

A plant split-ubiquitin system and TAP-tagging to
study *in vivo* protein interactions in the chloroplast
protein import machinery

A dissertation submitted to the
UNIVERSITY OF NEUCHÂTEL

For the degree of
Doctor of Natural Sciences

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the 16 December 2008

IMPRIMATUR POUR LA THESE

A plant split-ubiquitin system and TAP-tagging to study
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Keywords : chloroplast, protein import, Toc complex, protein interactions, split-ubiquitin, TAP-tag

Mots-clefs : chloroplaste, import des protéines, complexe Toc, interactions protéine-protéine, split-ubiquitin, TAP-tag

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Abstract

Most chloroplast proteins are synthesized as precursor proteins in the cytosol. The import of these precursor proteins 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 membrane) and Tic (translocon at the inner envelope membrane) respectively. In *Arabidopsis*, 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 dimerization of their GTP-binding domain (G-domain). Moreover, the crystal structure of the pea Toc33 ortholog, psToc34 indicates that it can stably homodimerize via its G-domain. However, neither Toc159/Toc33 heterodimers nor Toc33 homodimerization have been demonstrated *in planta*.

To get new insight into the *in vivo* interactions of Toc GTPases, we have developed a plant split-ubiquitin system. This method, originally developed for yeast, was adapted to study interactions between the Toc GTPases atToc159 and atToc33 in *Arabidopsis* and tobacco protoplasts. We also demonstrated that the peroxisomal membrane protein atPex11e, used initially as a model membrane protein in our system, self-interacts as does its yeast homolog. The plant split-ubiquitin system proves to be widely usable.

Another approach of this thesis was to get more information on the import mechanism via the identification of interaction partners of the Toc GTPase atToc33. atToc33 and proteins associated were isolated from *Arabidopsis* plants, using the tandem affinity purification (TAP) tag. We proved that this technique is suitable to purify Toc33, which encourages us to purify Toc proteins and complexes at a larger scale.

Résumé

Les protéines du chloroplaste sont, pour la majorité d'entre elles, synthétisées dans le cytosol sous la forme de précurseurs que l'on appelle également préprotéines. Ces protéines doivent, après leur transcription, être importées dans les chloroplastes. Cet import est possible grâce à la présence de complexes protéiques distincts situés sur la membrane externe et interne du chloroplaste, appelés Toc (Translocon de la membrane externe de l'enveloppe des chloroplastes) et Tic (Translocon de la membrane interne de l'enveloppe des chloroplastes). Chez la plante modèle *Arabidopsis thaliana*, le complexe Toc est composé principalement de deux GTPases homologues, atToc159 et atToc33, et de atToc75 qui forme un canal pour l'import des protéines.

Toc159 et Toc33 interviennent dans la reconnaissance du précurseur, mais les mécanismes exacts de reconnaissance et d'interactions de ces deux GTPases restent à découvrir. On sait cependant, grâce à des expériences menées *in vitro*, que Toc159 et Toc33 interagissent entre-elles par dimérisation par l'intermédiaire de leur domaine GTPasique (domaine G). De plus, la structure du cristal de psToc34, l'orthologue de Toc33 chez le pois, suggère que cette protéine a la possibilité de former un homodimère via son domaine G. Néanmoins, aucun homodimère de Toc33 ni aucune hétérodimerisation de Toc159 et Toc33 n'ont été encore observés *in planta*.

L'un des objectifs de mon travail était d'étudier ces interactions *in vivo*. Pour cela, nous avons développé chez *Arabidopsis* et le tabac, un système d'interaction protéine-protéine initialement créé dans la levure : le système "split-ubiquitin".

Afin d'en savoir plus sur les mécanismes d'import dans la plante, nous avons essayé dans un deuxième temps, d'identifier de nouvelles protéines participant à cet import. Pour cela, on a obtenu des plantes exprimant la protéine Toc33 fusionnée à une étiquette TAP-(Tandem affinity purification) afin d'isoler, purifier puis identifier les protéines qui interagissent avec cette Toc GTPase.

Abbreviations

α -protein	antibodies recognizing the specified protein
A-domain	acidic domain
atToc159G	GTP binding domain of atToc159
atToc33G	GTP binding domain of atToc33
atToc159A	acidic-domain of atToc159
at, <i>A. thaliana</i> ,	
<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
bp	base pairs
CaMV	cauliflower mosaic virus
CBP	Calmodulin binding protein
cDNA	complementary DNA
Col	Columbia
Cub	C- terminal half of ubiquitin
DNA	deoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
DUBs	deubiquitinating enzymes
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraaceticacid
EGTA	ethyleneglycoltetraaceticacid
FITC	fluorescein isothiocyanate
G-domain	GTP-binding domain
GFP	green fluorescent protein
HA	hemagglutinin
His6	hexahistidinyI-tag
IgG	immunoglobulin G
kb	kilo base pairs
kDa	kilo Dalton
LB	Luria Bertani
M-domain	Membrane domain
MS	Murashige and Skoog
Nub	N-terminal half of ubiquitin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pex	Peroxisomal protein
ppi	plastid protein import
protA	protein A
PRK	phosphoribulokinase

ps	<i>Pisum sativum</i>
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
SRP	signal recognition particle
TAP tag	tandem affinity purification tag
TBS	tris-buffered saline
T-DNA	transfer DNA
TEV	Tobacco Etch Virus
Tic	translocon at the inner chloroplast membrane
TM	transmembrane helix
Toc	translocon at the outer chloroplast membrane
Tris	tris (hydroxymethyl) aminomethane
TRITC	tetramethylrhodamine isothiocyanate
T0 lines	Arabidopsis plants that were transformed by floral dip
T1 lines	first generation of seeds after plant transformation by floral dip
U	unit
ub	ubiquitin
UBPs	ubiquitin specific proteases
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...	designates the Toc-, Tic-protein of <i>P. sativum</i>
Toc..., Tic...	refers to Toc-and Tic-proteins of both <i>A. thaliana</i> and <i>P. sativum</i> unless specified otherwise
upper case, italic	gene, wild type allele (e.g. <i>TOC33</i>)
lower case, italic	mutant allele(s) (e.g. <i>ppi1</i>)
regular case	protein (e.g. atToc33)

Introduction

1 Chloroplasts, the green plastids

1.1 Characteristics and distribution of plastids

Plastids are specialised organelles hosted by plant cells. They provide carbon and energy to the plant as a result of photosynthetic assimilation (Pyke, 1999). Biosynthesis events such as the synthesis of lipids, nucleic acids and amino acids take place in plastids (Galili, 1995, Ohlrogge and Browse, 1995). Originally, plastids were categorized in three groups by A.F.W Schimper in 1885 (Mühlethaler, 1971) according to their colour: leucoplasts (white plastids), chromoplasts (yellow plastids) and chloroplasts (green plastids). Chloroplasts are located in the plant green tissues as they contain chlorophyll pigments and are the site of photosynthesis. Chloroplasts are also present during the first days of embryo development (Mansfield and Briarty, 1992). Leucoplasts store starch (amyloplasts), proteins (proteinoplasts) or lipids (elaioplasts) and are present in roots and non photosynthetic tissues but also in petals (Pyke and Page, 1998). Chromoplasts are red-, orange, and yellow-coloured plastids which contain carotenoid pigments. They are located in fruits and flowers (Marano, *et al.*, 1993).

All these different groups of plastids derived from proplastids, which are present in meristematic cells (Leon *et al.*, 1998). One meristematic cell is proposed to contain 10 to 20 proplastids (as reviewed by Pyke, 1999). In the presence of light, proplastids synthesise chlorophyll and become chloroplasts. Alternatively, if seedlings are grown in the dark, proplastids are differentiated to etioplasts.

1.2 Structure of the chloroplast

Chloroplasts are about 5-10 μ m in diameter and 3-4 μ m in thickness, present in each cell at numbers of 10 to more than 100 depending on the plant species (as reviewed by Lopez-Juez and Pyke, 2005). They are bounded by two envelope membranes (Fig.1). The photosynthetic apparatus is localised in an internal system, the thylakoids, also enclosed by a membrane. Thylakoids usually forms sacks which are arranged in stacks, called grana, but individual thylakoids are also found (stromal thylakoids). They are embedded in the stroma, the chloroplast aqueous matrix, together with DNA, ribosomes, starch and lipid droplets called plastoglobuli.

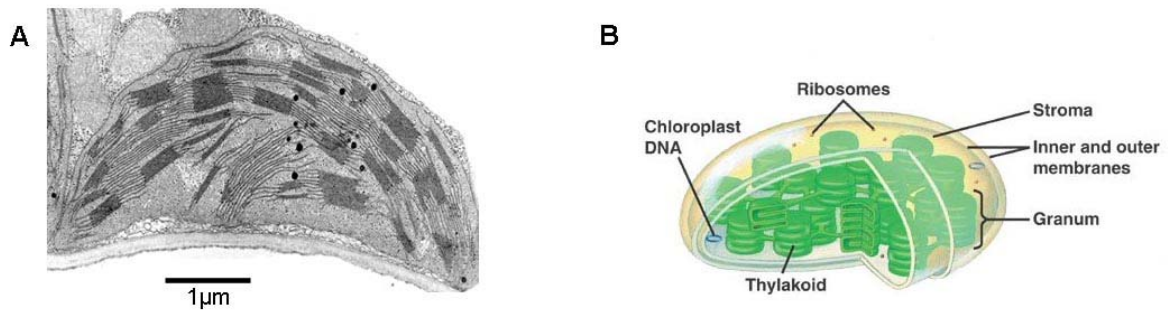


Fig.1: The chloroplast

A : Transmission electron microscopy of the chloroplast (University of Wisconsin Botany website <http://botit.botany.wisc.edu/>)

B : Schematic representation of the chloroplast (Campbell, N.A, 1996)

Chloroplasts have two lipid membranes separated by an intermembrane space that enclose the stroma. The stroma contains the thylakoids, arranged in stacks called grana.

The proteins controlling photosynthesis (photosystems I and II, the cytochrome b_6/f complex and the ATP synthase) are positioned in the thylakoid membranes. The grana disposition of thylakoids is delimiting the two photosystems: the photosystem I is in contact with the stroma whereas the photosystem II is limited to the granal membranes only.

Chloroplast and thylakoid envelope membranes are rich in galactolipids, whereas the other plant membranes are mainly composed of phospholipids (Jarvis *et al.*, 2000). The chloroplast outer envelope is permeable to molecules up to 10kDa while the inner envelope is more selective (Lopez-Juez and Pyke, 2005).

2 Evolution

2.1 “Little workers, green slaves”

The hypothesis that plastids have an endosymbiont origin already arose in 1905, when Mereschkowsky suggested that plastids have cyanobacterial origins and act as “little workers, green slaves” within the cell (Martin and Kowallik, 1999). It is now commonly accepted that plastids emerged more than 1 billion years ago from one symbiogenetic event when a single ancestral cyanobacterium (Martin and Hermann, 1999; Palmer and Delwiche, 1998) integrated into the ancestral host cell (Cavallier-Smith, 2000).

This was confirmed by studies and comparison of genes coding for chloroplast proteins with genes that have a cyanobacterial origin (Sugita *et al.*, 1997). Proteins sequence comparisons also demonstrate that plants and red algae are phylogenetically related (Moreira *et al.*, 2000; McFadden and van Dooren, 2004).

Due to their endosymbiont origins, plastid division is similar to bacterial fission. Plastids replicate from pre-existing plastids and plastid division is the result of a series of events which include plastid division components of both plant and cyanobacterial origin (Pyke, 1999).

2.2 Genome evolution

The plant genome was also affected by this endosymbiotic event. Indeed, analysis of the nuclear genome of *Arabidopsis* showed that about 18% of the protein-coding genes have a cyanobacterial origin (Martin *et al.*, 2002). Compared to cyanobacteria that has approximately 3000 genes (Kaneko *et al.*, 1996), chloroplast genomes contain between 120 and 135 genes which encode less than 80 proteins, mainly components of the

photosynthesis protein complexes (Lopez-Juez and Pyke, 2005). Some of the genes that were not essential anymore for the endosymbiont disappeared during evolution and the other genes of the endosymbiont were transferred to the plant nuclear genome (Martin *et al.*, 1998, Timmis *et al.*, 2004).

The majority of the proteins required for plastid functions is thus encoded in the nucleus and translated on cytoplasmic ribosomes. These nuclear encoded proteins need to be targeted to the chloroplast surface. Proteins destined to be imported have to pass through the two envelope membranes before carrying on their journey inside the chloroplast.

3 Chloroplast protein import machinery

3.1 Targeting of the preproteins

The majority of the imported proteins are synthesised as precursor proteins (preproteins) with a cleavable N-terminal transit peptide. This peptide is essential and sufficient to address the preprotein to the plastid outer membrane and to transfer it across the chloroplast translocation machinery (Becker *et al.*, 2005). Proteins targeted to the thylakoid membrane or lumen bear bipartite N-terminal presequences, with two domains, one for each compartment the protein will be targeted to (Robinson *et al.*, 2001).

However, most of the outer envelope membrane proteins are not synthesised with a transit peptide. This is also true for some chloroplast-imported proteins (Kleffmann *et al.*, 2004).

3.2 The chloroplast transit peptides

Compared to other transit peptides that belong, for example, to the secretory pathway, or mitochondrial proteins, chloroplasts targeting signals lack consensus features. They are made up of 20 to 80 amino acids, contain a low amount of acidic and hydrophobic residues, but many of them are hydroxylated and positively charged. Transit peptides are rich in serine and threonine that may act as phosphorylation sites. The presence of such sites raised the hypothesis that transit peptides could interact with cytosolic and chloroplastic chaperones required for targeting and import (von Heijne and Nishikawa, 1991). Indeed more than 75% of transit peptides are predicted to have at least one binding site for DnaK, the 70 kDa heat shock protein (Hsp70) from *E. coli*. *In vitro*

protein binding experiments detected the interaction between DnaK and the transit peptide of two chloroplastic imported proteins: ferredoxin NADP oxidoreductase and the small subunit of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) (Ivey and Bruce, 2000; Rial *et al.*, 2000). Hsp70s are ATPase proteins that play a key role in protein folding and may bind to the transit peptides of preproteins to prevent aggregation and give them the spatial conformation (unfolded state) necessary for import through the chloroplast membrane. However altering the binding capacity of precursor proteins to the Hsp70 chaperone does not affect import (Rial *et al.*, 2003). This indicates that the exact role of chaperones during chloroplast protein translocation remains to be solved.

3.3 Guidance complex

Transit peptides are rich in the hydroxylated amino acids serine and threonine. These residues are known to be a site for phosphorylation and are also binding sites for 14-3-3 proteins that belong to a family of highly conserved proteins of 30 kDa expressed in all eukaryotic cells. 14-3-3 proteins play a role in cell signalling pathways and are proposed to be phospho-dependent chaperones (as reviewed by Yaffe, 2002 and by Ferl, 2004). It has been proposed that precursors would bind to 14-3-3 proteins after phosphorylation of their transit peptide, together with Hsp70 and maybe unidentified components to form a guidance complex (May and Soll, 2000). However, the role of the guidance complex in preprotein targeting remains hypothetical, as the mutation of the phosphorylation sites did not impair preprotein import (Nakrieko *et al.*, 2004).

3.4 The existence of a cytosolic receptor

An alternative to the guidance complex involves a cytosolic form of Toc159, one of the preprotein receptors (that form the Toc complex) located at the outer membrane of the chloroplast. This complex and its components will be described later.

It has been suggested that Toc159 exists in a cytosolic, soluble form as well as in a chloroplast membrane bound form (Hiltbrunner *et al.*, 2001b). The soluble form of Toc159 would then recognise and bind to the preprotein and “guide” it to the chloroplast membrane to be translocated. Recent findings have indeed shown that soluble Toc159 directly interacts with preproteins (Smith *et al.*, 2004). *In vivo* data are still missing to confirm this hypothesis and to identify the components that are involved in targeting of the soluble Toc159-preprotein complex to the membrane.

