana* on Murashige and Skoog (MS) medium

Seeds were incubated 10 minutes in 70% ethanol, 20 minutes in absolute ethanol and finally rinse with sterile ddH_2O . Sterile seeds were spread on 1% (w/v) Phyto-Agar (Duchefa) containing 0.5x MS medium (MS; Duchefa) supplemented with 0.1% (w/v) sucrose and appropriate antibiotic (kanamycin and/or phosphinotricin). Germination was synchronised by exposing the seeds 2 days at 4°C in the dark. Plants were grown under short-day conditions (8 hours light [$120 \cdot 10^{-6} \text{ mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$], 22°C; 16 hours dark, 17°C).

4.2.1.2- Growing *Arabidopsis thaliana* on soil

Seeds were set on sandy soil (Top Dressing, Ricoter AG, Aarberg, Switzerland). Germination was synchronised as above. Plants were grown under short-day (8 hours light [$120 \cdot 10^{-6} \text{ mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$], 22°C, 16 hours dark, 17°C) or long-day (16 hours light [$120 \cdot 10^{-6} \text{ mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$], 22°C, 8 hours dark, 17°C) conditions.

4.2.2- Molecular cloning

4.2.2.1- PCR, restriction digest and ligation reaction

Standard protocols were used for cloning (Sambrook, 2001). DNA fragments for cloning were PCR amplified using Taq-polymerase (New England Biolabs, Inc., Beverly MA, USA) according to the supplier's recommendation. Suitable restriction sites were introduced into PCR primers. Pre-existing plasmid constructs were used as templates for PCR.

PCR fragments and vectors were digested with the respective restriction enzymes (New England Biolabs, Inc., Beverly MA, USA) and purified from agarose gels using the QIAquick kit (Quiagen). Vectors were dephosphorylated using Phosphatase Antarctica (New England Biolabs, Inc., Beverly MA, USA). T4 DNA ligase (New England Biolabs) was used according manufacturer's recommendation to ligate vectors and inserts.

4.2.2.2- Transformation of thermo-competent *Escherichia coli* cells

Ligation reactions were subsequently transformed by heat shock into competent *E.coli* DH5- α or BL21 cells (Sambrook, 2001). Competent *E.coli* cells were prepared as described (Inoue *et al.*, 1990). Selection was done on LB medium (25g.l⁻¹ LB Broth Miller [Becton Dickinson Diagnostic Systems], 1% (w/v) Agar Bacteriological grade [ICN Biomedicals]) supplemented with appropriate antibiotics. Clones were selected by PCR and restriction digestion.

4.2.2.3- Plasmid isolation and purification

For 5 ml culture small-scale plasmid isolation, the GenElute Plasmid MiniPrep Kit (Sigma) was used according to the supplier's instructions. The PureYield™ Plasmid MidiPrep System (Promega) was used for plasmid isolation from 50 ml culture volume.

4.2.2.4- DNA sequencing

DNA sequences were verified by sequencing (Microsynth GmbH, Balgach, Switzerland).

4.2.2.5- Transformation of Yeast

Yeast transformation was performed as described in the Yeast Protocols Handbook (Clontech Laboratories Inc.).

4.2.2.6- Transformation of electro-competent *Agrobacterium tumefaciens*

A.tumefaciens, strain C58, were grown in YEB medium (0.5% (w/v) Bacto-Trypton, 0.5% (w/v) Bacto-Peptone, 0.1% (w/v) yeast extract (from Difco), 2 mM MgCl₂), supplemented with appropriate antibiotics, until an optical density of 0.6, at 526 nm, is reached. Cells are pelleted (4000 g, 10 min, 4°C) and washed with 10% glycerol. Fifty ng of plasmid DNA were used for electroporation in a MicroPulser (Biorad laboratories) according to manufacturer instructions. Transformants were selected on YEB medium, 1% (w/v) Agar supplemented with appropriate antibiotics.

4.2.3- DNA constructs

4.2.3.1- pGADT7 constructs

The cloning of pGADT7-Toc33G (Toc33₁₋₂₆₅) has been described previously (Rahim *et al*, 2009).

4.2.3.2- pGBKT7 constructs

The cloning of pGBKT7-Toc159G (Toc159₇₂₈₋₁₀₉₃) has been described previously (Rahim *et al*, 2009).

The plasmid pGBKT7-Toc159G was the template to introduce the D919V point mutation along with an additional PstI restriction site using complementary primers qc_D919V_s (5'-GGG TTT GAA GTC TGC AGC GAT GGT TCA AAG TAC AAA TGC-3') and qc_D919V_r (5'- GCA TTT GTA CTT TGA ACC ATC GCT GCA GAC TTC AAA CCC-3') via QuikChange® Site-Directed Mutagenesis (Stratagene) resulting in pGBKT7-Toc159G D919V. The construct was verified by sequencing.

4.2.3.3- pET21d constructs

The cloning of pET21d-Toc33G (Toc33₁₋₂₆₅) has been described previously (Hiltbrunner *et al*, 2001b).

The cloning of pET21d-Toc33G-R130A has been described previously (Weibel *et al*, 2003).

The cloning of pET21d-Toc159 has been described previously (Bauer *et al*, 2000).

The plasmid pET21d-Toc159 was the template to introduce the D919V point mutation along with an additional PstI restriction site via QuikChange® Site-Directed Mutagenesis (Stratagene) resulting in pET21d-Toc159–D919V. The construct was verified by sequencing.

4.2.3.4- pGEX4-T1 constructs

The cloning of pGEX4-T1-Toc159G (Toc159₇₂₇₋₁₀₉₃) has been described previously (Rahim *et al*, 2009).

Point mutations D919V was introduced via QuikChange® Site-Directed Mutagenesis (Stratagene) into the plasmid pGEX4-T1-Toc159G resulting in pGEX4-T1-Toc159G D919V. The construct was verified by sequencing.

4.2.3.5- pCHF7 constructs

The cloning of pCHF7-NTAPi-Toc159GM has been described previously (Agne *et al*, 2009).

For cloning of pCHF7-NTAPi-Toc159GM D919V, plasmid pGBKT7-Toc159G D919V was digested NcoI/StuI and the fragment has been ligated into BspHI/StuI digested pCHF7-NTAPi-Toc159GM, resulting in pCHF7-NTAPi-Toc159GM D919V. The construct was verified by sequencing.

4.2.4- Bacterial expression and purification of recombinant protein

4.2.4.1- pET21d constructs

For bacterial expression, the pET21d constructs were transformed into *E.coli* BL21(DE3) cells. Bacteria were grown till OD₆₀₀ achieved a value of 0.6 and expression was induced during 4 hours with 0.4 mM IPTG at 37°C with moderate shaking. Following over-expression, the protein was purified from the soluble fraction of bacterial lysate under non-denaturing conditions using pre-packed HisTrap HP chromatography column along with AKTA PrimePlus from GE Healthcare. The bound protein was eluted on a gradient from 0 to 100% of French Press Buffer (50 mM Tris/HCl pH 8, 300 mM NaCl) containing 500 mM imidazole and 1 mM β -mercaptoethanol. Fractions containing the protein were dialysed overnight against dialysis buffer (50 mM Tris/HCl, 25 mM KOAc, 1 mM DTT, 1 mM MgCl₂ and 10% (v/v) glycerol). The dialysate was subsequently aliquoted and stored at -80°C.

4.2.4.2- pGEX4-T1 constructs

For bacterial expression, the pGEX4T1 constructs were transformed into *E.coli* BL21(DE3) cells. Bacteria were grown till OD₆₀₀ achieved a value of 0.6 and expression was induced during 4 hours with 0.4 mM IPTG at 37°C with moderate shaking. Following over-expression, the protein was purified from the soluble fraction of bacterial lysate under non-denaturing conditions using pre-packed GSTrap HP chromatography column along with AKTA PrimePlus from GE Healthcare. The bound protein was eluted with elution buffer (50 mM Tris/HCl pH 8 and 10 mM reduced glutathione). Fractions containing the protein were dialysed overnight against dialysis buffer (50 mM HEPES/KOH pH 7.5, 25 mM KOAc, 1 mM Mg(OAc)₂, 1 mM DTT and 10% (v/v) glycerol). The dialysate was subsequently aliquoted and stored at -80°C.

4.2.5- Stable transformation of *Arabidopsis thaliana*

Stable transformation of heterozygous *Arabidopsis thaliana* (Ws) *ppi2* plant was carried out using the floral dip method (Clough & Bent, 1998). Transformants selection was performed by growing plants on selective medium and segregation analysis. Genotype determination and presence of the transgene were verified according to (Agne *et al*, 2009) with primers pairs specific for the *WT* (prA3R 5'-CAG TGC ATG TTG ATG TGG CA-3' and pr159int 5'-GAA TAG GGT TTT AAT CGG AAG-3') or *ppi2* (prLB5 5'- GAT GCA ATC GAT ATC AGC CAA TTT TAG AC-3' and pr159int) alleles. A specific primer set was used to confirm the presence of the T-DNA construct (pCL60_35S 5'-TCT CCA CTG ACG TAA GGG AT-3' and pr86H6 5'-CAA CAG TCT ATA GGA CAG GC-3'). The presence of D919V mutation was confirmed by digestion of the transgene PCR product with PstI. Protein expression of the transgene was detected after total protein extraction (Rensink *et al*, 1998a) and methanol/chloroform precipitation for separation by SDS-PAGE and immunoblotting with IgG and α Toc159A (Agne *et al*, 2009).

4.2.6- SDS-PAGE, Western blotting and immuno-detection

4.2.6.1- SDS-PAGE and Western blotting analysis

Protein analysis was made by SDS-PAGE (Laemli, 1970) with the Mini-PROTEAN apparatus from Biorad (Biorad Laboratories, Hercules CA, USA). The percentage of polyacrylamide was chosen according the mass of proteins to be separated, ranging from 8 to 12%. The separating gel consists of 8-12% (w/v) acrylamide/bis-acrylamide (37.5:1, Biorad), 0.4 M Tris/HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (v/v) TEMED (Biorad) and 0.08% (w/v) APS. The stacking gel, poured on top of the separating gel, consist of 4% (w/v) acrylamide/bis-acrylamide (37.5:1, Biorad), 0.06 M Tris/HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (v/v) TEMED (Biorad) and 0.08% (w/v) APS. The proteins, prior to their separation on SDS-PAGE, were taken up in 1x SDS-PAGE sample buffer (0.04 M Tris/HCl pH 6.8, 0.1 M DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% glycerol (v/v)) and heated at 65°C for 15 minutes. Following proteins separation, the gel was either stained with Coomassie blue according standard protocol (Sambrook & Russel, 2001) and dried or used for western blot transfer on Protran nitro-cellulose membrane (Schleicher and Schuell, Dassel, Germany) and stained with AmidoBlack (Naphthol Blue Black) according standard protocol(Sambrook & Russel, 2001).

4.2.6.2- Immuno-detection using specific antibodies

To block unspecific binding of antibodies, nitro-cellulose membranes were incubated 1h at ambient temperature or, o/n at 4°C, in blocking buffer (PBS buffer consisting in 150 mM NaCl, 7.5 mM Na₂HPO₄, 1.5 mM NaH₂PO₄ containing 5% (w/v) skim milk powder). Membranes were then incubated for 1h at ambient temperature with adequate primary antibody (either IgG dilution 1:5000 or anti-Toc159A dilution 1:3000) in PBS with 5% (w/v) skim milk powder. After incubation with the primary antibody, nitro-cellulose membranes are washed extensively with PBS or PBS-tween (0.05% (v/v) Tween R 20). To reveal primary antibodies, membranes were incubated 1h at ambient temperature with a 3'000x dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Bio-Rad Laboratories) in PBS buffer containing 5% (w/v) skim milk powder. After extensive wash with PBS or PBS-T buffer, signals were detected by enhanced chemiluminescence. Incubation for 1 minute in ECL buffer (0.1 M Tris/HCl pH 8.5, 1.25 mM 3-aminophthalhydrazide (luminol), 0.2 mM p-coumaric acid, 0.009% (v/v) H₂O₂) and exposed to high performance chemiluminescence camera (Biorad).

Antibodies were produced into rabbit organisms.

For re-use of the blot with another primary antibody, the nitrocellulose membrane is stripped with stripping buffer (PBS, 100 mM β-mercaptoethanol, 2% (v:v) SDS) during 30 min at ambient temperature with moderate shaking, modified from (Kaufmann *et al*, 1987) and extensively washed with PBS.

4.2.7- Yeast two-hybrid library

In order to screen for mutants able to suppress the loss of interaction phenotype of atToc33G R130A, a mutant library has been generated in yeast. For this purpose a 30 cycles mutagenic PCR was performed using 60 pmol of primers 159GB5' NcoI and pETR in a 50 µl reaction with 1 ng of pET21d-Toc159G, 0.5 mM MnCl₂, 0.2 mM dATP and dGTP, 1 mM dTTP and dCTP, goTaq buffer and 0.25 µl of goTaq polymerase, modified from (Cadwell & Joyce, 1992). Plasmid pGBKT7-Toc159G (Rahim *et al.*, 2009) has been digested by PstI/StuI resulting in a linearized plasmid. Both mutagenized amplicons and linearized plasmid were co-transformed in yeast and plated on SD medium -Trp -Leu for selection of positive clones. Plasmids were subsequently isolated and sequenced.