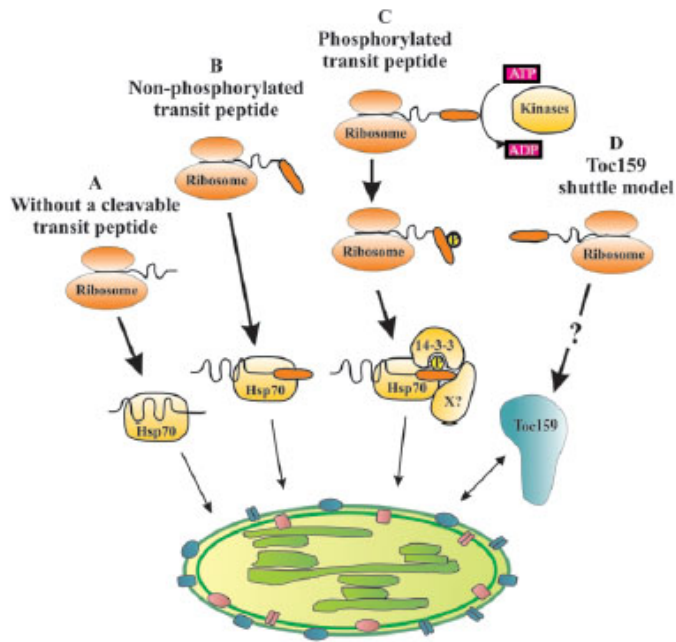


Fig.2: Targeting of chloroplast precursor proteins (from Becker *et al.*, 2005)

Four different pathways are proposed for targeted proteins that have no transit peptide (A), a non-phosphorylated transit peptide (B) a phosphorylated transit peptide (C), or are guided by the soluble form of Toc159 (D).

3.5 Four different ways of preprotein targeting

To conclude, there are four different hypotheses concerning the targeting of preproteins to the chloroplast membrane (as reviewed by Becker *et al.*, 2005 and in Fig 2). Chloroplastic proteins without transit peptides are directly inserted into the membrane. Non-phosphorylated preproteins may be imported via cytosolic compounds that remain to be identified, whereas phosphorylated ones may bind to the guidance complex. The soluble form of the outer envelope receptor Toc159 is also proposed to be one of the cytosolic components necessary for the addressing of precursors.

4 The Chloroplast translocation machinery

The import of preproteins is facilitated by translocon complexes located in both chloroplast envelope membranes. These complexes are called Toc (translocon at the outer envelope membrane) and Tic (translocon at the inner envelope membrane) respectively. The numbers of the different Toc and Tic components correspond to their molecular mass in kDa.

4.1 The Toc complex

In pea, the initial plant model that was used to study chloroplast protein import, the Toc complex consists of Toc159, Toc34, Toc75, Toc12 and possibly Toc64 (Fig.3), but only the three first proteins are considered as the core components of the Toc complex as they were sufficient to allow protein translocation using artificial lipid vesicles (Schleiff *et al.*, 2003a). The core complex is proposed to have a molecular weight of 500kDa and a stoichiometry of 1/4/4 between Toc159/Toc75/Toc34 (Schleiff *et al.*, 2003b). These three proteins that form this hetero-oligomeric complex in pea will be described in more detail below.

Toc64 was characterised in pea by co-purification with Toc159, Toc34 and Toc75 (Sohrt and Soll, 2000), however in a later work performed by the same group, Toc64 did not co-purify with the other Toc components suggesting that the interaction between Toc64 and the Toc core component is transient (Schleiff *et al.*, 2003b). Toc64 is made of three tetratricopeptide repeat (TPR) motifs that are known to mediate protein-protein interactions and interaction between proteins and chaperones (reviewed by Bedard and Jarvis, 2005). The role of Toc64 remains elusive, but because of its TPR motifs, it could

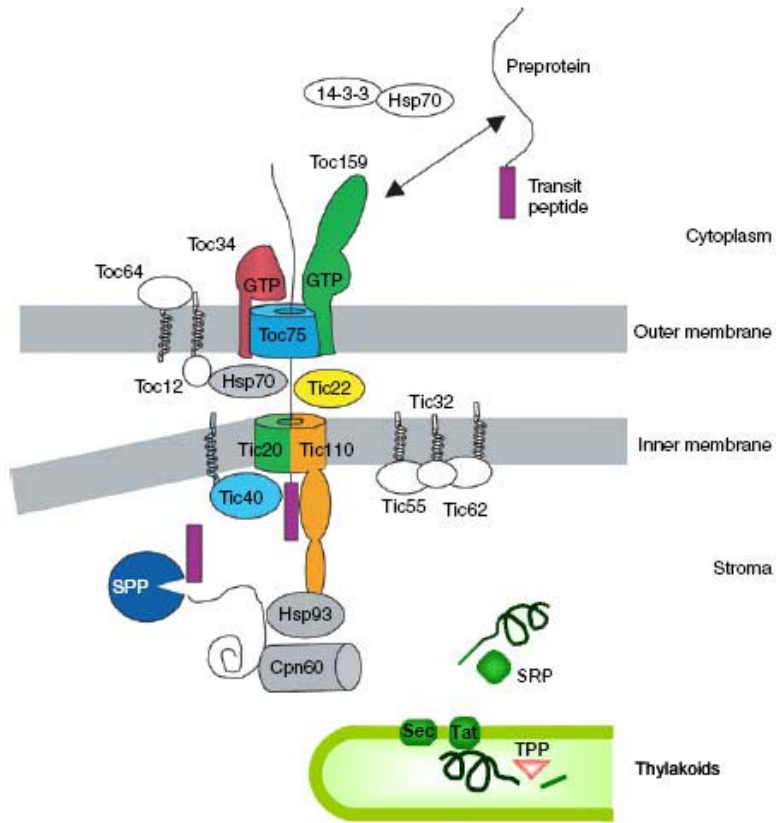


Fig.3. Components of the chloroplast protein import machinery (adapted from Kessler and Schnell, 2006, and Lopez-Juez and Pyke, 2005).

Targeting of preproteins to the chloroplast surface is still not well known, but could involve a guidance complex composed of 14-3-3 proteins and a chaperone Hsp70 as well as Toc64. The Toc core components consist of two GTPase receptors, Toc159, Toc34 and a channel protein Toc75. Preproteins are translocated across the Toc complex, and then bind to another chaperone, Hsp70, and Tic22 in the intermembrane space, to guide them towards the Tic complex. Tic110 and (or) Tic20 form the Tic channel. The stromal part of Tic110 is proposed to bind preproteins and recruit chaperones Hsp93 and Cpn60 with the help of Tic40. Tic55, Tic62 and Tic 32 may regulate the protein import via a redox cycle.

Once in the stroma, the transit peptide is cleaved by a stromal processing peptidase (SPP). Import into the thylakoids involves different pathways depending on the nature of the protein imported. Once in the thylakoid lumen, the “thylakoidal” signal peptide is cleaved by a thylakoid processing peptidase (TPP).

play a role in the guidance complex as a docking site for preproteins (Qbadou *et al.*, 2006; Qbadou *et al.*, 2007; Schlegel *et al.*, 2007; Sohr and Soll, 2000). However, in recent studies in the moss *Physcomitrella patens*, Toc64 is proposed to have no role in protein import but to be involved in chloroplast morphology, as mutants were not impaired in import but had a difference in plastid shape (Hofmann and Theg, 2005). In *Arabidopsis*, efficiency of import is also not affected by Toc64 mutations (Aronsson *et al.*, 2007).

Once the precursor proteins reach the chloroplast outer membrane, their recognition and binding are performed by Toc159 and Toc34, two homologous GTPases. Hydrolysis of GTP occurs during import but the precise mechanisms of the two GTPases (Toc159 and Toc34) are still unknown (Kessler and Schnell, 2004). Once recognized by the Toc complex, preproteins are translocated across the outer membrane passing through Toc75, the protein import channel.

ATP and GTP are required for the irreversible binding of preprotein to the translocon complex (Olsen *et al.*, 1989; Young *et al.*, 1999) and ATP is necessary for its translocation (Theg *et al.*, 1989).

In the intermembrane space, Toc12 has been proposed to recruit Hsp70, which will increase the chaperone affinity for the preprotein that will be addressed to the Tic complex with the help of Tic22 (Becker *et al.*, 2004a).

Toc12 is anchored to the outer envelope membrane, but its C-terminal part is located in the intermembrane space. Toc12 contains a J-domain and stimulates the ATPase activity of DnaK *in vitro* (Becker *et al.*, 2004a).

4.2 The Tic complex

Toc and Tic proteins are believed to interact and associate with each other. It has been suggested that these Toc-Tic super-complexes are formed during preprotein import across the chloroplast membrane (Kessler and Schnell, 2006).

The Tic complex components are: Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20 (as reviewed by Keegstra and Cline, 1999) (Fig.3).

Tic110 is proposed to be the inner envelope membrane protein import-channel (Heins *et al.*, 2002) together with Tic20 (Kouranov *et al.*, 1998). Recently, Tic21, a protein with topological similarity to Tic20, was identified as a third putative channel component (Teng *et al.*, 2006), although the role of this component in chloroplast protein import has been disputed (Duy *et al.*, 2007). The stromal part of Tic110 is supposed to bind preproteins as this region of Tic110 contains a transit peptide binding site (Inaba *et al.*, 2003). While binding to preprotein, Tic110 would recruit chaperones Hsp93 and Cpn60 with the help of Tic40 (Kessler and Schnell, 2006). These chaperones probably keep the preprotein in a correct folding conformation and give the driving force to complete the

translocation into the stroma. The role of Tic22 is still not known but as it is facing the inter membrane space, this protein could be a preprotein receptor or it could help formation of Toc-Tic super-complexes during translocation (Kouranov *et al.*, 1998).

Tic62, Tic55 and Tic32 are believed to be redox components that are proposed to serve as regulators of the translocation reaction (Caliebe *et al.*, 1997; Kuchler *et al.*, 2002; Hormann *et al.*, 2004; Bédard and Jarvis, 2005; Jarvis, 2008). Indeed, Tic62 interacts with a ferredoxin-NAD(P)⁺ oxidoreductase that can be regulated by photosynthetic electron transfer (Küchler *et al.*, 2002) and Tic55 is predicted to contain a Rieske-type iron-sulfur cluster that catalyses electron transfer reactions (Caliebe *et al.*, 1997). The role of Tic32 is still unclear but this protein is essential as *Arabidopsis* mutant plants that do not express this protein are embryo lethal (Hormann *et al.*, 2004).

4.3 Import towards the thylakoids

Once in the stroma, a stromal processing peptidase (SPP) cleaves the transit peptide. The preprotein becomes a mature protein unless its fate is to be targeted to the thylakoid.

Different pathways are involved in the targeting of thylakoid preproteins (as reviewed by Robinson *et al.*, 2001). Thylakoid membrane proteins either integrate via the signal recognition particle pathway (SRP), or, integrate spontaneously without the assistance of any known apparatus. Proteins addressed to the lumen are imported by the Sec pathway or the Twin-arginine translocation (Tat) pathway. The Sec pathway is comparable to the way prokaryotes export or secrete proteins. The Sec pathway involves a stromal SecA, ATP hydrolysis and a Sec complex that binds to the membrane. The Tat pathway is also called the Δ pH-dependent pathway, as the pH gradient across the thylakoid drives the translocation. It translocates fully folded proteins into the thylakoids; ATP, and maybe GTP, are involved in this import. When the proteins are in the lumen, the thylakoid targeting signal peptide is cleaved by a thylakoid processing peptidase (TPP).

4.4 Chloroplast protein import can be divided into three stages

The first step of import into chloroplast does not require energy and consists of reversible precursor protein association with the chloroplast outer membrane (Perry and Keegstra, 1994; Ma *et al.*, 1996).

The second step consists of the binding of the precursor protein to the chloroplast import machinery to form an “early import intermediates”. This binding is irreversible

and requires low concentrations of either hydrolyzable GTP or ATP (100 μ M) (Olsen *et al.*, 1989; Olsen and Keegstra, 1992; Schnell and Blobel, 1993). The required ATP is hydrolysed in the intermembrane space (Olsen and Keegstra, 1992).

The ultimate stage is the complete translocation of the precursor protein through both outer and inner envelope membrane and, when arrested, is called late translocation intermediate. This step requires concentrations of ATP of 1 to 3mM (Pain et Blobel, 1987; Theg *et al.*, 1989), which is probably consumed by ATP hydrolyzing chaperones such as Hsp60, Hsp70 or Hsp100 (Kessler and Blobel 1996).

4.5 Import of proteins independent of the translocon complexes

Further pathways lead to import of chloroplast proteins. The majority of outer membrane proteins that do not possess a transit peptide insert spontaneously, or, by interaction with lipids of the outer envelope membrane (Keegstra and Cline, 1999).

Some proteins transported to the inner envelope membrane also lack a transit peptide. For example, the targeting information of Tic32 (Nada and Soll, 2004) and the chloroplast envelope quinone oxidoreductase protein (Miras *et al.*, 2007), is present within the mature protein. These two proteins are not targeted through the Toc complex apparatus (Nada and Soll, 2004; Miras *et al.*, 2007).

Interestingly, the secretory pathway also plays a role in chloroplast import as some proteins are N-glycosylated before being imported (Villajero *et al.*, 2005), which is not so surprising as some chloroplast proteins were predicted to have a signal peptide for ER targeting (Kleffmann *et al.*, 2004).

A substrate dependent import also exists. This is the case for one of the light-dependent enzymes that control chlorophyll synthesis: NADPH-protochlorophyllide (PChlide) oxidoreductase A (PORA). PORA import is suggested to be dependent of its substrate PChlide (Reinbothe *et al.* 1995) and to involve atToc33, the *Arabidopsis* homologue of psToc34 (Reinbothe *et al.* 2005). However, some studies have shown that the import is substrate-independent (Aronsson *et al.*, 2000) and may involve the guidance complex (Schemenewitz *et al.*, 2007). Thus, it has been proposed that both pathways are used depending on the plant development stage (Schemenewitz *et al.*, 2007).

5 The different components of the Toc core complex

5.1 Toc75

Although Toc75 is located at the outer membrane of the chloroplast, it is synthesised with a large bipartite transit peptide (Tranel and Keegstra, 1996). The N-terminal part targets the protein and is cleaved by a stromal processing peptidase; the C-terminal part of the transit peptide prevents targeting to the stroma and mediates envelope targeting (Tranel and Keegstra, 1996; Inoue and Keegstra, 2003).

Toc75 is one of the protein import components (Perry and Keegstra, 1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995) that is predicted to form a β -barrel structure and to act as a cation selective conducting channel (Hinnah *et al.*, 1997; Hinnah *et al.*, 2002).

In the plant model *Arabidopsis thaliana*, three genes encode proteins homologous to pea Toc75 (psToc75): *atTOC75-I*, *atTOC75-III*, and *atTOC75-IV* but only *atTOC75-III* seems to code for the ortholog of psToc75 (Jackson-Constant and Keegstra, 2001).

The discovery of syn75, an homolog of pea Toc75 in the cyanobacterium *Synechocystis* PCC6803 (Bölter *et al.*, 1998; Reumann *et al.*, 1999) support the fact that Toc75 has a prokaryotic origin, on the opposite, no homolog of Toc159 and Toc34 are found in *Synechocystis*, consequently these two GTPases are believed to have an eukaryotic origin (Reumann and Keegstra, 1999; Dyall *et al.*, 2004).

5.2 Toc GTPases

The common feature of the two GTPases of the Toc complex, Toc159 and Toc34, is a conserved GTP binding domain (G-domain). These GTPases of eukaryotic origin belong to a new family of GTPases (Sun *et al.*, 2002, Aronsson *et al.*, 2003) that also belong to the paraseptin family (as reviewed by Weirich *et al.*, 2008).