4.2.7.1- Two-hybrid colony-lift filter assay

The filter assay has been performed according to the Yeast Protocol Handbook (Clontech Laboratories, Inc.)

4.2.7.2- ONPG assay

The liquid culture assay using ONPG as substrate has been performed according to the Yeast Protocol Handbook (Clontech Laboratories, Inc.)

4.2.8- Pull-down assay

Recombinant 6xHis-tagged Toc33WT or Toc33R130A were purified as described in 4.2.4.2 and incubated at 0, 2.5, 5 and 10 μ M in presence of 2.5 μ M of recombinant purified GST-Toc159G WT or GST-Toc159G D919V (see 4.2.4.3) in binding buffer (25 mM HEPES-KOH pH 7.5, 40 mM KOAc, 2 mM Mg(OAc)₂, 20 μ M DTT, 0.1% (v/v) Triton X-100) under moderate agitation at 4°C. After 10 minutes, 10 μ l of pre-equilibrated Ni-NTA column was added and incubation at 4 °C, under moderate agitation, continued for 1 hour to re-isolate 6xHis-tagged proteins. The resin was then washed 6 times with 0.5 ml of washing buffer (binding buffer complemented with 0.4mM GTP) and 2 times with 0.5 ml of binding buffer containing 40 mM imidazole. Elution was performed by adding 200 μ l of elution buffer (binding buffer containing 200 mM imidazole). Eluted proteins were precipitated by chloroform/methanol, resolved by SDS-PAGE followed by Coomassie blue staining. Quantitation was performed with QuantityOne software (Biorad).

4.2.9- Guanosine Tri-Phosphate binding and hydrolysis

4.2.9.1- GTP binding assay

One μ M of purified recombinant protein, GST-Toc159G WT or GST-Toc159G D919V (section 4.2.4.3), was incubated with 0.1 μ M [α ³³P]-GTP on ice for 60 min in binding buffer (20 mM Tris-HCl pH8, 50 μ M MgCl₂, 0.3% [v/v] Tween 20 and 0.4 mM GTP) in a final volume of 20 μ l. A 2 μ l aliquot of the reaction has been spotted onto nitrocellulose membrane, which

has been pre-incubated in binding buffer and air dried. The spotted membrane was subsequently washed 3 times with 10 ml ice cold washing buffer (20 mM Tris-HCl pH8, 5 mM MgCl₂ and, 0.3% [v/v] Tween 20) and air dried. Bound [$\alpha^{33}\text{P}$]-GTP was detected and quantified using a PhosphorImager and QuantityOne software (Biorad laboratories, Hercules CA, USA). Modified after (Weibel *et al*, 2003).

4.2.9.2- GTP hydrolysis assay

GTP hydrolysis rate of purified recombinant protein GST-Toc159G WT or GST-Toc159G D919V (section 4.2.4.3) was measured using a method adapted from (Liang *et al*, 2000). The recombinant protein, at a concentration of 2.5 μM , was incubated at 25°C in hydrolysis buffer (20 mM Tris/Cl pH 8, 25 mM KOAc, 2 mM MgCl₂, 0.1 g/l BSA and 50 nM [$\alpha^{33}\text{P}$]-GTP) containing increasing concentration (0, 10, 100, 200, 500, 1000, 2000, 5000 and 10000 μM) of non-radioactive GTP in a final volume of 50 μl . After 0 min, 30 min, 60 min and 120 min incubation, 10 μl of the reaction was removed and stopped by the addition of 10 μl of stopping buffer (0.4 % SDS, 20 mM EDTA, 8 mM GTP, 8 mM GDP) and heated to 65°C for 2 min. Two μl of the samples were spotted onto PEI cellulose F TLC plates (Macherey Nagel). GTP and GDP were separated by chromatography using 0.75 M KH₂PO₄ pH 3.5 as the solvent. The plates were air dried and spots corresponding to GTP and GDP were detected and quantified using a Phosphorimager (Biorad).

For each concentration of unlabelled GTP, the amount of hydrolysed GTP has been plotted as a function of time. A linear regression has been fitted for each plot in order to calculate the initial rate of GTP hydrolysis. From this, moles of GTP hydrolysed per minute per mole of enzyme were calculated and plotted against the concentration of GTP according Michaelis-Menten representation. Data were modelised using SigmaPlot for fitting a non-linear regression curves according Michaelis-Menten equation for enzymatic parameters (V_M and K_M) determination.

4.2.10- Insertion and import into the Chloroplasts

4.2.10.1- *In vitro* translation/transcription

[^{35}S -Met] Toc159 WT/D919V to be used in the insertion assay were synthesised *in vitro* from plasmids described in section 4.2.3.3. The synthesis was done using the

reticulocyte based TNT T7 Quick-coupled transcription/translation system (Promega) according to the supplier's instructions.

4.2.10.2- Insertion assay

Targeting experiment of [³⁵S-Met]-Toc159 WT or [³⁵S-Met]-Toc159 D919V was performed as described in (Bauer *et al*, 2002) on isolated chloroplasts (Fitzpatrick & Keegstra, 2001) according to modifications of (Agne *et al*, 2009). Prior to perform the assay, chloroplasts were incubated in the dark in order to deplete them of endogenous ATP. After chloroform/methanol protein precipitation of thermolysine treated and untreated chloroplasts, proteins were resolved on SDS-PAGE. The gel was dried and expose on PhosphorImager (Biorad) for detection and quantification of radioactive labelled Toc159.

4.2.10.3- Import assay

The import experiment of the small subunit of RubisCO into intact isolated chloroplast has been described previously (Agne *et al*, 2009).

4.2.11- Electron microscopy

Leaf tissue from 21 day-old *in vitro* grown control and mutant plants were fixed in fixative buffer [5% (w/v) glutaraldehyde and 4% (w/v) formaldehyde in 100 mM phosphate buffer (pH 6.8)] overnight at 4°C, rinsed several times in phosphate buffer, and post-fixed for 2 h with 1% (w/v) osmium tetroxide in phosphate buffer at 20°C. After two washing steps in phosphate buffer and distilled water, the pieces were dehydrated in ethanol and embedded in Spurr's low-viscosity resin (Polyscience). Ultrathin sections of 50–70 nm were cut with a diamond knife (Ultracut-E microtome – Reichert-Jung), mounted on uncoated copper grids and contrasted with uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Sections were observed with a Philips CM100 transmission electron microscope operating at 60 kV (Philips Electron Optics BV, Eindhoven, the Netherlands).

Bibliography

Agne B, Andres C, Montondan C, Christ B, Ertan A, Jung F, Infanger S, Bischof S, Baginsky S, Kessler F (2010) The acidic A-domain of Arabidopsis Toc159 occurs as a hyperphosphorylated protein. *Plant Physiology* **153**: 1016-30

Agne B, Infanger S, Wang F, Hofstetter V, Rahim G, Martin M, Lee D, Hwang I, Schnell D, Kessler F (2009) A Toc159 import receptor mutant, defective in hydrolysis of GTP, supports preprotein import into chloroplasts. *The Journal of Biological Chemistry* **284**: 8670-9

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. *J Cell Biol* **136**: 983-94

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) *Molecular biology of the cell*, new York: Garland Science.

Aldrige C, Cain P, Robinson C (2009) Protein transport in organelles: Protein transport into and across the thylakoid membrane. *FEBS* **276**: 1177-86

Anderson J, Melis A (1983) Localization of different photosystems in separate regions of chloroplast membranes. *PNAS* **80**: 745-9

Andersson S, Zomorodipour A, Andersson J, Sicheritz-Ponten T, Alsmark U, Podowski R, Naslund A, Eriksson A, Winkler H, CG K (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**: 133-40

Armbruster U, Hertle A, Makarenko E, Zuhlke J, Pribil M, Dietzmann A, Schliebner I, Aseeva E, Fenino E, Scharfenberg M, Voigt C, Leister D (2009) Chloroplast proteins without cleavable transit peptides: rare exceptions or a major constituent of the chloroplast proteome? *Molecular Plant* **2**: 1325-35

Aronsson H, Combe J, Patel R, Agne B, Martin M, Kessler F, Jarvis P (2010) Nucleotide binding and dimerization at the chloroplast pre-protein import receptor, atToc33, are not essential *in vivo* but do increase import efficiency. *The Plant Journal* **63**: 297-311

Aronsson H, Combe J, Patel R, Jarvis P (2006) *In vivo* assessment of the significance of phosphorylation of the Arabidopsis chloroplast protein import receptor, atToc33. *FEBS Lett* **580**: 649-55

Aronsson H, Jarvis P (2011) Dimerization of TOC receptor GTPases and its implementation for the control of protein import into chloroplasts. *Biochem J* **436**: e1-e2

Bae W, Lee YJ, Kim DH, Lee J, Kim S, Sohn EJ, Hwang I (2008) AKR2A-mediated import of chloroplast outer membrane proteins is essential for chloroplast biogenesis. *Nat Cell Biol* **10**: 220-7

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403**: 203-7

Bauer J, Hiltbrunner A, Weibel P, Vidi PA, Alvarez-Huerta M, Smith MD, Schnell DJ, Kessler F (2002) Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. *J Cell Biol* **159**: 845-54

Becker T, Jelic M, Vojta A, Radunz A, Soll J, Schleiff E (2004) Preprotein recognition by the Toc complex. *EMBO J* **23**: 520-30

Bedard J, Kubis S, Bimanadham S, Jarvis P (2007) Functional similarity between the chloroplast translocon component, Tic40, and the human co-chaperone, Hsp70-interacting protein (Hip). *J Biol Chem* **282**: 21404-14

Bhushan S, Kuhn C, Berglund AK, Roth C, Glaser E (2006) The role of the N-terminal domain of chloroplast targeting peptides in organellar protein import and miss-sorting. *FEBS Lett* **580**: 3966-72

Block M, Douce R, Joyard J, Rolland N (2007) Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. *Photosynth Res* **92**: 225-44

Bolter B, May T, Soll J (1998) A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. *FEBS Lett* **441**: 59-62

Bourne H, Sanders D, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349**

Bourne HR, Sanders DA, McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348**: 125-32

Bowsher C, Tobin A (2001) Compartmentation of metabolism within mitochondria and plastids. *J Exp Bot* **52**: 513-27

Brehelin C, Kessler F, van Wijk J (2007) Plastoglobules: versatile lipoprotein particles in plastids. *Trends in Plant Science* **12**

Brenner S, Jacob F, Meselson M (1961) An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature* **190**

Bruce BD (2001) The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. *Biochim Biophys Acta* **1541**: 2-21

Cadwell R, Joyce G (1992) Randomization of genes by PCR mutagenesis. *Genomes Res* **2**: 28-33

Caliebe A, Grimm R, Kaiser G, Lubeck J, Soll J, Heins L (1997) The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. *EMBO J* **16**: 7342-50

Cavalier-Smith T (1981) Eukaryote kingdoms: seven or nine? *Biosystems* **14**: 461-81

Cavalier-Smith T (2000) Membrane heredity and early chloroplast evolution. *Trends in Plant Science* **5**: 174-82

Chen D, Schnell D (1997) Insertion of the 34-kDa chloroplast protein import component, IAP34, into the chloroplast outer membrane is dependant on its intrinsic GTP-binding capacity. *The Journal of Biological Chemistry* **272**: 6614-20

Chen K, Chen X, Schnell DJ (2000) Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. *Plant Physiol* **122**: 813-22

Chen X, Schnell DJ (1999) Protein import into chloroplasts. *Trends Cell Biol* **9**: 222-7

Chen X, Smith MD, Fitzpatrick L, Schnell DJ (2002) In vivo analysis of the role of atTic20 in protein import into chloroplasts. *Plant Cell* **14**: 641-54

Chou ML, Fitzpatrick LM, Tu SL, Budziszewski G, Potter-Lewis S, Akita M, Levin JZ, Keegstra K, Li HM (2003) Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. *EMBO J* **22**: 2970-80

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735-43

Colicelli J (2004) Human RAS superfamily proteins and related GTPses. *Sci STKE* **2004**: re13

Constan D, Patel R, Keegstra K, Jarvis P (2004) An outer envelope membrane component of the plastid protein import apparatus plays an essential role in *Arabidopsis*. *Plant J* **38**: 93-106

Douce R, Joyard J (1990) Biochemistry and function of the plastid envelope. *Annu Rev Cell Biol* **6**: 173-216

Dutta S, Mohanty S, Tripathy B (2009) Role of temperature stress on chloroplast biogenesis and protein import in pea. *Plant Physiology* **150**: 1050-61

Economou A, Wickner W (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* **78**: 835-43

Egea I, Barsan C, Bian W, Purgatto E, Latche A, Chervin C, Bouzayen M, Pech J (2010) Chromoplast differentiation: current status and perspectives. *Plant Cell Physiol* **51**: 1601-11

Farnsworth C, Feig L (1991) Dominant inhibitory mutations in the Mg^{2+} -binding site of Ras^H prevent its activation by GTP. *Molecular and cellular biology* **11**: 4822-29