Toc159 is composed of an N-terminal acidic domain (A-domain), followed by a central G-domain and by a C-terminal domain (M-domain) that is anchored to the membrane. Toc34 is constituted almost exclusively of its G-domain, and is anchored in the outer membrane by its C-terminal part which is composed of a stretch of hydrophobic amino acids.

In *Arabidopsis*, two genes encode proteins homologous to pea Toc34 (Fig.4): *atTOC33* and *atTOC34* (Jarvis *et al.*, 1998; Hiltbrunner *et al.*, 2001a, Jackson-Constant and Keegstra, 2001), four genes encode homologs of psToc159: *atTOC159*, *atTOC132*, *atTOC120* and *atTOC90* (Bauer *et al.*, 2000; Hiltbrunner *et al.*, 2001a; Hiltbrunner *et*

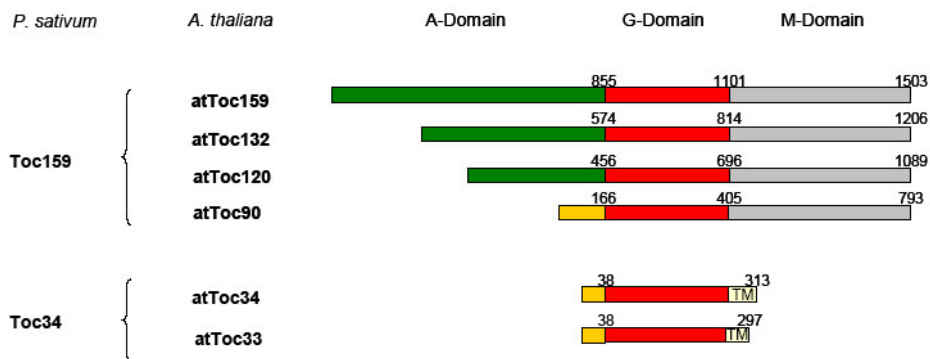


Fig.4: GTP binding proteins of the Toc-complex in *A. thaliana*: (from Hiltbrunner *et al.*, 2001a and Weibel, 2005)

The members of the family of GTP binding proteins of *Arabidopsis* fall into two subgroups, the homologues of pea Toc159 (atToc159, atToc132, atToc120 and atToc90) and the homologues of pea Toc34 (atToc34 and atToc33). They all share a GTP binding-domain (G-domain). Toc159 family proteins have an additional N-terminal acidic domain (A-domain) with the exception of Toc90 and a C-terminal membrane domain (M-domain), whereas the Toc34 family has a C-terminal transmembrane helix (TM). The numbers indicate amino acids.

al., 2004). *atToc159* and *atToc33* are the orthologs of *psToc159* and *psToc34* respectively. All the homologs of *psToc159*, apart from *atToc90*, share an N-terminal A-domain.

Arabidopsis is not the only plant where homologs of pea *Toc159* and *Toc34* proteins are present. Analysis of genomic databases identified two or three *Toc34* homologs in at least seven plant species such as spinach, rape seed, seed, potato, tomato, poplar tree, maize, and in the moss *Physcomitrella patens*, moreover, homologs of *Toc159*, *Toc132* and *Toc120* are known in rice and *Physcomitrella patens* (Voigt *et al.*, 2005).

5.2.1 *Toc159* and its homologs

Due to its proteolytic sensibility, *Toc159* was first identified as an 86kDa protein (Waegemann and Soll, 1991) and was then initially named *Toc86* (Hirsch *et al.*, 1994; Kessler *et al.*, 1994). *Toc159* is believed to be the primary receptor of preproteins (Hirsch *et al.*, 1994, Perry and Keegstra, 1994), and, as discussed earlier, binding to preproteins may happen when *Toc159* is in a soluble cytosolic form.

The function of the A-domain of *Toc159* is still not known, but in *Arabidopsis*, proteins lacking this domain can complement the *atToc159* knock-out mutant, *ppi2* (Lee *et al.*, 2003). In *Arabidopsis* protoplasts, import is not affected if proteins are lacking this domain (Lee *et al.*, 2003) and *Toc159* is still located at the chloroplast outer membrane, even when this domain is missing (Bauer *et al.*, 2002).

The M-domain anchors the protein to the chloroplast outer membrane, and is sufficient to complement the defect in protein import in *Arabidopsis* protoplasts (Lee *et al.*, 2003). This complementation is not complete though, as complemented plants have 50% less chlorophyll than wild type plants (Lee *et al.*, 2003).

The G-domain contains GTP binding motifs as well as a dimerisation motif (Sun *et al.*, 2002). The G-domain binds GTP and is involved in the targeting of *Toc159* to the membrane in the presence of GTP (Bauer *et al.*, 2002; Smith *et al.*, 2002, Lee *et al.*, 2003). Indeed, only this domain is sufficient to target the protein to the chloroplast outer membrane in *Arabidopsis* protoplasts (Bauer *et al.*, 2002). Targeting and insertion of *Toc159* needs GTP binding and hydrolysis (Bauer *et al.*, 2002; Smith *et al.*, 2002).

It has been proposed that both the G- and the M-domain play a role in *Toc159* targeting to the chloroplast membrane (Lee *et al.*, 2003).

In *Arabidopsis*, the *atToc159* knock-out mutant, *ppi2* (*ppi* stands for plastid protein import), has an albino phenotype, plastids do not develop and the plants cannot grow autotrophically, moreover, this mutation is seedling lethal (Bauer *et al.*, 2000). This mutant is the result of a T-DNA insertion that disrupts the *atTOC159* gene. The other members of the import apparatus are expressed and functional in this mutant; it appears

that Toc159 is essential for chloroplast biogenesis, especially as accumulation of photosynthetic proteins is dramatically reduced. However, import of non-photosynthetic proteins does not seem to be affected in *ppi2* plants. It has been suggested that the three other homologs of psToc159, atToc132, atToc120 and atToc90 partially compensate for the absence of atToc159, or that these proteins are dedicated to the import of non-photosynthetic proteins (Bauer *et al.*, 2000).

Knock-out mutants of either atToc132, atToc120 or atToc90 show no specific phenotype, this suggests that their function is not essential and that they are functionally redundant (Hiltbrunner *et al.*, 2004; Ivanova *et al.*, 2004; Kubis *et al.*, 2004). Preprotein binding studies confirmed that atToc132 and atToc120 have specificity for non-photosynthetic proteins (Ivanova *et al.*, 2004).

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. atToc159 cannot complement the atToc132/atToc120 mutant phenotype and the double mutant atToc159/atToc132 is embryo lethal (Kubis *et al.*, 2004); these findings support the fact that the members of the Toc159 family in *Arabidopsis* are involved in different import pathways.

Double knock out atToc90/atToc132, atToc90/atToc120 have no phenotype which is surprising as the *atTOC90* gene is expressed with high levels within the plant (Kubis *et al.*, 2004). Therefore, the role of atToc90 still has to be defined, but atToc90 definitely does not seem to be essential for import and maybe even is not acting in chloroplast protein import (Hiltbrunner *et al.*, 2004). These results also show that there is no functional redundancy between atToc90/atToc132 and atToc90/atToc120.

5.2.2 Toc34

Toc34 is composed of one C-terminal transmembrane helix and of a G-domain similar to the G-domain of Toc159. Toc34 is synthesized with no cleavable presequence and inserts directly into the lipid layer of the chloroplast outer envelope membrane with the help of GTP (Qbadou *et al.*, 2003). Crystal structure of the G-domain of pea Toc34 and *in vitro* studies, suggests homodimerisation of Toc34 in a GDP-bound form but also heterodimerisation with the G-domain of Toc159 (Sun *et al.*, 2002; Weibel *et al.*, 2003).

Integration and assembly of Toc159 in the Toc complex is proposed to be mediated by Toc34 GTPase activity with the help of Toc75 (Wallas *et al.*, 2003). During this process, the M-domain of Toc159 seems to interact with the G-domain of Toc34.

psToc34 functions as a GTP-dependent receptor for preproteins, and is controlled by protein phosphorylation in *in vitro* experiments (Sveshnikova *et al.*, 2000): GTP-bound Toc34 shows higher affinity for phosphorylated preproteins. This binding stimulates GTP hydrolysis, which causes the release of GDP and the precursor protein (Jelic *et al.*,

2003). In *Arabidopsis*, atToc33 may be phosphorylated whereas atToc34 is not (Jelic *et al.*, 2003). However, mutation of the atToc33 protein phosphorylation site *in vivo*, does not seem to have any effect on protein import (Aronsson *et al.*, 2006).

In *Arabidopsis*, the knock out mutant of atToc33, the ortholog of psToc34, was generated from a T-DNA insertion within the *atTOC33* gene. This mutant, called *ppi1*, has pale green leaves during the first weeks of development, thereafter, this phenotype disappears (Jarvis *et al.*, 1998). This indicates that atToc33 may be involved during early stage of plant development and that atToc34 can partially complement the loss of Toc33 expression (Jarvis *et al.*, 1998). *ppi1* mutant plants also show a defect in expression and import of photosynthetic proteins (Kubis *et al.*, 2003) which suggests that atToc33 is mainly involved in import of photosynthetic proteins.

On the other hand, *Arabidopsis* plants that do not express atToc34, (this mutant is named *ppi3*) due to T-DNA insertion, have no specific phenotype apart from delayed root growth. These results confirm the hypothesis that atToc33 can substitute atToc34 and that atToc34 may have a more important role for plastid biogenesis in roots (Constan *et al.*, 2004). The double mutant *ppi1/ppi3* is embryo lethal. Therefore, it can be concluded that the function of these two proteins is essential for protein import (Constan *et al.*, 2004).

atToc33 and atToc34 antisense *Arabidopsis* plants were created. The phenotype for antisense *atTOC33* plants is the same as *ppi1* plants, but antisense *atTOC34* plants have a paler phenotype compared to the wild type when seedlings are 4 days-old, but then the phenotype disappears to get similar to wild type plants (Gutensohn *et al.*, 2000). One possible explanation is that antisense *atTOC34* plants may have an effect on *atTOC33* expression during early development (Constan *et al.*, 2004), or that atToc33 and atToc34 specificity for photosynthetic or non-photosynthetic proteins is more complex than initially stated (Gutensohn *et al.*, 2000).

5.3 Specificity of import

As mentioned before, in *Arabidopsis*, atToc159 seems to be involved in a different pathway than atToc132, atToc120 and atToc90, likely, atToc33 and atToc34 also have a different substrate (preprotein) specificity. atToc159 and atToc33 are thought to be dedicated to import photosynthetic proteins, while the others would import non-photosynthetic proteins. In addition, co-immunoprecipitation and *in vitro* binding experiments showed that atToc159 is binding preferentially to atToc33 rather than atToc34 (Ivanova *et al.*, 2004). On the other hand atToc132 and atToc120 bind equally to atToc33 and atToc34. It is proposed that by competition, atToc159 binds preferentially to atToc33, which leads atToc132 and atToc120 to bind more likely to atToc34.

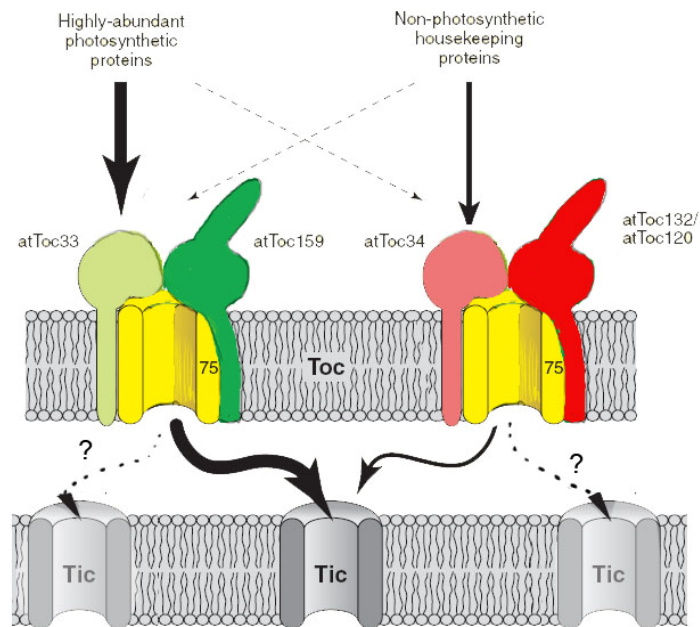


Fig.5 : An alternative pathway to import non-photosynthetic proteins. (Adapted from Jarvis and Robinson, 1994; Kessler and Schnell, 2006)

In *Arabidopsis*, Toc GTPases are encoded by a small gene family. The different isoforms are involved in at least two different pathways. atToc159 and atToc33 import photosynthetic proteins which constitute the majority of imported proteins. atToc132, atToc120 and atToc34 are involved in the import of non-photosynthetic proteins, also called “housekeeping” proteins. Afterwards, preproteins are imported through the Tic complex. Whether the components of this Tic complex are identical or not for the import of photosynthetic and housekeeping proteins is not known.

These data suggest at least two different pathways of protein import depending on the nature of the substrate (Fig. 5). Photosynthetic proteins could be imported by the Toc complex composed of atToc159, atToc33 and atToc75. Import of non-photosynthetic proteins could rely on atToc132/atToc120 and atToc75. Whether different Tic complexes exist depending on the imported protein still remains to be deciphered (as reviewed by Kessler and Schnell, 2006).

6 Two models for preprotein import

GTP is believed to play an important role in protein translocation due to the presence of the two Toc GTPases, Toc159 and Toc34. Moreover, *in vitro* import and precursor protein binding experiments require GTP (Olsen and Keegstra, 1992; Kessler *et al.*, 1994). Also, heterodimerisation of Toc159 and Toc34 is favoured in the presence of GDP (Weibel *et al.*, 2003), which confirms the role of GTP hydrolysis in this complex (Kessler *et al.*, 1994; Young *et al.*, 1999).

However, the mechanisms of protein import through the Toc complex and the exact function of the Toc GTPases remain unknown and currently, two models are proposed in pea, the initial plant model for Toc import studies (Fig. 6): the “motor” model and the “targeting” hypothesis. These two models acknowledge the importance of GTP during import, but disagree on the primary Toc receptor for precursor protein.

6.1 The motor model

This model (proposed by Becker *et al.*, 2004b) considers that preproteins are targeted to the chloroplast membrane through the guidance complex (May and Soll, 2000) and that the transit peptide of these precursor proteins is phosphorylated. Becker and associates observed that Toc34 binds to phosphorylated proteins whereas Toc159 does not, thus, Toc34 is proposed to be the initial receptor. More precisely, Toc34 in a GTP bound form (Toc34_{GTP}) is binding to the phosphorylated C-terminal part of the transit peptide, then, the N-terminal part of the transit peptide binds to Toc159_{GTP}. The interaction with the transit peptide stimulates GTP activity of Toc34 (Jelic *et al.*, 2002) and Toc34 dissociates from the preprotein. Subsequently, the transit peptide is dephosphorylated by an unknown phosphatase since dephosphorylation is required for complete translocation into isolated chloroplast (Waegmann and Soll, 1996). Then dephosphorylation induces GTP hydrolysis of Toc159 and transfers the precursor

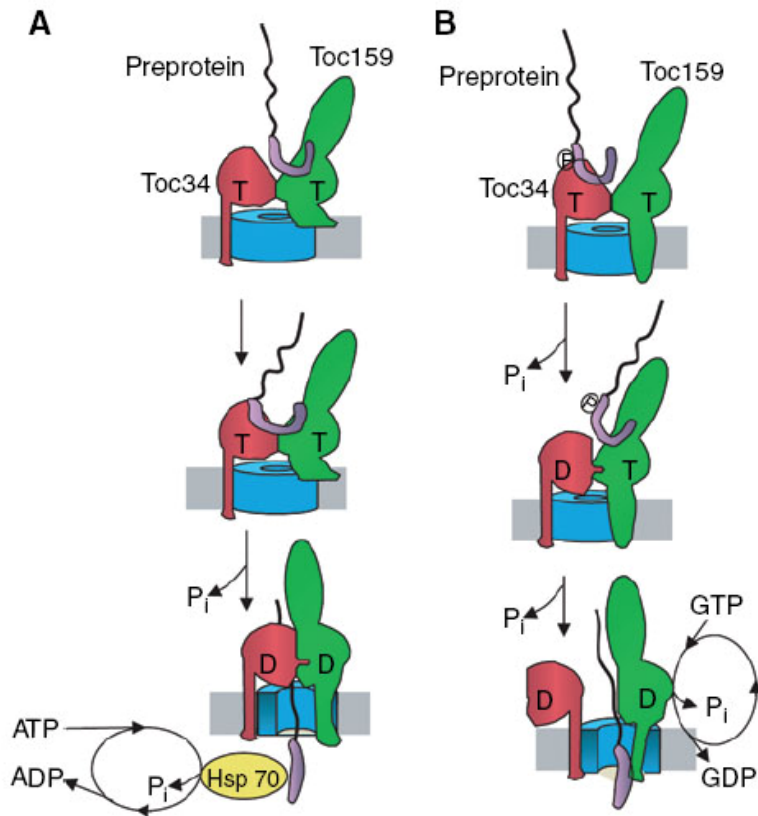


Fig.6: Two models of preprotein import in pea chloroplasts (from Kessler and Schnell, 2006)

A. The “Targeting” model proposes that Toc159 is the primary receptor of the preprotein. Toc159 and Toc34 interact via their G-domain which causes GTP hydrolysis. This GTP hydrolysis changes the conformational state of the Toc complex leading to the import of the preprotein.