Faso C, Boulafloous A, Brandizzi F (2009) The plant Golgi apparatus: last 10 years of answered and open questions. *FEBS Lett* **583**: 3752-7

Felsenstein J (1974) The evolutionary advantage of recombination. *Genetics* **78**: 737-56

Fitzpatrick LM, Keegstra K (2001) A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. *Plant J* **27**: 59-65

Frigerio L, Hinz G, Robinson D (2008) Multiple vacuoles in plant cells: rule or exception? *Traffic* **9**: 1564-70

Gasper R, Meyer S, Gotthardt K, Sirajuddin M, Wittinghofer A (2009) It takes two to tango: regulation of G proteins by dimerization. *Nat Rev Mol Cell Biol* **10**: 423-9

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett* **261**: 455-8

Gould S, Waller R, McFadden G (2008) Plastid evolution. *Annu Rev Plant Biol* **59**: 491-517

Groenendyk J, Sreenivasaiah P, Kim do H, Michalak M (2010) Biology of endoplasmic reticulum stress in the heart. *Circ Res* **107**: 1185-97

Grossman A, Bartlett S, Schmidt G, Chua N (1980) Post-translational uptake of cytoplasmically synthesized proteins by intact chloroplasts in vitro. *Ann N Y Acad Sci* **343**: 266-74

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-9

Heins L, Mehrle A, Hemmler R, Wagner R, Kuchler M, Hormann F, Sveshnikov D, Soll J (2002) The preprotein conducting channel at the inner envelope membrane of plastids. *EMBO J* **21**: 2616-25

Heldt H, Piechulla B (2004) *Plant Biochemistry*, 3rd edn.: Elsevier.

Hernandez Torres J, Maldonado M, Chomilier J (2007) Tandem duplications of a degenerated GTP-binding domain at the origin of GTPase receptors Toc159 and thylakoids SRP. *Biochem Biophys Res Commun* **364**: 325-31

Hiltbrunner A, Bauer J, Alvarez-Huerta M, Kessler F (2001a) Protein translocon at the Arabidopsis outer chloroplast membrane. *Biochem Cell Biol* **79**: 629-35

Hiltbrunner A, Bauer J, Vidi PA, Infanger S, Weibel P, Hohwy M, Kessler F (2001b) Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. *J Cell Biol* **154**: 309-16

Hiltbrunner A, Grunig K, Alvarez-Huerta M, Infanger S, Bauer J, Kessler F (2004) AtToc90, a new GTP-binding component of the Arabidopsis chloroplast protein import machinery. *Plant Mol Biol* **54**: 427-40

Hinnah SC, Hill K, Wagner R, Schlicher T, Soll J (1997) Reconstitution of a chloroplast protein import channel. *EMBO J* **16**: 7351-60

Hinnah SC, Wagner R, Sveshnikova N, Harrer R, Soll J (2002) The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. *Biophys J* **83**: 899-911

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. *Science* **266**: 1989-92

Hofmann N, Theg S (2004) *Physcomitrella patens* as a model for the study of chloroplast protein transport: conserved machineries between vascular and non-vascular plants. *Plant Molecular Biology* **53**: 643-54

Hopkins W (2003) *Physiologie végétale*, Bruxelles: De Boeck Université.

Hormann F, Kuchler M, Sveshnikov D, Oppermann U, Li Y, Soll J (2004) Tic32, an essential component in chloroplast biogenesis. *J Biol Chem* **279**: 34756-62

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmerman P (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics* **2008**

Inaba T, Li M, Alvarez-Huerta M, Kessler F, Schnell DJ (2003) atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. *J Biol Chem* **278**: 38617-27

Infanger S, Bischof S, Hiltbrunner A, Agne B, Baginsky S, Kessler F (2011) The chloroplast import receptor Toc90 partially restores the accumulation of Toc159 client proteins in the *Arabidopsis thaliana* *ppi2* mutant. *Molecular Plant* **4**: 252-63

Ingle R, Collett H, Cooper K, Takahashi Y, Farrant J, Illing N (2008) Chloroplast biogenesis during rehydration of the resurrection plant *Xerophyta humilis*: parallels to the etioplast-chloroplast transition. *Plant Cell Environ* **31**: 1813-24

Inoue H, Rounds C, Schnell D (2010) The molecular basis for distinct pathways for protein import into *Arabidopsis* chloroplasts. *Plant Cell* **22**: 1947-60

Ivanova Y, Smith MD, Chen K, Schnell DJ (2004) Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. *Mol Biol Cell* **15**: 3379-92

Jackson-Constan D, Keegstra K (2001) *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol* **125**: 1567-76

Jarvis P, Chen LJ, Li H, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* **282**: 100-3

Jarvis P, Dormann P, Peto C, Benning C, Chory J (2000) Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis* MGD synthase 1 mutant. *PNAS* **97**: 8175-9

Jelic M, Soll J, Schleiff E (2003) Two Toc34 homologues with different properties. *Biochemistry* **42**: 5906-16

Jelic M, Sveshnikova N, Motzkus M, Horth P, Soll J, Schleiff E (2002) The chloroplast import receptor Toc34 functions as preprotein-regulated GTPase. *Biol Chem Hoppe Seyler* **383**: 1875-83

Kahlau S, Bock R (2008) Plastid transcriptomics and translomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. *Plant Cell* **20**: 856-74

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirose M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence

determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* **3**: 109-36

Kaufmann S, Ewing C, Shaper J (1987) The erasable Western Blot. *Analytical Biochemistry* **161**: 89-95

Keenan RJ, Freymann DM, Stroud RM, Walter P (2001) The signal recognition particle. *Annu Rev Biochem* **70**: 755-75

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries of the chloroplast. *Proc Natl Acad Sci U S A* **93**: 7684-9

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* **266**: 1035-9

Kessler F, Schnell DJ (2004) Chloroplast protein import: solve the GTPase riddle for entry. *Trends Cell Biol* **14**: 334-8

Kessler F, Schnell DJ (2006) The function and diversity of plastid protein import pathways: a multilane GTPase highway into plastids. *Traffic* **7**: 248-57

Kikuchi S, Hirohashi T, Nakai M (2006) Characterization of the preprotein translocon at the outer envelope membrane of chloroplasts by blue native PAGE. *Plant Cell Physiol* **47**: 363-71

Kleffmann T, von Zychlinski A, Russenberger D, Hirsh-Hoffmann M, Gehrig P, Gruissem W, Baginsky S (2007) Proteome dynamics during plastid differentiation in rice. *Plant Physiology* **143**: 912-23

Kleine T, Voigt C, Leister D (2009) Plastid signalling to the nucleus: messengers still in the mists? *Trends Genet* **25**: 185-92

Klumperman J (2000) Transport between ER and Golgi. *Curr Opin Cell Biol* **12**: 445-9

Koenig P, Oreb M, Hofle A, Kaltofen S, Rippe K, Sinning I, Schleiff E, Tews I (2008) The GTPase cycle of the chloroplast import receptors Toc33/Toc34: implications from monomeric and dimeric structures. *Structure* **16**: 585-96

Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *J Cell Biol* **139**: 1677-85

Kovacheva S, Bedard J, Wardle A, Patel R, Jarvis P (2007) Further *in vivo* studies on the role of the molecular chaperone, Hsp93, in plastid protein import. *Plant J* **50**: 364-79

Kovacs-Bogdan E, Soll J, Bolter B (2010) Protein import into chloroplasts: The Tic complex and its regulation. *Biochimica et Biophysica Acta* **1803**: 740-7

Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D, Jarvis P (2003) The Arabidopsis *ppi1* mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. *Plant Cell* **15**: 1859-71

Kubis S, Patel R, Combe J, Bedard J, Kovacheva S, Lilley K, Biehl A, Leister D, Rios G, Koncz C, Jarvis P (2004) Functional specialization amongst the Arabidopsis Toc159 family of chloroplast protein import receptors. *Plant Cell* **16**: 2059-77

Kuchler M, Decker S, Hormann F, Soll J, Heins L (2002) Protein import into chloroplasts involves redox-regulated proteins. *EMBO J* **21**: 6136-45

Laemli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**: 848-53

Lamond A, Earnshaw W (1998) Structure and function in the nucleus. *Science* **280**: 547-53

Lee D, Lee S, Lee G, Lee K, Kim S, Cheong G, Hwang I (2006) Functional characterization of sequence motifs in the transit peptide of Arabidopsis small subunit of Rubisco. *Plant Physiology* **140**: 466-83

Lee DW, Kim JK, Lee S, Choi S, Kim S, Hwang I (2008) Arabidopsis Nuclear-Encoded Plastid Transit Peptides Contain Multiple Sequence Subgroups with Distinctive Chloroplast-Targeting Sequence Motifs. *Plant Cell* **20**: 1603-22

- Lee J, Wang F, Schnell D** (2009) Toc receptor dimerization participates in the initiation of membrane translocation during protein import into chloroplasts. *The Journal of Biological Chemistry* **284**: 31130-41
- Lee KH, Kim SJ, Lee YJ, Jin JB, Hwang I** (2003) The M domain of atToc159 plays an essential role in the import of proteins into chloroplasts and chloroplast biogenesis. *J Biol Chem* **278**: 36794-805
- Leister D** (2005) Genomic-based dissection of the cross-talk of chloroplasts with the nucleus and mitochondria in Arabidopsis. *Gene* **354**: 110-6
- Liang Z, Mather T, Li G** (2000) GTPase mechanism and function: new insights from systematic mutational analysis of the phosphate-binding loop residue Ala30 of Rab5. *Biochem J* **346**: 501-8
- Lippincott-Schwartz J** (1993) Membrane cycling between the ER and Golgi apparatus and its role in biosynthetic transport. *Subcell Biochem* **21**: 95-119
- Lopez-Juez E, Pyke KA** (2005) Plastids unleashed: their development and their integration in plant development. *Int J Dev Biol* **49**: 557-77
- Lubeck J, Soll J, Akita M, Nielsen E, Keegstra K** (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. *EMBO J* **15**: 4230-8
- Lycett G** (2008) The role of Rab GTPases in cell wall metabolism. *J Exp Bot* **59**: 4061-74
- Ma Y, Kouranov A, LaSala SE, Schnell DJ** (1996) Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *J Cell Biol* **134**: 315-27
- Margulis L** (1971) Symbiosis and evolution. *Sci Am* **225**: 48-57
- Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M, Penny D** (2002) Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc Natl Acad Sci U S A* **99**: 12246-51

Martin W, Stoebe B, Goremykin V, Hapsmann S, Hasegawa M, Kowallik K (1998) Gene transfer to the nucleus and the evolution of chloroplasts. *Nature* **393**: 162-5

Matsuo M, Yamauchi R, Obokata J (2005) The rice nuclear genome continuously integrates, shuffles and eliminates the chloroplast genome to cause chloroplast-nuclear DNA flux. *Plant Cell* **17**: 665-75

May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell* **12**: 53-64

McFadden G, van Dooren G (2004) Evolution: red algal genome affirms a common origin of all plastids. *Current Biology* **14**: 514-6

Nakamura Y, Tsuchiya M, Ohta H (2007) Plastidic phosphatidic acid phosphatases identified in a distinct subfamily of lipid phosphate phosphatases with prokaryotic origin. *The Journal of Biological Chemistry* **282**: 29013-21

Nakrieko KA, Mould RM, Smith AG (2004) Fidelity of targeting to chloroplasts is not affected by removal of the phosphorylation site from the transit peptide. *Eur J Biochem* **271**: 509-16

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO J* **16**: 935-46

Obbard D, Gordon K, Buck A, Jiggins F (2008) The evolution of RNAi as a defence against viruses and transposable elements. *Phil Trans R Soc* **364**: 99-115

Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. *J Biol Chem* **267**: 433-9

Oreb M, Hofle A, Mirus O, Schleiff E (2008) Phosphorylation regulates the assembly of chloroplast import machinery. *J Exp Bot* **59**: 2309-16

Oreb M, Zoryan M, Vojta A, Maier UG, Eichacker LA, Schleiff E (2007) Phospho-mimicry mutant of atToc33 affects early development of *Arabidopsis thaliana*. *FEBS Lett* **581**: 5945-51

Padmanabhan M, Dinesh-Kumar S (2010) All hands on deck - the role of chloroplast, endoplasmic reticulum and the nucleus in driving plant innate immunity. *Mol Plant Microbe Interact* **23**: 1368-80

Pain D, Blobel G (1987) Protein import into chloroplasts requires a chloroplast ATPase. *Proc Natl Acad Sci U S A* **84**: 3288-92

Papp S, Dziak E, Michalak M, Opas M (2003) Is all of the endoplasmic reticulum created equal? The effects of the heterogeneous distribution of endoplasmic reticulum Ca²⁺-handling proteins. *J Cell Biol* **160**: 475-9

Park P, De Boni U (1999) Dynamics of structure-function relationship in interphase nuclei. *Life Sci* **64**: 1703-18

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell* **6**: 93-105

Pinnaduwege P, Bruce B (1996) *In vitro* interaction between a chloroplast transit peptide and chloroplast outer envelope lipids is sequence-specific and lipid class-dependent. *J Biol Chem* **271**: 32907-15