B. The “motor” model suggests that Toc34 is the primary receptor in a GTP-bound state of the precursor protein that is in a phosphorylated form. This binding to the precursor induces GTP hydrolysis and the preprotein is transferred to Toc159 in a GTP-bound form. Then after dephosphorylation of the precursor, Toc159 plays a motor role and the precursor is translocated through Toc75

protein through Toc75. Thereafter the Toc complex regains its GTP bound state, following GDP-GTP exchange, to accept another round of preprotein import.

In this model, Toc159 is a GTP driven motor protein that translocates the preprotein to the intermembrane space (Schleiff *et al.*, 2003b). According to the stoichiometry of the Toc complex of 1/4/4 between Toc159/Toc75/Toc34 (Schleiff *et al.*, 2003b), Toc159 is proposed to be located in the center of this multimeric complex.

However, the fact that phosphorylation is dispensable for protein import (Nakrieko *et al.*, 2004), and that the M-domain alone of Toc159 can partially complement the defect in protein import in the *ppi2* mutant (Lee *et al.*, 2003), indicates that this model may not correspond exactly to the import of preproteins *in planta*. Moreover, some data show that GTP is required early during the import process but not during precursor protein translocation to the intermembrane space (Young *et al.*, 1999; Kessler and Schnell, 2006).

6.2 The targeting model

In this model, Toc159, in its cytosolic form, is thought to be the primary receptor of precursor proteins (Hiltbrunner *et al.*, 2001b). Precursor proteins bind to the G-domain of Toc159_{GTP} but the M-domain of Toc159 may also play a role in this binding process (Smith *et al.*, 2004, Lee *et al.*, 2003).

Toc159 would then interact and bind to Toc34, resulting in insertion of Toc159 to the membrane and GTP hydrolysis. This scheme is supported by the fact that Toc159 and Toc34 are likely to heterodimerise in a GDP-bound conformation (Sun *et al.*, 2002) and that stable association of atToc159 to atToc33 requires the GDP conformation *in vitro* (Smith *et al.*, 2002). Besides, the activity of both GTPases is required for proper insertion of Toc159 to the membrane (Bauer *et al.*, 2002; Smith *et al.*, 2002; Wallas *et al.*, 2003)

The heterodimer formed by Toc159_{GDP} and Toc34_{GDP} may acquire, due to its GDP-bound form, an adapted conformation to allow the precursor protein translocation through the protein channel, Toc75. Once the precursor is translocated, the Toc GTPases can regain their initial GTP-bound state, and Toc159 is released into the cytosol.

However, this model is not universally accepted because the existence of a soluble form of Toc159 is not widely accepted: some researchers consider that soluble Toc159 is an artefact due to membrane disruption during fractionation experiments (Becker *et al.*, 2004). More *in vivo* experiments would be necessary to confirm or infirm the targeting model as well as the motor hypothesis.

To resume, GTPase activity is important in both models. In the “motor” model, GTP hydrolysis is involved in precursor protein translocation from one Toc component to another. In the “targeting” hypothesis GTP hydrolysis is involved in the “stability” of the Toc complex, allowing Toc159 insertion to the membrane and interaction of the two GTPases.

7 Aims of the thesis

The exact mechanisms that involve the two Toc GTPases of the Toc complex during import remain unknown. Still, many experiments on *in vitro* interaction of the two GTPases Toc159 and Toc34 showed that these proteins dimerise via their G-domain (Hiltbrunner, *et al.*, 2001; Sun *et al.*, 2002; Smith *et al.*, 2002; Bauer *et al.*, 2002; Wallas *et al.*, 2003; Weibel *et al.*, 2003). But heterodimerisation of Toc159 and Toc34 still has not been demonstrated *in vivo*, nor have been observed stable heterodimers (Li *et al.*, 2007).

Today different methods are available to study protein-protein interaction *in vivo* (as reviewed by Lalonde *et al.*, 2008) many of them are based on the reconstitution of a function of the two halves of a split protein.

The yeast two hybrid system (Fields and Song, 1989) is a powerful heterologous system that is quite efficient to screen many potential candidates but cannot be applied to study membrane proteins. In this case the yeast split ubiquitin assay (Johnsson and Varshavsky, 1994; Stagljar *et al.*, 1998) is more appropriate as it is suitable for studies on membrane and soluble proteins and allows to detect transient interactions. These two methods are mainly used to detect direct protein interactions. To detect secondary interaction within a complex, affinity purification of complexes followed by identification of the proteins in the different complexes by mass spectroscopy (MS) (as reviewed by Miernyk and Thelen, 2008) is a good alternative.

In order to gain more insight into protein import into chloroplasts in the plant itself, one of the goals of my work is to study the interaction between the Toc GTPases using a system for the detection of protein-protein interactions *in vivo*; for that purpose a system based on the yeast split-ubiquitin *in Arabidopsis* and Tobacco protoplasts was developed.

It is very likely that still unknown proteins are involved in the mechanism of the Toc complex. To identify such partner proteins, an affinity purification using *in vivo* tagging followed by MS is a suitable method. For that purpose, the Tandem affinity purification tag (TAP tag) method (Rigaut *et al.*, 1999) was chosen to isolate Toc GTPases and associated complexes from *Arabidopsis*. Consequently, the second aim of this thesis is

to confirm existing partners of atToc33 and identify new ones with the TAP-tag method.

Results and Discussion

Chapter 1

Use of plant split-ubiquitin to study the interaction between Toc33 and Toc159 GTP-binding domains in vivo

1 Presentation of the system

Ubiquitin is a highly conserved protein of 76 amino acids that is present in all eucaryotes. It is often found covalently attached to other proteins to form monoubiquitin or polyubiquitin chains. Polyubiquitin chains are formed by an isopeptide linkage between the residue at the carboxy-terminus Gly-76 of ubiquitin and lysine residues of proteins ubiquitin is linked to. This chain is a degradation signal for the 26S proteasome (Hershko *et al.*, 2000). The targeted protein is degraded by a proteasome-related protease. During this process, ubiquitin is released from its substrate by deubiquitinating enzymes (DUBs) to be recycled. DUBs are divided into two groups: smaller (10-30kDa) ubiquitin C-terminal hydrolase (UCH) and larger (50-300kDa) ubiquitin specific proteases (UBPs). Besides its function in proteasome-mediated proteolysis, ubiquitin serves as a multi-purpose regulatory modification and is known to have a role during ribosome biogenesis (Finley *et al.*, 1989; Schnell and Hicke, 2003; Stavreva *et al.*, 2006).

In the split ubiquitin system, ubiquitin is spliced and expressed in two separate parts, an N-terminal part (termed Nub, consisting of amino acids 1-37) and a C-terminal part (Cub, amino acids 35-76) fused to a gene coding for a reporter protein (Johnsson and Varshavsky, 1994; Stagljar *et al.*, 1998) (Fig.1). Proteins of interest are fused either to Nub or Cub. If the two proteins interact, the two halves of ubiquitin are brought into close proximity and a quasi-native ubiquitin is reconstituted and recognised by UBPs, resulting in the cleavage of the Cub fusion and the release of the reporter protein.

However, Nub and Cub can reassemble quite easily. To limit unspecific reassociation, two mutations were engineered into Nub in position 13; at this position wild type Nub carries an isoleucine. If isoleucine is replaced by an alanine (Nub^A) or a glycine (Nub^G), affinity of the mutated Nub and Cub is lower, (the lowest affinity to Cub is with Nub^G, Johnsson and Varshavsky, 1994).

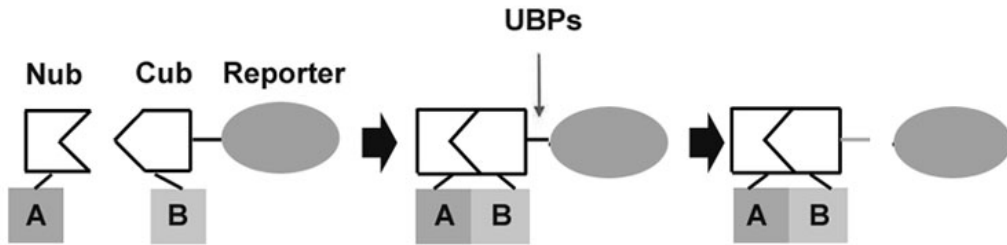


Fig.1 Schematic representation of the split ubiquitin system

Ubiquitin is split in N-terminal (Nub) and C terminal halves (Cub). Each half is fused to a protein of interest. If proteins interact, ubiquitin is reconstituted and recognised by ubiquitin-specific proteases (UBPs). The reporter gene is then cleaved. In the yeast system, the reporter is a nutritional marker or a transcription factor. In the plant system, GFP was chosen as reporter protein.

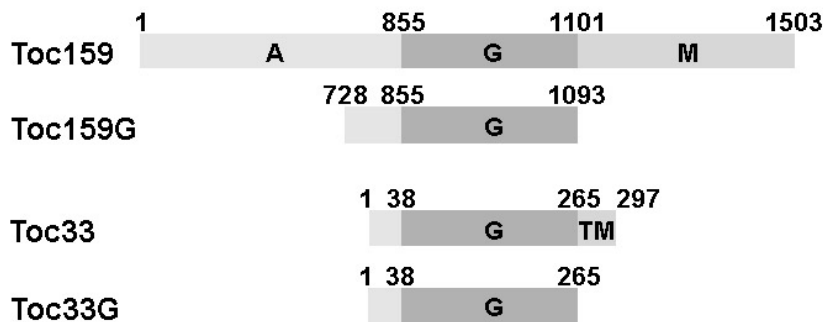


Fig.2 Schematic representation of the Toc GTPase proteins atToc159 and atToc33

atToc159 and atToc33 have conserved GTP binding-domains (G-domain, shown in dark grey). In addition, atToc159 has an N-terminal acidic domain (A domain) and a C-terminal membrane anchoring domain (M domain). atToc33 has a short C-terminal hydrophobic transmembrane sequence (TM). In this work, the coding sequence for the G-domain only of atToc159 (Toc159G, Toc159₇₂₈₋₁₀₉₃) was introduced into the different constructs. The atToc33 constructs used contain the coding sequence for G-domain (Toc33G, Toc33₁₋₂₆₅) or for the entire protein (Toc33).

The yeast split-ubiquitin system has been successfully applied for the study of numerous protein-protein interaction pairs as well as for genome-wide library interaction screens (Lehming, 2002; Miller *et al.*, 2005). Proteins of higher eukaryotes were among the proteins tested, including several, mainly plasma membrane located, plant proteins (Bregante *et al.*, 2008; Deslandes *et al.*, 2003; Ihara-Ohori *et al.*, 2007; Ludewig *et al.*, 2003; Obrdlik *et al.*, 2004; Orsel *et al.*, 2006; Pandey and Assmann 2004; Park *et al.*, 2005; Pasch *et al.*, 2005; Reinders *et al.*, 2002a; Reinders *et al.*, 2002b; Schulze *et al.*, 2003; Tsujimoto *et al.*, 2003; Yoo *et al.*, 2005). One disadvantage of the yeast split-ubiquitin system for the study of plant protein interactions is the lack of plant specific factors which might influence the interaction, as observed for the protoplast two-hybrid system (Ehlert *et al.*, 2006). Moreover, for the study of chloroplast outer membrane proteins, yeasts are lacking plastid organelles.

2 Suitability of the yeast split-ubiquitin system to study Toc GTPase interactions

2.1 Toc GTPase interaction studies

Studying Toc GTPases interaction in a yeast system was already successfully attempted in our lab; interaction among the G-domains of atToc33 and atToc159 was demonstrated using the yeast two hybrid system (Dr Birgit Agne, unpublished data). In this thesis it was determined whether Toc159G and Toc33G interacted in the yeast split-ubiquitin system. In the following constructs we engineered, Toc159G corresponds to amino acids 728-1093 of atToc159, and Toc33G to amino acids 1-265 of atToc33 (Fig.2).

Split-ubiquitin fusion constructs were generated by replacing *STE14* or *PEX11* in the constructs *STE14-Cub-RURA3* (Wittke *et al.*, 1999) or *Nub-PEX11* (Eckert and Johnsson, 2003) by Toc33G or Toc159G, respectively (Fig.3A). To allow for subsequent Western Blot analyses, two hemagglutinin epitope (HA) tags were introduced upstream of Cub and a Myc epitope downstream of Nub. Constructs encoding for Nub-Pex11p and Pex11p-2HA-Cub-RUra3p were used as a positive control in our experiments as these two fusion proteins were shown to homodimerise using yeast split ubiquitin system (Eckert and Johnsson, 2003). Originally, the arginine-URA3 (RURA3) element was designed to serve as metabolic marker for the interaction between the Nub- and Cub-fusion proteins in growth assays. If the two proteins of interest interact, RUra3p is rapidly degraded by the enzymes of the N-end rule pathway (Varshavsky, 1997), preventing the yeast to grow on media lacking uracil, but allowing them to grow on a 5-Fluoroorotic acid (FOA) supplemented media (Wittke *et al.*, 1999). However, in this thesis, the interactions were monitored by Western blotting.