Qbadou S, Tien R, Soll J, Schleiff E (2003) Membrane insertion of the chloroplast outer envelope protein, Toc34: constraints for insertion and topology. *J Cell Sci* **116**: 837-46

Rahim G, Bischof S, Kessler F, Agne B (2009) *In vivo* interaction between *atToc33* and *atToc159* GTP-binding domains demonstrated in a plant split-ubiquitin system. *The Journal of Experimental Botany* **60**: 257-67

Ratcliff F, Harrison B, Baulcombe D (1997) A similarity between viral defense and gene silencing in plant. *Science* **276**: 1558-60

Reddick LE, Vaughn MD, Wright SJ, Campbell IM, Bruce BD (2007) *In vitro* comparative kinetic analysis of the chloroplast toc GTPases. *J Biol Chem* **282**: 11410-26

Rensink WA, Pilon M, Weisbeek P (1998) Domains of a transit sequence required for *in vivo* import in Arabidopsis chloroplasts. *Plant Physiol* **118**: 691-9

Reumann S, Davila-Aponte J, Keegstra K (1999) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. *Proc Natl Acad Sci U S A* **96**: 784-9

Reumann S, Keegstra K (1999) The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. *Trends Plant Sci* **4**: 302-7

Reyes-Prieto A, Bhattacharya D (2007) Phylogeny of Calvin cycle enzymes supports Plantae monophyly. *Molecular Phylogenetics and Evolution* **45**: 384-91

Reyes-Prieto A, Weber A, Bhattacharya D (2007) The origin and establishment of the plastid in algae and plants. *Annu Rev Genet* **41**: 147-68

Reynolds E (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* **17**: 208-12

Richardson L, Jelokhani-Niaraki M, Smith M (2009) The acidic domains of Toc159 chloroplast preprotein receptor family are intrinsically disordered protein domains. *BMC Biochem* **10**

Richter S, Lamppa G (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. *PNAS* **95**: 7463-8

Richter S, Lamppa G (1999) Stromal Processing Peptidase binds transit peptides and initiates their ATP-dependent turnover in chloroplasts. *J Cell Biol* **147**: 33-43

Rintamaki E, Lepisto A, Kangasjarvi S (2009) Implication of chlorophyll biosynthesis on chloroplast-to-nucleus retrograde signaling. *Plant Signaling & behavior* **4**: 545-7

Ruppel N, Logsdon C, Whippo C, Inoue K, Hangarter R (2011) A mutation in *Arabidopsis thaliana* SEEDLING PLASTID DEVELOPMENT 1 affects plastid differentiation in embryo-derived tissues during seedling growth. *Plant Physiology* **155**: 342-53

Sambrook J, Russel D (2001) *Molecular cloning: a laboratory manual*, New-York.

Sanchez-Pulido L, Devos D, Genevrois S, Vicente M, Valencia A (2003) POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem Sci* **28**: 523-6

Sandhu A, Randhawa G, Dhugga K (2009) Plant cell wall matrix polysaccharide biosynthesis. *Mol Plant* **2**: 840-50

Scheffzek K, Ahmadian MR (2005) GTPase activating proteins: structural and functional insights 18 years after discovery. *Cell Mol Life Sci* **62**: 3014-38

Schleiff E, Eichacker L, Eckart K, Becker T, Mirus O, Stahl T, Soll J (2003a) Prediction of the plant beta-barrel proteome: A case study of the chloroplast outer envelope. *Protein Science* **12**: 748-59

Schleiff E, Jelic M, Soll J (2003b) A GTP-driven motor moves proteins across the outer envelope of chloroplasts. *Proc Natl Acad Sci U S A* **100**: 4604-9

Schleiff E, Soll J, Kuchler M, Kuhlbrandt W, Harrer R (2003c) Characterization of the translocon of the outer envelope of chloroplasts. *J Cell Biol* **160**: 541-51

Schleiff E, Soll J, Sveshnikova N, Tien R, Wright S, Dabney-Smith C, Subramanian C, Bruce BD (2002) Structural and guanosine triphosphate/diphosphate requirements for transit peptide recognition by the cytosolic domain of the chloroplast outer envelope receptor, Toc34. *Biochemistry* **41**: 1934-46

Schnell DJ, Blobel G (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J Cell Biol* **120**: 103-15

Schnell DJ, Blobel G, Keegstra K, Kessler F, Ko K, Soll J (1997) A consensus nomenclature for the protein-import components of the chloroplast envelope. *Trends Cell Biol* **7**: 303-4

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. *Science* **266**: 1007-12

Shi L, Theg S (2010) A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella patens*. *The Plant Cell* **22**: 205-20

Simpson A, Roger A (2004) The real "kingdoms" of eukaryotes. *Current Biology* **14**

Smith M (2006) Protein import into chloroplasts: an ever-evolving story. *Canadian Journal of Botany* **84**: 531-42

Smith MD, Rounds CM, Wang F, Chen K, Afithile M, Schnell DJ (2004) atToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. *J Cell Biol* **165**: 323-34

Smith MD, Hiltbrunner A, Kessler F, Schnell DJ (2002) The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP. *J Cell Biol* **159**: 833-43

Stahl T, Glockmann C, Soll J, Heins L (1999) Tic40, a new "old" subunit of the chloroplast protein import translocon. *J Biol Chem* **274**: 37467-72

Stiller J, Reel D, Johnson J (2003) A single origin of plastid revisited: convergent evolution in organellar genome content. *Journal of Phycology* **39**: 95-105

Su P, Li H (2010) Stromal Hsp70 is important for protein translocation into Pea and Arabidopsis chloroplasts. *The Plant Cell* **22**: 1516-31

Sun YJ, Forouhar F, Li Hm HM, Tu SL, Yeh YH, Kao S, Shr HL, Chou CC, Chen C, Hsiao CD (2002) Crystal structure of pea Toc34, a novel GTPase of the chloroplast protein translocon. *Nat Struct Biol* **9**: 95-100

Suzuki K, Miyagishima S (2009) Eukaryotic and eubacterial contributions to the establishment of plastid proteome estimated by large-scale phylogenetic analyses. *Mol Biol Evol* **27**: 581-90

Sveshnikova N, Soll J, Schleiff E (2000) Toc34 is a preprotein receptor regulated by GTP and phosphorylation. *Proc Natl Acad Sci U S A* **97**: 4973-8

Teng YS, Su YS, Chen LJ, Lee YJ, Hwang I, Li HM (2006) Tic21 is an essential translocon component for protein translocation across the chloroplast inner envelope membrane. *Plant Cell* **18**: 2247-57

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. *J Biol Chem* **264**: 6730-6

Thompson J, Higgins D, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-80

Thomson W (1980) Development of nongreen plastids. *Ann Rev Plant Physiol* **31**: 375-94

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *Embo J* **14**: 2436-46

Tranel PJ, Keegstra K (1996) A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. *Plant Cell* **8**: 2093-104

Vesteg M, Vacula R, Krajcovic J (2009) On the origin of chloroplasts, import mechanisms of chloroplast-targeted proteins, and loss of photosynthetic ability. *Folia Microbiol* **54**: 303-21

Vojta A, Alavi M, Becker T, Hormann F, Kuchler M, Soll J, Thomson R, Schleiff E (2004) The protein translocon of the plastid envelopes. *J Biol Chem* **279**: 21401-5

Waegemann K, Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. *Plant Journal* **1**: 149-58

Waegemann K, Soll J (1996) Phosphorylation of the transit sequence of chloroplast precursor proteins. *J Biol Chem* **271**: 6545-54

Wallas TR, Smith MD, Sanchez-Nieto S, Schnell DJ (2003) The roles of toc34 and toc75 in targeting the toc159 preprotein receptor to chloroplasts. *J Biol Chem* **278**: 44289-97

Wang F, Agne B, Kessler F, Schnell D (2008) The role of GTP binding and hydrolysis at the atToc159 preprotein receptor during protein import into chloroplast. *The Journal of Cell Biology* **183**: 87-99

Waters M, Langdale J (2009) The making of chloroplast. *The EMBO Journal* **28**: 2861-73

Weibel P, Hiltbrunner A, Brand L, Kessler F (2003) Dimerization of Toc-GTPases at the chloroplast protein import machinery. *J Biol Chem* **278**: 37321-9

Whittaker R (1969) New concepts of kingdoms of organisms. *Science* **10**: 150-60

Woese C, Kandler O, Wheelis M (1990) Towards a natural system of organisms: proposal for the domains Archaea, bacteria, and Eucarya. *PNAS* **87**: 4576-9

Woodson J, Chory J (2008) Coordination of gene expression between organellar and nuclear genomes. *Nature reviews genetics* **9**: 383-9

Young ME, Keegstra K, Froehlich JE (1999) GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. *Plant Physiol* **121**: 237-44

Zhang XP, Glaser E (2002) Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. *Trends Plant Sci* **7**: 14-21

Zhong R, Wan J, Jin R, Lamppa G (2003) A pea antisense gene for the chloroplast stromal processing peptidase yields seedling lethals in Arabidopsis: survivors show defective GFP import in vivo. *Plant J* **34**: 802-12

ANNEX

List of primers

Primer	Sequence 5'-3'
prA3R	CAG TGC ATG TTG ATG TGG CA
pr159int	GAA TAG GGT TTT AAT CGG AAG
prLB5	GAT GCA ATC GAT ATC AGC CAA TTT TAG AC
pCL60_35S	CCA CTG ACG TAA GGG AT
pr86H6	CAG TCT ATA GGA CAG GC
pBKrev	CTC AAG ACC CGT TTA GAG G
pT7Terminator	GCT AGT TAT TGC TCA GCG G
prpET Upstream	ATG CGT CCG GCG TAG A
pDuetUP2	TTG TAC ACG GCC GCA TAA TC
pDuetDOWN1	GAT TAT GCG GCC GTG TAC AA
p159G Duet1	CCA TAG AGC TCG AGT CAG GAT GGT ACG
p33G Duet1	GGT ATG TCG ACC TTT CCT TTA TCA TCA G
pGST159G Duet	CTA TGG CCG GCC ACT CCC CTA TAC TAG GTT ATT GG
pGST159G Duet Rev	GAT GGT ACC TTA AAC TCG GAA ACC
qc_D919V_s	TTT GAA GTC TGC AGC GAT GGT TCA AAG TAC AAA TGC
qc_D919V_r	GCA TTT GTA CTT TGA ACC ATC GCT GCA GAC TTC AAA CCC
p159GBD946NXbaF	GAT ATT GTA CTA TAT GTA AAT CGT CTA GAC ACC CAG ACC AGG G
p159GBD946NXbaR	CCC TGG TCT GGG TGT CTA GAC GAT TTA CAT ATA GTA CAA TAT

List of plants

Complemented plants		
TAP-Toc159GM	TAP-Toc159	w/o TAP-tag
WT	WT	WT
K868R	D919V	D946N
D919V	D946N	D949V
D946N	D949V	
D949V		

List of plasmids

DNA Constructs	
Vector	Insert
pCHF7	TAP-Toc159GM WT
	TAP-Toc159GM K868R
	TAP-Toc159GM Sup1
	TAP-Toc159GM Sup2
	TAP-Toc159GM D919V
	TAP-Toc159GM D946N
	TAP-Toc159GM D949V
	TAP-Toc159 D919V
	TAP-Toc159 D946N
	TAP-Toc159 D949V
pET21d+	Toc159 WT
	Toc159 D919V
	Toc159 D946N
	Toc159 D949V
pETDuet	Toc33
	Toc159G
	Toc33-Toc159G
	Toc33-Toc159G D919V
	Toc33 R130A-Toc159G
	Toc33 R130A-Toc159G D919V
pGADT7	Toc33
	Toc33 R130A
pGBKT7	Toc159G
	Toc159G Sup1
	Toc159 Sup2
	Toc159 D919V
pGEMT	Toc159G D919V
pGEX4T1	Toc159G WT
	Toc159G K868R
	Toc159G D919V
	Toc159G D946N
	Toc159G D949V

ACKNOWLEDGMENTS

First and foremost, I would like to thank Pr. Felix KESSLER, my PhD thesis director, for giving me the opportunity to be a member of his group in the laboratory of Plant Physiology at the Faculty of Sciences of the University of Neuchâtel.

Special thanks to Dr. Birgit Agne, AKA MidiBoss, for sharing her knowledge and for fruitful discussion and collaboration.

Furthermore, I would like to address many thanks to the other members of the laboratory of Plant Physiology, past and present members, whom provided with a warm and friendly atmosphere during the time together.

Thanks to my parents who made all this possible and for their continuous love and support. A special thanks for my dad and its Photoshop/Illustrator hotline remote service.

Thanks to Binôme although we did not make our PhD thesis as a binome well: "binome forever!"

A great thanks to all my friends from Neuchâtel and more remote ones for their patience, their support and their friendship.

Meryll's experience has seen him work with international, house-hold names and cutting edge, upcoming scientists alike. Ever-evolving and being at the forefront of creating new science, his work is tantamount to quality and will continue to be so, for years to come.

Adapted after Dom T