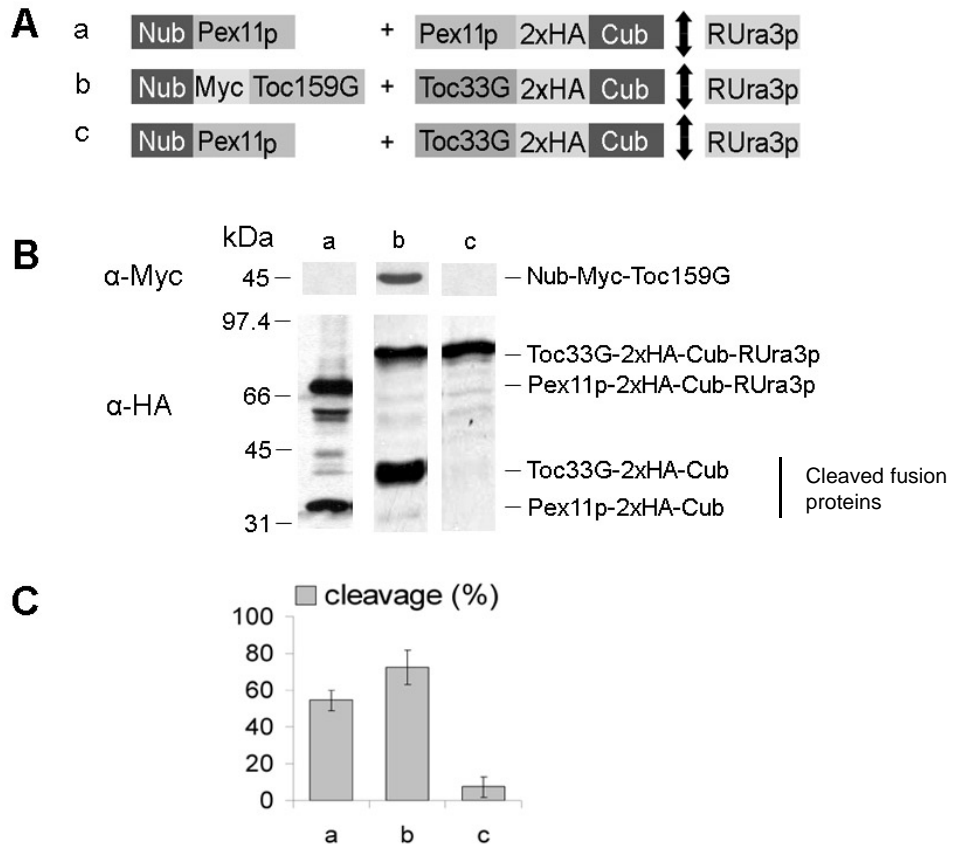


Fig.3 Yeast split-ubiquitin interaction of Toc159G and Toc33G.

A. Yeast cells were co-transformed with different combinations of Nub and Cub constructs (a-c). B. Total cellular protein was extracted and analysed by Western blotting using antibodies against the Myc or the HA epitope tag to detect Nub-myc-Toc159G or the Cub-fusion proteins, respectively. Co-expression of Nub-Pex11p and Pex11p-2HA-RUra3p (a) or Nub-myc-Toc159G and Toc33G-2HA-Cub-RUra3p (b) lead to partial cleavage of the RUra3p reporter, indicating interaction of these protein pairs. No cleavage was observed upon co-expression of Nub-Pex11p and Toc33G-2HA-Cub-RUra3p (c). C. Estimation of reporter gene cleavage via chemiluminescence quantification of three independent experiments.

Yeast cells (strain JD53) were co-transformed with the different constructs as shown in Fig.3A. Equal amounts of cellular protein of the transformants were subjected to Western blot analysis with anti-Myc (α -Myc) antibodies to check the presence of Nub-Myc-Toc159G; and with α -HA antibodies to detect the cleavage of the Cub-fusion proteins as indicator of the interaction (Fig.3B). Cleavage (partial) of the Toc33G-Cub fusion protein was observed (Toc33G-Cub-HA, 38kDa) when it was expressed in the presence of Nub-Myc-Toc159G (Fig.3C lane b) whereas no cleavage was observed upon co-expression with a Nub fusion of the peroxisomal protein Nub-Pex11p (Fig.3C, lane c) only a single band corresponding to the Toc33G-Cub-HA-RURA3 (73kDa) fusion protein was detected (negative control). In the positive control experiment, Nub Pex11p fusion protein induced cleavage of Pex11p-2HA-Cub-RUra3p (Fig.3C, lane a), consistent with Pex11p homodimerisation (Eckert and Johnsson, 2003). This cleavage is incomplete like it was for the interaction Nub-myc-Toc159G with Toc33G-Cub-HA-RURA3.

These results suggest that split-ubiquitin is suitable to study Toc protein interactions in yeast and encouraged us to establish the system in plants. In parallel, more experiments on Toc GTPase-precursor protein interactions were carried out in the yeast system as shown below.

2.2 The effects of mutations on Nub upon Toc GTPase

As mentioned at the beginning of the result part, it is possible to mutate one amino acid of ubiquitin (Ile13) to avoid unspecific assembly of the two ubiquitin parts. In order to investigate the effect of Nub mutations upon the interaction of Toc159G and Toc33G, the interaction experiment (Fig.3) was repeated using Nub wt (Fig.4A construct a), Nub^G (Fig.4A,b), and Nub^A (Fig.4A,c). While there is cleavage when co-transforming yeast cells by Nub-Myc-Toc159G and Toc33G-HA-Cub-RURA3 (Fig.4B lane a), this cleavage pattern disappears when using the mutated form of the Nub half (Fig.4B lanes b and c).

2.3 Detection of the interaction between Toc159G and a chloroplast precursor protein

Interaction between Toc159G and a chloroplast precursor protein was also studied in the yeast split ubiquitin system. The coding sequence of the small sub-unit of RuBisCO in its precursor form (pre-ats1B, amino acids 1-181) and mature form (ats1B, amino acids: 55-181) were fused to the Cub-HA-RURA3 cassette (Fig.5A). Yeast cells were co-transformed with Nub-Myc-Toc159G and preats1B-HA-Cub-RURA3 (Fig.5A,b) or

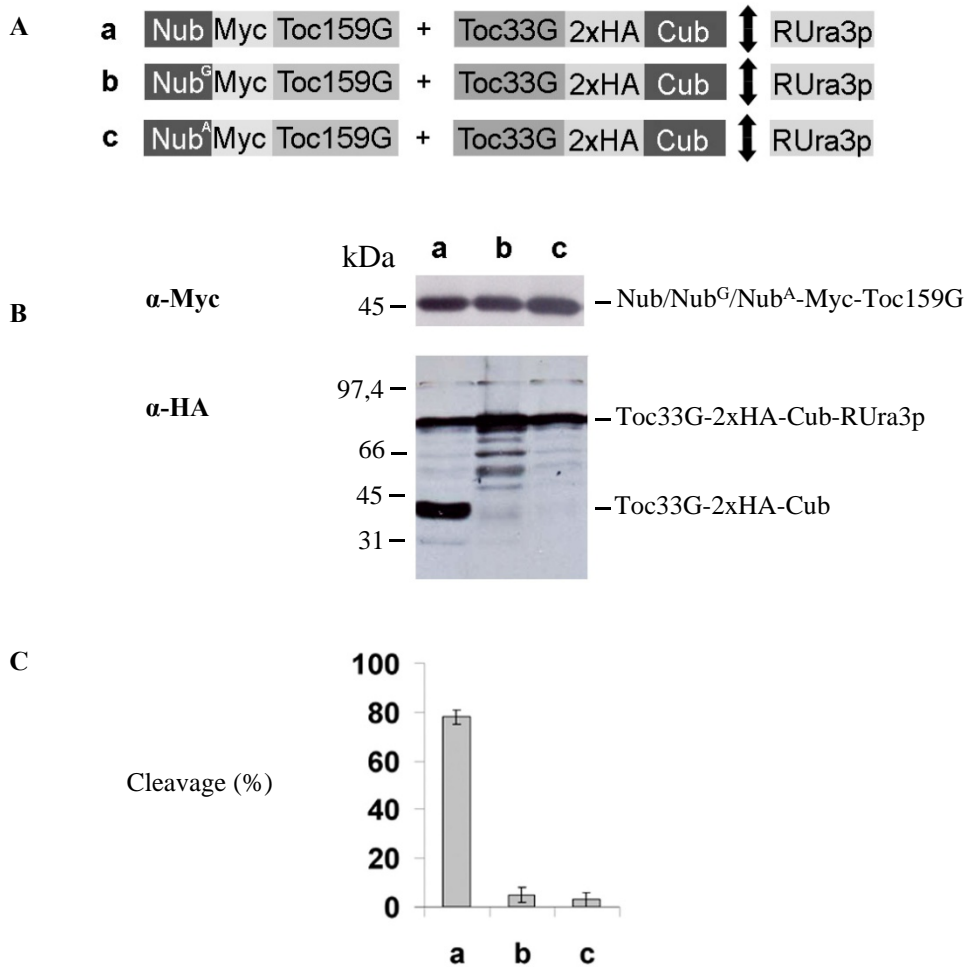


Fig.4 Yeast split-ubiquitin interaction of Toc159G and Toc33G using wt Nub and Nub^G (replacement Ile13 by Gly) or Nub^A (Ile13-Ala).

A. Yeast cells were co-transformed with different combinations of Nub and Cub constructs (a-c). B. Total cellular protein was extracted and analysed by Western blotting using antibodies against the Myc or the HA epitope tag to detect Nub-myc-Toc159G or the Cub-fusion proteins, respectively. Co-expression Nub-myc-Toc159G and Toc33G-2HA-Cub-RUr3p using Nub wt (a), lead to partial cleavage of the RUr3p reporter, indicating interaction of these protein pairs. No cleavage was observed upon co-expression of Nub^G-myc-Toc159G and Toc33G-2HA-Cub-RUr3p (b) or Nub^A-myc-Toc159G and Toc33G-2HA-Cub-RUr3p (c). C. Quantification of reporter gene cleavage via chemiluminescence detection of three independent experiments.

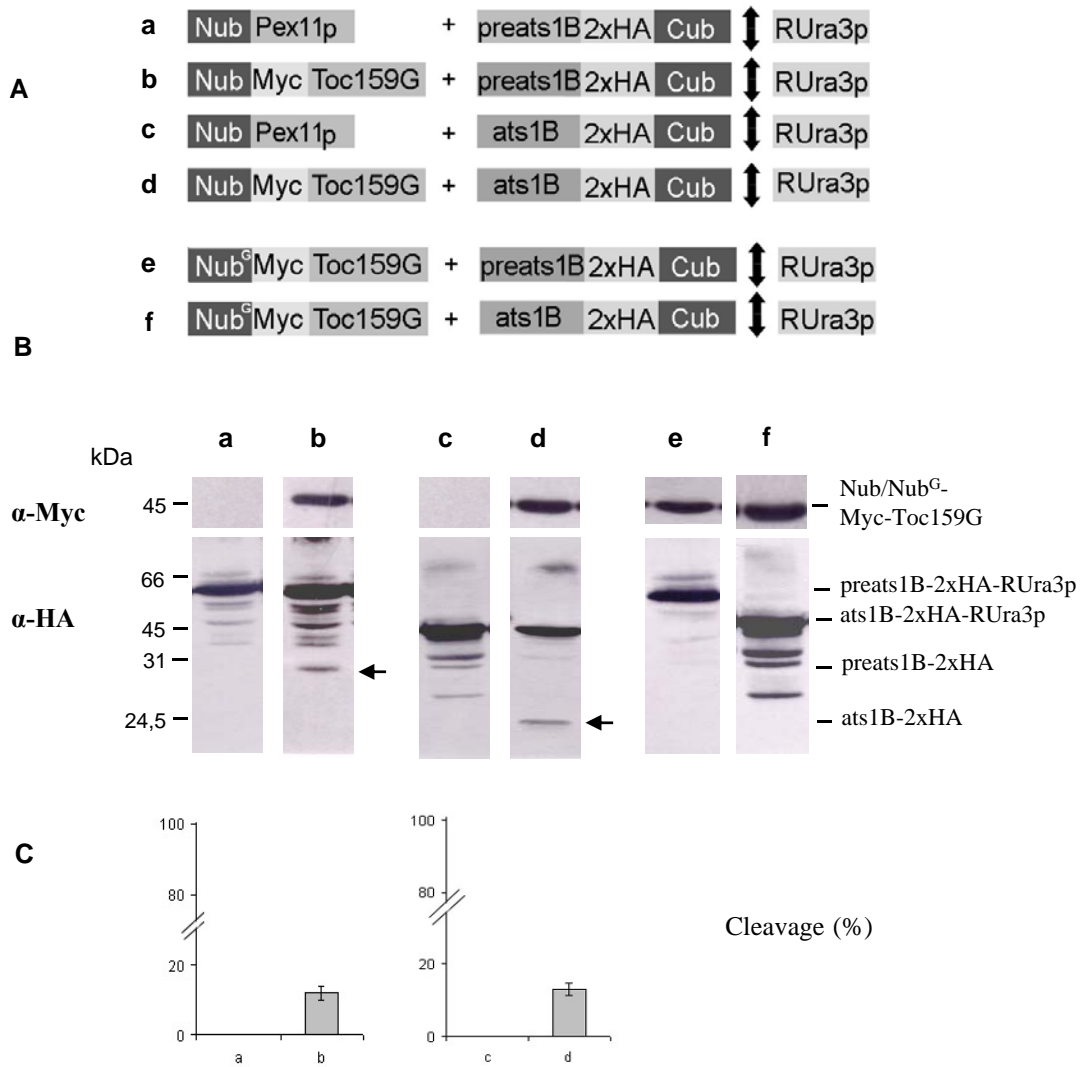


Fig.5 Yeast split-ubiquitin interaction of Toc159G and the small subunit of RuBisCO in its mature (*ats1B*) and precursor form (*preats1B*).

A. Yeast cells were co-transformed with different combinations of Nub and Cub constructs (a-f). B. Total cellular protein was extracted and analysed by Western blotting using antibodies against the Myc or the HA epitope tag to detect Nub-myc-Toc159G or the Cub-fusion proteins, respectively. Co-expression of Nub-Toc159G and *ats1B*-2HA-RUr3p (d) or Nub-myc-Toc159G and *preats1B*-2HA-Cub-RUr3p (b) lead to little cleavage of the RUr3p reporter, indicating interaction of these protein pairs. No cleavage was observed upon co-expression of Nub-Pex11p and *preat1B*-2HA-Cub-RUr3p (a) or Nub-Pex11p and *at1B*-2HA-Cub-RUr3p (c). No cleavage was observed when experiment was repeated using mutated Nub^G (replacement of Ile13 by Gly) (e and f). C. Quantification of reporter gene cleavage via chemiluminescence detection of three independent experiments.

ats1B-HA-Cub-RURA3 (Fig.5A,d). The interaction was determined by Western blot. Weak signals corresponding to the cleaved RURA3 were detected (Fig.5B lanes b and d). As negative controls yeast cells were co-transformed with Nub-Pex11p together with the preats1b- (Fig.5A constructs a) or ats1B-HA-Cub-RURA3 (Fig.5A,c) fusion proteins. No cleavage was observed.

The interaction between Nub-Myc-Toc159G and preats1B or ats1B-HA-Cub-RURA3 was repeated using the mutated Nub^G (Fig.5A,e,f). As already observed for Nub^A-/Nub^G-Myc-Toc159G and Toc33-Cub (Fig.4B), no cleavage was detected using the mutated Nub-fusions (Fig.5B lanes e and f).

3 Protein interaction analysis in *Arabidopsis* protoplasts

For the plant split-ubiquitin system we used plant ubiquitin *AtUBQ11* (At4g05050.1) instead of the yeast *ScUBI4* and enhanced green fluorescent protein (GFP) as reporter protein. *AtUBQ11* is 97% identical to yeast ubiquitin differing from *S. cerevisiae* Ubi4p by only two amino acids exchanges (S28A, S57A). The N- and C-terminal ubiquitin parts were defined as in yeast, Nub consisting of amino acids 1-37, Cub of amino acids 35-76. Constructs were engineered in the pCL60 vector (Bauer *et al.* 2000), containing a CaMV 35S promoter and a nopaline synthase (nos) terminator. A HA epitope tag was included in the Cub constructs for subsequent Western blot analysis. Isolated *Arabidopsis* protoplasts were transformed with constructs encoding for Nub alone, Nub-Toc159G and Toc33G or full length Toc33 fused to HA-Cub-GFP in different combinations (Fig.6A). The GFP reporter protein of the Cub constructs allowed assessing the protoplast transformation efficiency (around 30% in most of the experiments) by confocal microscopy (Fig.6B).

Western blots were performed on protein extracts of transformed protoplasts using α -HA antibodies to determine whether cleavage had occurred (Fig. 6C, lower panel) and α -Toc159G antibodies to monitor Nub-Toc159G expression (Fig. 6C, upper panel). When Nub-159G and Toc33G-HA-Cub-GFP were co-expressed, more than 80% cleavage of the GFP reporter was observed (Fig. 6C lane b). In a control experiment in which Nub alone was co-expressed together with Toc33G-HA-Cub-GFP, non-specific cleavage in the range of 40% of the GFP reporter gene was observed (Fig. 4C lane a)

One of our goals, using this method in plant, is to study Toc GTPase interactions in their target membrane. Therefore we performed the same experiment using a Cub construct containing the full length cDNA coding for Toc33 inclusive its C-terminal transmembrane helix (Toc33-HA-Cub-GFP). Around 80% cleavage of the GFP reporter was observed upon co-expression with Nub-159G (Fig.6C lane c) and around 30 to 40% upon co-expression with Nub alone (Fig.7 lane c and Fig.8 lane a).

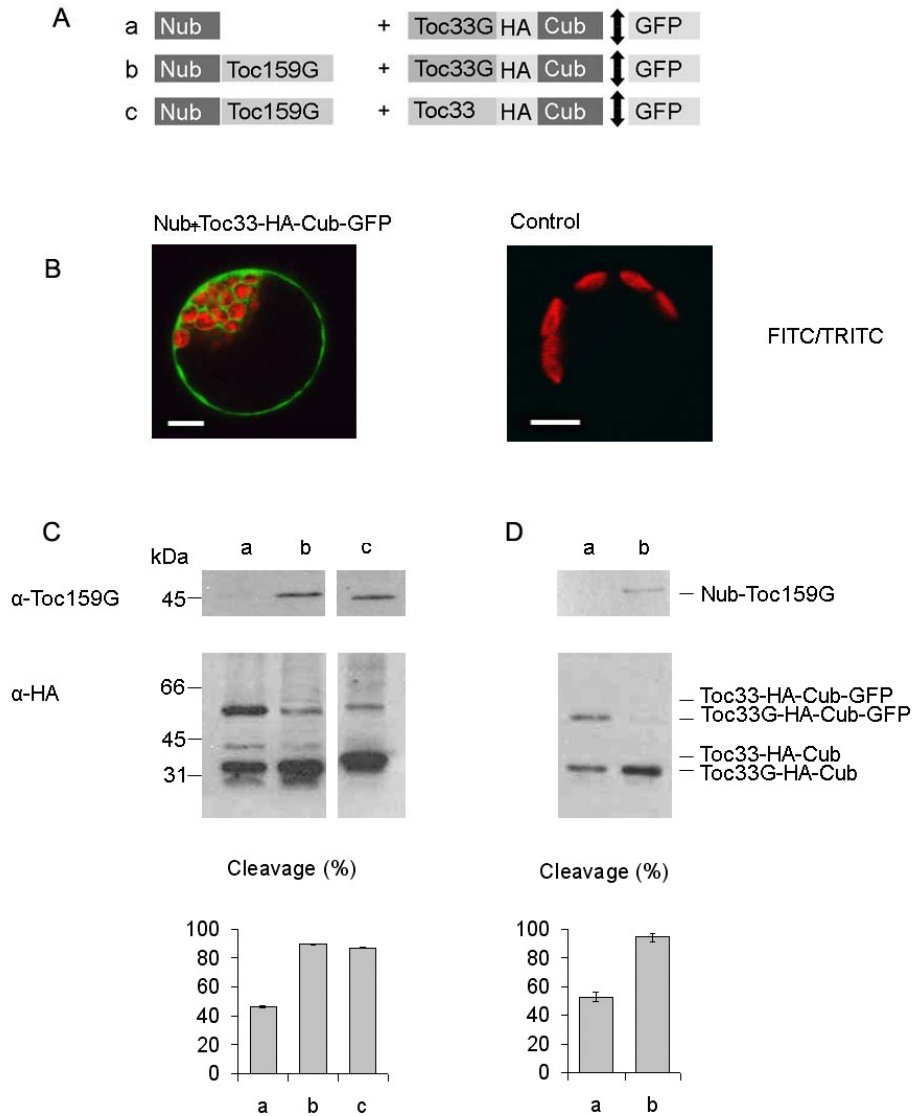


Fig.6 Plant split-ubiquitin interaction for Toc159G and Toc33

A. *Arabidopsis* protoplasts were co-transformed with Nub and Cub constructs as indicated (a-c). B. Use of the GFP reporter to assess protoplast transformation efficiency via confocal microscopy. Due to the background cleavage all Cub-GFP-fusions gave the same fluorescence pattern as exemplified here for Toc33-HA-Cub-GFP (bar = 5µm). C. Total proteins were extracted and analysed by Western blotting using antibodies raised against Toc159G and anti-HA to check for the presence of Nub-Toc159G and the HA-tagged Cub-fusion proteins, respectively. Co-expression of NubToc159G with Toc33G-Cub-GFP (b) or full-length Toc33-Cub-GFP (c) results in almost complete cleavage of the GFP reporter indicating interaction. The control experiment (a) reveals background cleavage of the reporter gene. D. Experiments (a) and (b) were carried out in tobacco protoplasts and gave similar cleavage patterns. The graphs below show the results of chemiluminescence quantification of three independent experiments.

3.1 The plant split-ubiquitin system is applicable to other plant species

To test whether the plant split-ubiquitin system could be used to study protein interactions in other plant species, the Nub-Toc159G and Toc33G-HA-Cub-GFP constructs were transformed into isolated tobacco protoplasts (Fig. 6D). In a control experiment in which Nub alone was co-expressed together with Toc33G-HA-Cub-GFP in tobacco protoplasts, non-specific cleavage in the range of 50% of the GFP reporter occurred. When Nub-Toc159G and Toc33G-HA-Cub-GFP were co-transformed, cleavage of the reporter gene rose to around 90%.

Taken together, the increase in cleavage of the Toc33-Cub fusion proteins in the presence of Nub-Toc159G indicated interaction of these proteins *in planta*.

3.2 The mutated Nub^G is not suitable to study Toc protein-protein interactions

In order to limit the background cleavage observed with non interacting proteins, the Nub^G mutation was introduced in the plant split-ubiquitin experiments (Fig.7). The background cleavage is still present whether protoplasts are co-transformed with Nub and Toc33-HA-Cub-GFP (Fig.7 lane b) or with Nub^G and Toc33-HA-Cub-GFP (Fig.7 lane c). However the complete cleavage pattern observed when protoplasts transiently express Nub-Toc159G and Toc33-HA-Cub-GFP (Fig.7 lane e) is strongly reduced when co-expressing the mutated Nub^G Toc159G and Toc33-HA-Cub-GFP (Fig.7 lane d). Using Nub^G gave exactly the same result as the one observed with the yeast system, interaction using Nub^A mutation was not tested. This result indicates that only wt Nub is suitable to study Toc protein interactions. One possible explanation could be that Toc proteins interact only transiently and a mutated ubiquitin is not suitable to reveal the interaction, because the mutation in the Nub may slow down reconstitution.

As a result of background cleavage, the GFP reporter served only as an indicator of the protoplast transformation rate, but could not be exploited to localize the Cub fusion proteins in the absence of an interacting protein. The transformation of all Cub-GFP fusion proteins used in this study resulted in essentially the same cytosolic fluorescence pattern as can be seen in Fig. 6B. The high rate of non-specific cleavage in the plant split-ubiquitin system is in contrast to the very low rate of background cleavage observed in the yeast split-ubiquitin assays (Fig.3) and could be due to a comparatively high activity of *Arabidopsis* UBPs (Yan *et al.*, 2000).

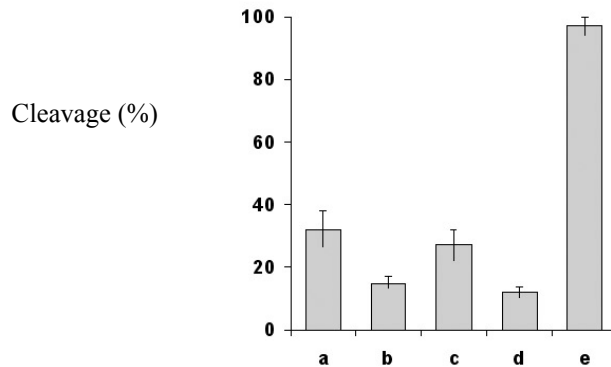
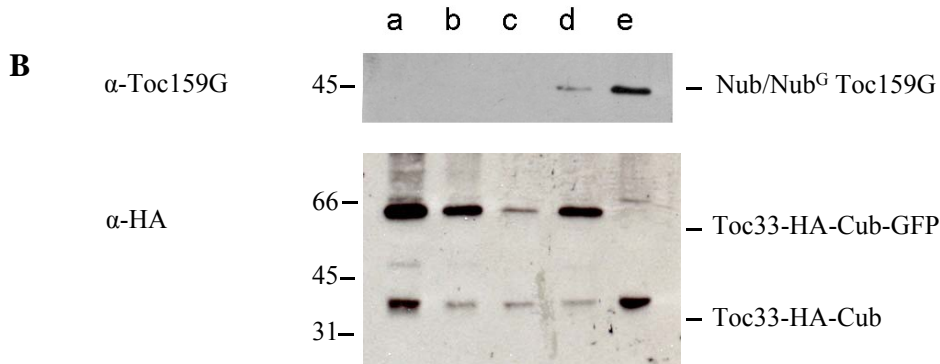
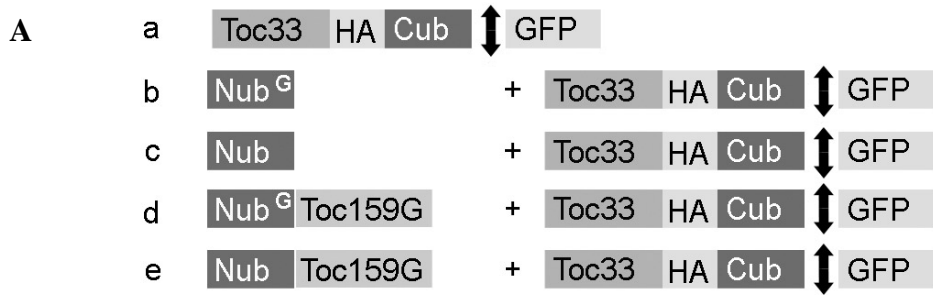


Fig.7 Plant split-ubiquitin interaction among Toc159G and Toc33 using wtNub and Nub^G

A. *Arabidopsis* protoplasts were co-transformed with Nub and Cub constructs as indicated (a-e). B. Total proteins were extracted and analysed by Western blotting using antibodies raised against Toc159G and anti-HA to check for the presence of Nub-Toc159G and the HA-tagged Cub-fusion proteins, respectively. Co-expression of NubToc159G with Toc33-Cub-GFP (e) results in almost complete cleavage of the GFP reporter indicating interaction. This complete cleavage disappears with coexpression of Nub^GToc159G with Toc33-Cub-GFP. The control experiments (b and c) reveal partial background cleavage of the reporter gene, as well as expression of Toc33-Cub-GFP alone (a). C. The graphs below show the results of chemiluminescence quantification of three independent experiments.

3.3 General suitability of the plant split-ubiquitin system to analyze protein interactions

To test whether the plant split-ubiquitin system is useful for protein pairs other than Toc33 and Toc159 constructs encoding Nub and Cub fusions to an *Arabidopsis* homolog of yeast Pex11 were engineered. Five PEX11 homologs were identified in *Arabidopsis* (atPEX11a-e), all representing peroxisomal membrane proteins involved in peroxisome proliferation (Lingard and Trelease, 2006; Orth *et al.*, 2007). Two out of these five homologs, atPEX11c and atPEX11e have been demonstrated to partially complement the *S. cerevisiae pex11* null mutant (Erdmann and Blobel, 1995) indicating a conserved function in peroxisome biogenesis and similar interaction patterns (Orth *et al.*, 2007). atPex11e was selected as the model protein and constructs were engineered encoding Nub-Pex11e and Pex11e-HA-Cub-GFP (Fig.8A) in order to test for atPex11e self-interaction in our newly developed plant split-ubiquitin system. Pex11e was not expected to interact with either Toc33 or Toc159. Therefore Pex11e may also be useful as a negative control for Toc GTPase interactions. Co-expression of Nub-Pex11e and Pex11e-HA-Cub-GFP in isolated tobacco protoplasts gave around 85% reporter GFP cleavage (Fig.8B lane d) indicating interaction whereas co-expression of Pex11e-HA-Cub-GFP with either Nub alone (Fig.8B lane c) or Nub-Toc159G (Fig.8B lane e) resulted in only 30% and 40% of cleavage. Similarly, co-expression of Nub-Pex11e with Toc33-HA-Cub-GFP resulted in around 45% cleavage of the GFP-reporter (Fig.8B lane f). Thus, the cleavage observed when co-expressing a Toc GTPase with Pex11e is similar to the one observed upon co-expression of Nub and Toc33-HA-Cub-GFP (Fig.8B lane a) and therefore most probably unspecific.

3.4 Toc protein-protein interactions in the protoplast cytosol

To test whether the fusions of the membrane proteins Toc33 and Pex11e insert into membranes the experiments depicted in Fig.8 were repeated with an additional cell fractionation step. Extracts of transformed tobacco protoplasts were centrifuged at 100000xg to separate soluble proteins (Fig.9, S “soluble”) from membrane proteins (Fig.9, P “pellet”). Western blot analysis with α -HA revealed that both the uncleaved and cleaved form of full length Toc33HACub-GFP were predominantly located in the soluble fraction (Fig.9 lanes a,b, S). Only upon co-expression of Nub-Toc159G, a small portion of cleaved Toc33-HA-Cub was detected in the 100'000xg pellet fraction (Fig.9 lane b, P). These data suggest that the C-terminal HA-Cub-GFP fusion prevents insertion of Toc33 into the membrane, and that only upon cleavage of the GFP, Toc33 membrane insertion is possible. Therefore, the interaction we observed between Nub-Toc159G and full length Toc33-HA-Cub-GFP in the plant split-ubiquitin system most likely occurs in the protoplast cytosol. The uncleaved and cleaved fusion of the second

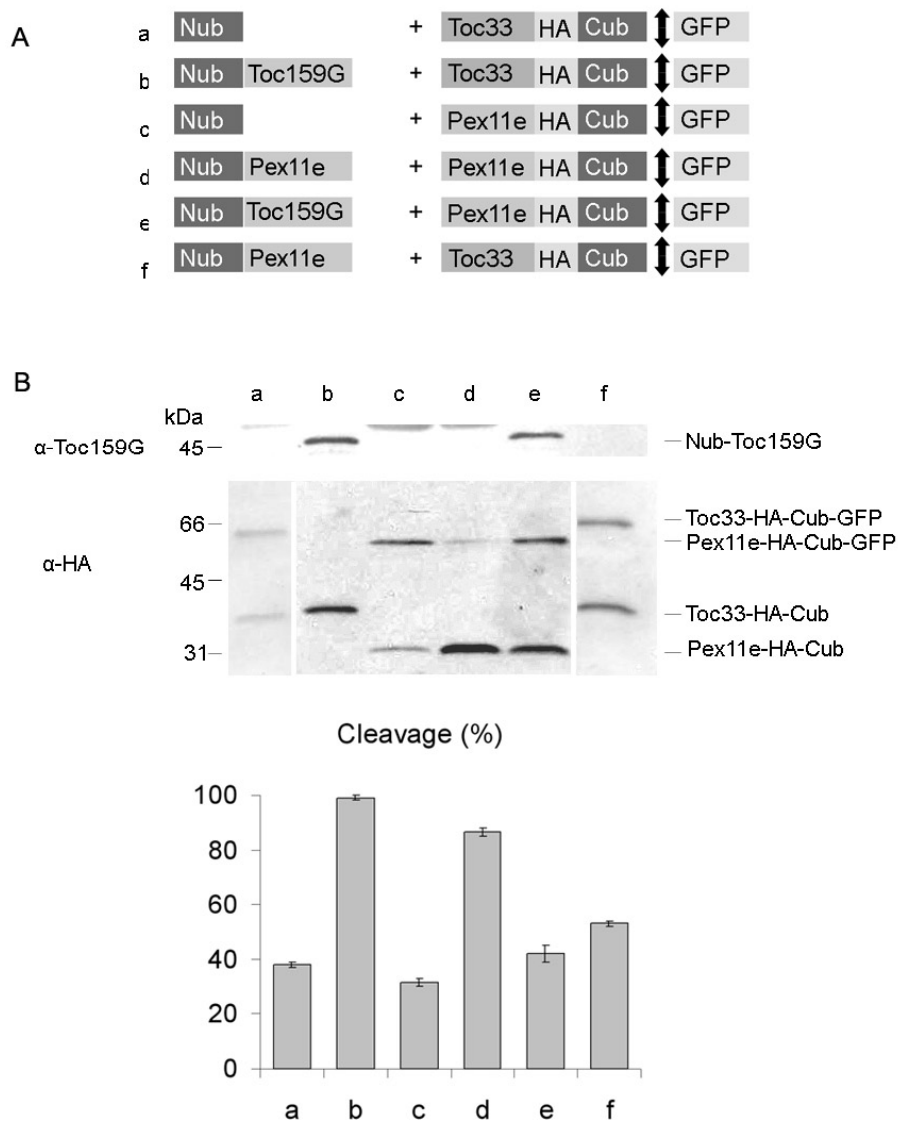


Fig.8 Toc and Pex protein interactions in the plant split-ubiquitin system

A. Tobacco protoplasts were co-transformed with Nub and Cub constructs. B. Total proteins were extracted and analysed by Western blotting using antibodies raised against Toc159G to check for the presence of Nub-Toc159G or anti-HA for the Cub-fusion proteins. C. Interacting protein pairs result in almost complete cleavage of GFP [NubToc159G and Toc33-HA-Cub-GFP (b), NubPex11e and Pex11e-HA-Cub-GFP (d)], non-interacting protein pairs result in partial background cleavage of the reporter gene [Nub and Toc33-HA-Cub-GFP (a), Nub and Pex11e-HA-Cub-GFP (c), NubToc159G and Pex11e-HA-Cub-GFP (e), NubPex11e and Toc33-HA-Cub-GFP (f)].

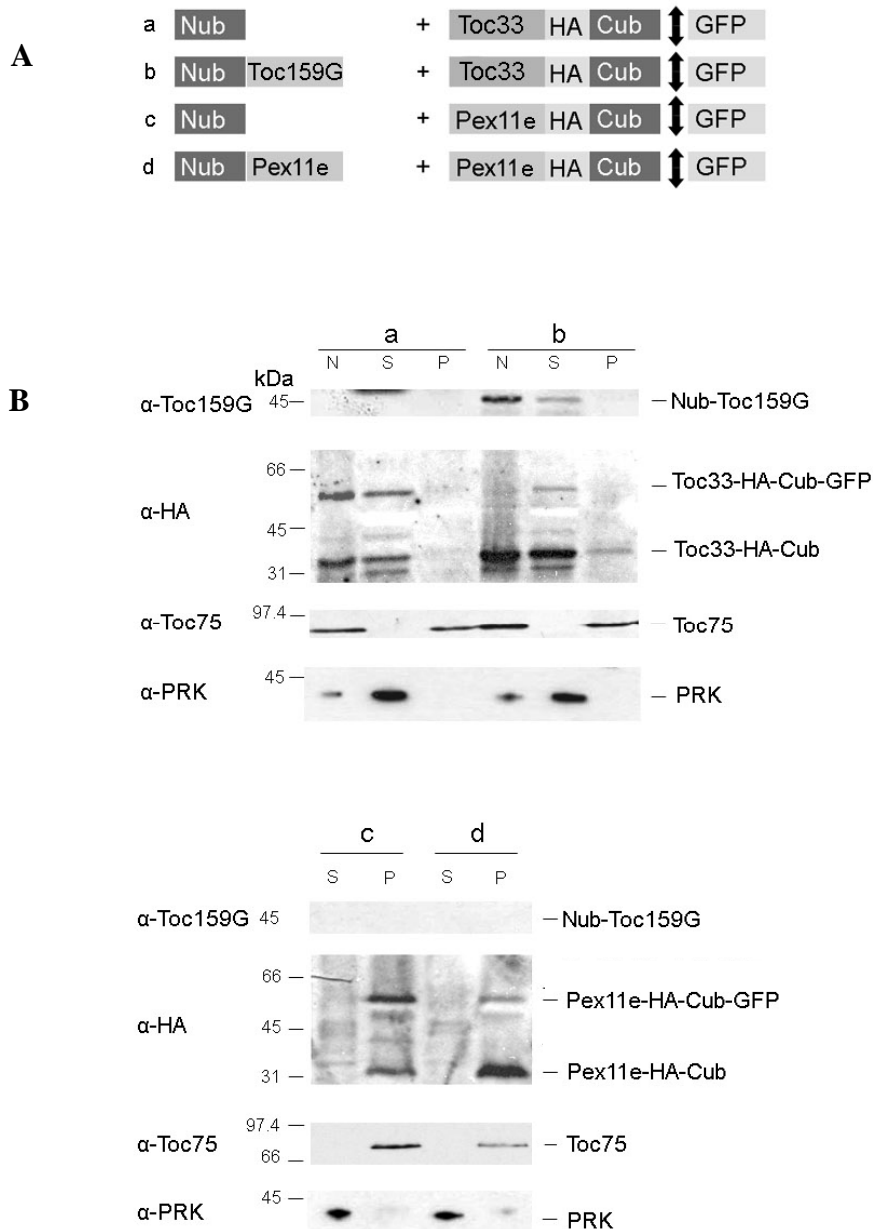


Fig.9 Membrane association of full length Toc33 and Pex11e in plant split-ubiquitin assays.

A. Tobacco protoplasts were co-transformed with Nub and Cub constructs. B. Protoplasts were lysed and separated into soluble and pellet fraction by centrifugation at 100.000 x g for 1h. Equal amounts of protein of non-treated protoplasts (N), soluble (S) and pellet (P) fraction were analysed by immunoblotting using antibodies against Toc159G, the HA epitope, Toc75 and phosphoribulokinase (PRK). Toc75 and phosphoribulokinase served as membrane and soluble marker, respectively. Co-expression of Nub-Toc159G with full length Toc33-HA-Cub-GFP (b) or Nub-Pex11e with Pex11e-HA-Cub-GFP (d) results increased cleavage. Uncleaved and cleaved form of the Toc33-HA-Cub fusion (a, b) are mainly present in the soluble fraction suggesting inhibition of Toc33 membrane insertion by the C-terminal fusion part. In marked contrast, uncleaved and cleaved fusions of the integral membrane protein Pex11e (c, d) are both located in the pellet fraction.

membrane protein tested, Pex11e, were mainly located in the insoluble fraction (Fig.9 lanes c,d). This indicates that in contrast to Toc33, membrane insertion of Pex11e is not affected by the C-terminal fusion part. Thus, in the plant split-ubiquitin system it is feasible to study interactions of membrane associated Pex11e.

4 Discussion

4.1 Comparison of the plant split-ubiquitin system with other available *in vivo* protein-protein interaction assays

In response to the emerging need for *in vivo* protein-protein interaction data several *in vivo* assay systems have been developed in the recent past. Most of these are based on protein fragment complementation and have been demonstrated to be applicable to plant cells as well. Examples are bimolecular fluorescence complementation (BiFC) (Bhat *et al.*, 2006; Hu *et al.*, 2002; Kerppola, 2008), fluorescence resonance energy transfer (FRET) (Miyawaki *et al.*, 1997), split-mDHFR (murine dihydrofolate reductase) (Subramaniam *et al.*, 2001) or split-luciferase (Fujikawa and Kato, 2007) protein complementation assays and the protoplast two-hybrid (P2H) system (Ehlert *et al.*, 2006).

The availability of different protein-protein interaction assay systems is advantageous as each has his inherent limitations and might not be applicable to all pairs of interacting proteins. Membrane receptor GTPases at the chloroplast outer surface are presumed to undergo short-lived and dynamic interactions with chloroplast precursor proteins and among themselves. For their study *in vivo*, it is necessary to use a system that allows the analysis of transient protein-protein interaction at the cytosolic face of organelles. For that purpose we adapted the yeast split-ubiquitin system possessing the desired characteristics for plant cells and demonstrate the interaction between Toc159 and Toc33 as well as Pex11self-interaction.

4.2 Toc GTPase heterodimerisation *in vivo*

In many *in vitro* studies homo- or heterodimerisation of the G-domains of Toc33 and Toc159 has been observed (Bauer *et al.*, 2002; Hiltbrunner *et al.*, 2001b; Reddick, *et al.*, 2007; Smith *et al.*, 2002; Sun *et al.*, 2002; Weibel *et al.*, 2003; Yeh *et al.*, 2007). Working with recombinant or *in vitro* translated proteins, stable homodimers of Toc159 and Toc33 are much more easily obtained than heterodimers leading to the assumption

that Toc159 and Toc33 do not form stable heterodimers or that heterodimers are formed only transiently *in vivo* (Li *et al.*, 2007).

A short-lived interaction among Toc159 and Toc33 fits well with a model of a highly dynamic, nucleotide dependent Toc GTPase cycle in chloroplast protein import. In our lab we have demonstrated for the first time the heterodimerisation among the Toc GTPases Toc159 and Toc33 in three different *in vivo* interaction assay systems: the yeast two-hybrid system (previous work done by Dr Birgit Agne), the yeast split-ubiquitin system and the plant split-ubiquitin system, the latter especially developed for this purpose. Surprisingly and in contrast to *in vitro* studies mentioned above, Dr Birgit Agne could not observe Toc33G-Toc33G or Toc159G-Toc159G homodimerisation in the yeast two-hybrid system. This is why we haven't studied homodimerisation by split-ubiquitin yet. However, homodimerisation in plant split ubiquitin system, will have to be tested in the future, moreover, our results supply evidence that heterodimerisation indeed occurs *in vivo*.

The yeast two-hybrid interaction is an excellent tool to screen for mutations altering the binding properties of Toc159G for Toc33G and vice versa. The resulting mutations could be subsequently assayed *in planta* by split-ubiquitin. By cell-fractionation via ultracentrifugation we demonstrated that, the interaction observed in the plant split-ubiquitin system among Toc159G and full-length Toc33, occurred in the cytosol and not at the chloroplast membrane. Apparently, the C-terminal GFP fusion interfered with Toc33 membrane insertion. The data suggest that the C-terminus of Toc33 must be freely accessible for membrane insertion. This is supported by the insertion of a small portion of Toc33-HA-Cub after cleavage. By switching orientation of the fusion parts and including full length Toc159 the system is currently improved for the study of Toc GTPase interactions at the chloroplast surface.

4.3 Interaction with precursor proteins

In the yeast split-ubiquitin system, we could demonstrate the interaction between Toc159G and the precursor protein of the small sub-unit of RuBisCO, preat1b. Surprisingly, Toc159G seems to interact also with the mature protein. But the cleavage observed is quite low, less than 20% (Fig.5), compared to the 70% of cleavage observed with the presence of Nub-Toc159G and Toc33G-Cub fusion proteins (Fig3). This very weak cleavage could be due to very short-lived interaction or a problem of mistargeting into mitochondria. Indeed in organisms that do not have chloroplasts such as yeast cells, chloroplastic proteins like RuBisCO (Rudhe *et al.*, 2002) and ferredoxin (Hirohashi *et al.*, 2001) can be mistargeted into mitochondria (Hurt *et al.*, 1986). To test this hypothesis, we could test in the near future the interaction between Toc proteins and preat1b in the plant split-ubiquitin system.

4.4 atPex11e self-interaction

By means of immunoprecipitation the mammalian PEX11 isoforms PEX11 α and PEX11 β have been shown to homo- but not heterodimerise (Li and Gould, 2003). No such data is available for the *Arabidopsis* PEX11 family comprising five members (named by letters a to e). The plant split-ubiquitin experiments carried out in this study show *in vivo* homodimerisation of atPex11e. In the case of atPex11e (in contrast to Toc33-HA-Cub-GFP) the C-terminal Cub-GFP fusion was almost entirely present in the membrane pellet after centrifugation at 100'000xg (Fig.9). The C-terminal GFP did therefore not appear to interfere with membrane insertion. This result demonstrates that plant split-ubiquitin may indeed be applied to determine and analyze interactions between integral membrane proteins. Specifically the plant split-ubiquitin system may be used to test homo- or heterodimerisation among the *Arabidopsis* PEX11 homologs *in vivo*.

4.5 Future modification and improvement of the plant split-ubiquitin system

Some improvement of the plant split-ubiquitin system will need to be done, particularly concerning the reduction of background cleavage and the reporter system.

A higher level of background cleavage of the Toc33-Cub-fusion was observed in the absence of an interacting protein in the plant than in the yeast split-ubiquitin assays. This is not due to a higher rate of spontaneous *in vivo* association of the Nub and Cub fragments in plants as the same level of background cleavage was observed when the Cub-fusion proteins were expressed in the absence of free Nub or Nub-fusion proteins (Fig.7). Possible explanations are that substrate recognition by plant UBPs is less dependent on a complete ubiquitin moiety or that the overall activity of UBPs in plants is higher than in yeast.

The latter appears likely as about twice as many DUBs have been identified in *Arabidopsis thaliana* compared to *S. cerevisiae* (Yang *et al.*, 2007). For a further development of the plant split-ubiquitin system reduction of the background-cleavage would be desirable. Potentially, this could be achieved by performing the assays in protoplasts derived from mutant plants in which selected, nonessential UBPs are knocked-out. Furthermore, optimization of assay duration or other parameters could improve the ratio between unspecific background and interaction induced cleavage.

The yeast split-ubiquitin system is available with different reporter molecules. For the study of interactions between membrane proteins a system with a transcription factor reporter was created (Stagljar *et al.*, 1998). In this system the Cub-reporter is fused to a membrane-associated protein and transcriptional activation of a secondary reporter only

occurs upon cleavage and liberation of the primary reporter (transcription factor). A similar system is conceivable for the plant split-ubiquitin system combining it for example with the transcription activation system described by Moore (Moore *et al.*, 1998): using the chimeric transcription factor LhG4 as cleavable reporter fused to a plant membrane protein and protoplasts of transgenic plants with an pOp- β -glucuronidase (GUS) insertion would allow for quantification of interaction via GUS activity assays. This system would be similar to the protoplast two-hybrid system with the advantage that membrane protein interactions could be monitored. A problem in obtaining quantitative data from transient expression experiments in protoplasts is the variability of transformation rates. As in the protoplast two-hybrid system, variant protoplast transformation rates could be corrected by co-expression of the GUS compatible reporter NAN (synthetic neuraminidase gene) (Ehlert *et al.* 2006).

Thus, this work presented in this manuscript provides an ideal starting point for the optimization and further development of split-ubiquitin system to exploit its full potential in plant systems.

(The results presented on this first chapter have been accepted for publication: Rahim *et al.*, 2008(?))

Chapter 2

Isolation of atToc33 and associated complexes from *Arabidopsis*.

1 Presentation of the system

The TAP (tandem affinity purification) tag (Rigaut *et al.*, 1999) is composed of two IgG binding domains of *Staphylococcus aureus* Protein A (ProtA), and a calmodulin binding peptide (CBP) separated by a TEV (Tobacco Etch Virus) protease cleavage site (Fig.1B). These two tags are designed to isolate and purify fusion proteins under native conditions. In this thesis, a modified TAP tag (TAPi) lacking a plant nuclear localization signal initially present in the CBP domain (Rohila *et al.*, 2004) has been employed.

The method consists of two sequential purification steps (Fig.1B). First, the ProtA of the fusion protein is bound to IgG coated-beads. The fusion protein is eluted under native conditions using TEV protease. Second, the eluted protein is incubated with a calmodulin matrix. Elution is performed under native conditions using EGTA.

Alternatively to TEV protease cleavage, protein complexes can be eluted from IgG beads with glycine at pH3. This acidic elution elutes the entire TAP-tag-fusion protein and complexes from the IgG beads.

2 Generation of transgenic plants

The cDNAs of atToc33 and the G domain of atToc33 (Toc33G, aa 1-265) were fused to the coding sequence of the N-terminal TAPi tag (NTAPi) (Fig.1A) and ligated into a binary vector (pCAMBIA) containing the cauliflower mosaic virus 35S promoter, the nopaline synthase terminator and a phosphinotricin (BASTA)-resistance gene for selection of the transformants. Wild type (Col0) and atToc33 knockout mutant *ppi1* plants were transformed with these two constructs using the floral dip method (Clough and Bent, 1998) (generation T0). This resulted in the following lines: Col0 NTAPiToc33, Col0 NTAPiToc33G, *ppi1* NTAPiToc33 and *ppi1* NTAPiToc33G.

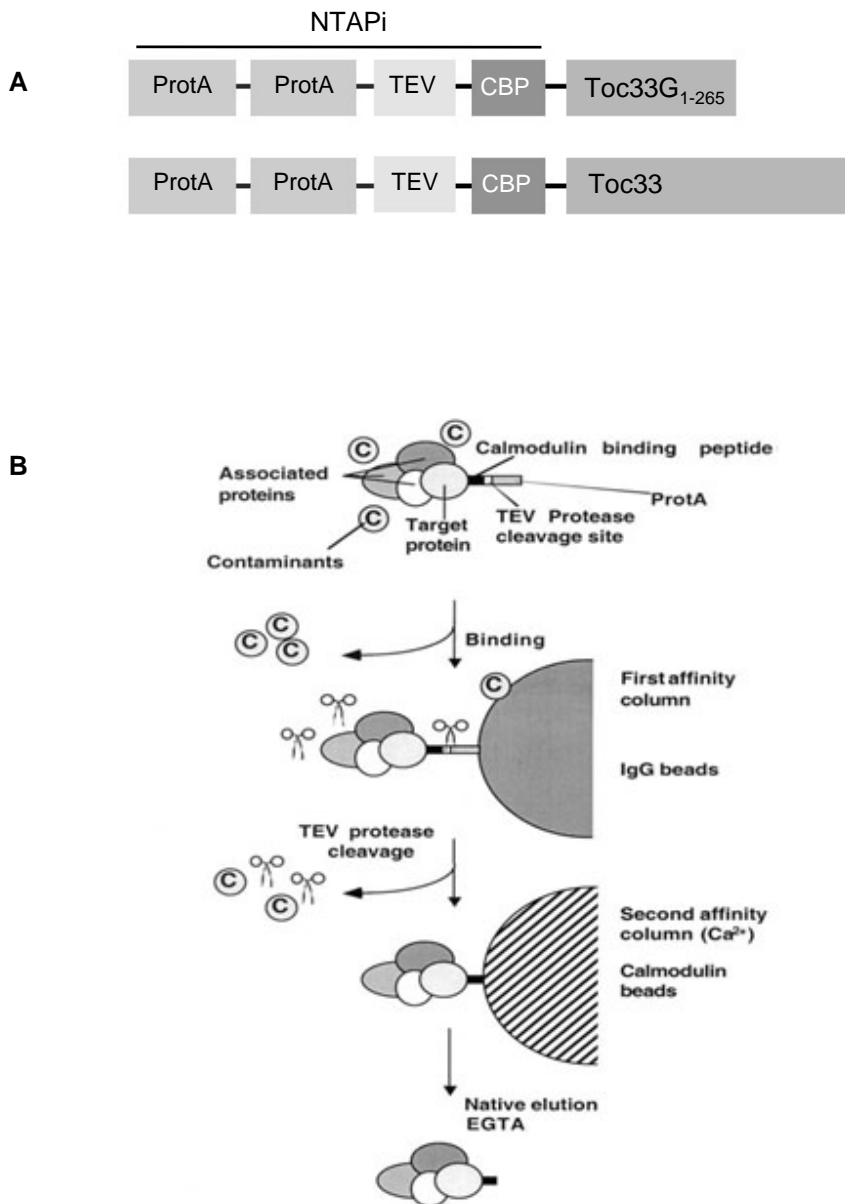


Fig.1 Structure of the TAP tag expression cassette and overview of the different TAP purification steps (from Puig *et al.*, 2001)

A. Schematic representation of the two NTAPi-Toc33/Toc33G constructs.

B. Overview of the TAP protocol. During the first step, TAP-tagged proteins are sequestered by IgG sepharose and released by TEV-protease cleavage. TEV protease-cleaved proteins are then bound to a Calmodulin matrix and eluted using EGTA.

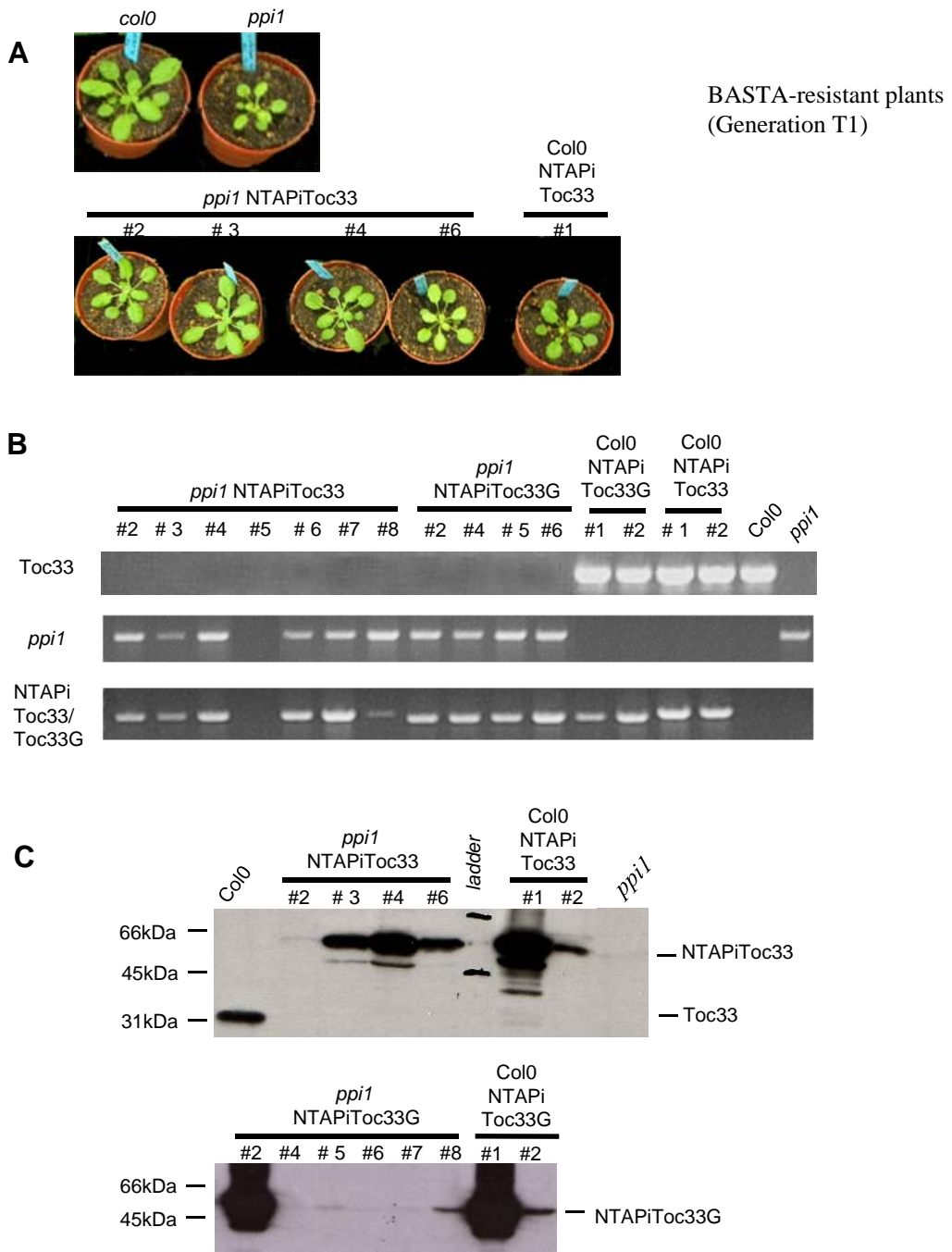


Fig. 2 Analysis of transgenic plants expressing NTAPi-Toc33 and NTAPi-Toc33G.

A. BASTA resistant plants were selected and DNA was extracted from 4 weeks-old-plant leaves. **B.** PCR was performed with primers specific for the T-DNA insertion in TOC33 (“*ppi1*”), as well as for the transgenes NTAPi-Toc33 and NTAPi-Toc33G (“NTAPiToc33/Toc33G”). **C.** Tap-tagged protein expression was monitored by Western blotting using specific antibodies raised against Toc33. Each lane corresponds to a different plant line. 25µg of protein were applied per lane.

Wild type Col0 and *ppi1* plants were used as controls.

Phosphinotricin resistant seedlings (generation T1) (Fig.2A) were screened by PCR to detect both the *ppi1* T-DNA insertion (Jarvis *et al.*, 1998) and the transgene (Fig.2B). A Western blot using α -atToc33 antibodies was also performed on total proteins extract to check the expression levels of the TAP-tagged Toc33 proteins (Fig.2C). This immunoblot produced a signal at the expected sizes: 54kDa and 49kDa for NTAPi-Toc33 and NTAPi-Toc33G respectively. The strong signal observed in some lanes (*ppi1* NTAPi-Toc33G#2, Col0 NTAPi-Toc33G#1, Col0 NTAPi-Toc33#1) suggests a very high expression level of the fusion protein. T1 generation plants expressing NTAPi-Toc33 or NTAPi-Toc33G according to these Western blots were selected and grown to produce seeds. Resulting T2 phosphinotricin-resistant lines were grown on soil under short day conditions to perform a small-scale purification of the TAP tag complex.

3 Characteristics of plants expressing NTAPi-Toc33/Toc33G fusion proteins

3.1 Phenotype and chlorophyll measurements

Plants grown on soil that expressed NTAPi-Toc33 or NTAPi-Toc33G showed different phenotypes (Fig.3B). *ppi1* NTAPi-Toc33G plants were much paler than Col0 NTAPi-Toc33G plants. *ppi1* NTAPi-Toc33 plants were slightly paler than Col0 NTAPi-Toc33 plants but had a stronger green phenotype compared to untransformed *ppi1* plants. This phenotype was also observed on plants that were grown in vitro (Fig.3A).

Chlorophyll was measured on the four different transgenic lines grown on plates (Fig.3C) using Col0 and *ppi1* plants as controls (*ppi1* mutant plants contain reduced levels of chlorophyll at early developmental stages (Jarvis *et al.*, 1998)). These measurements revealed similar chlorophyll amounts in Col0, Col0 NTAPi-Toc33 and Col0 NTAPi-Toc33G plants. The chlorophyll amount in *ppi1* NTAPi-Toc33G plants is similar to that found in *ppi1* plants. In *ppi1* NTAPi-Toc33 plants, chlorophyll is slightly weak compared to Col0 NTAPi-Toc33 plants, but definitely higher than *ppi1* plants. These results suggested that NTAPi-Toc33 complemented the *ppi1* phenotype and defect on chlorophyll levels, whereas NTAPi-Toc33G did not.

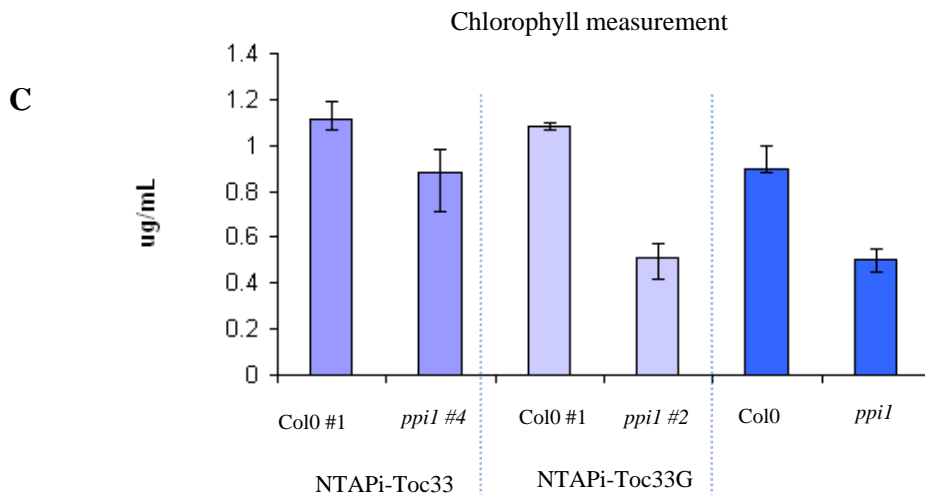
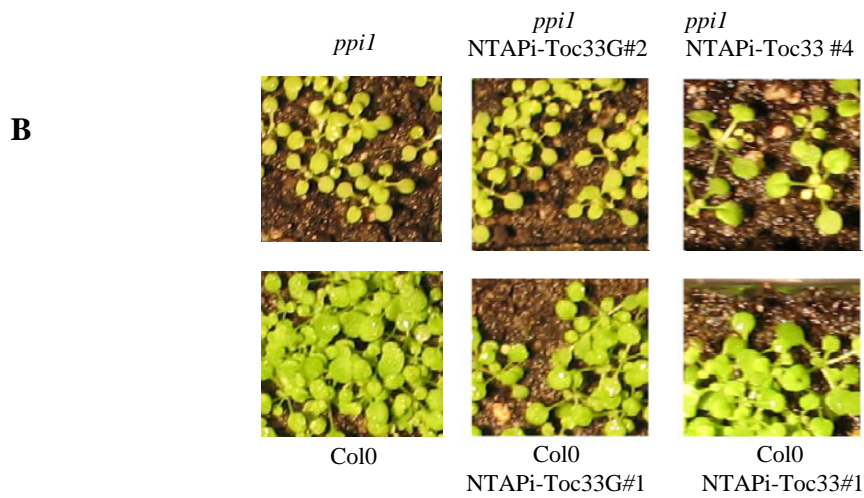
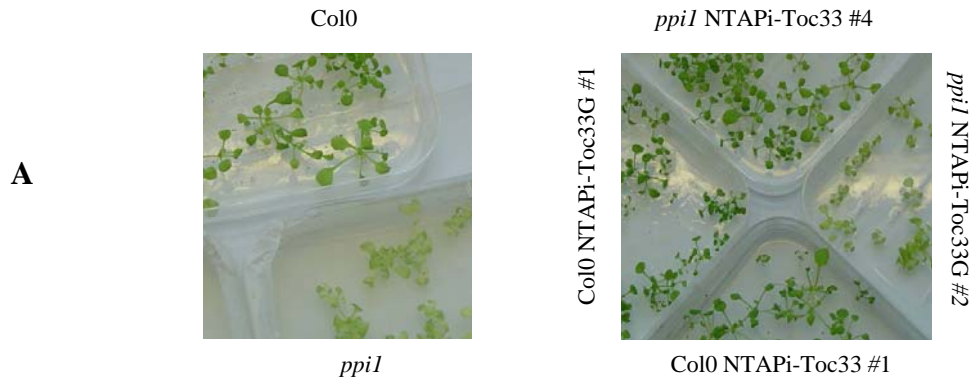


Fig.3 : Phenotype of plants expressing TAP tagged Toc33

Pictures of 3 weeks old plants growing on plate (A) or on soil (B) under short day conditions. Chlorophyll measurement of plants expressing TAP tagged Toc33 grown *in vitro* (C) .

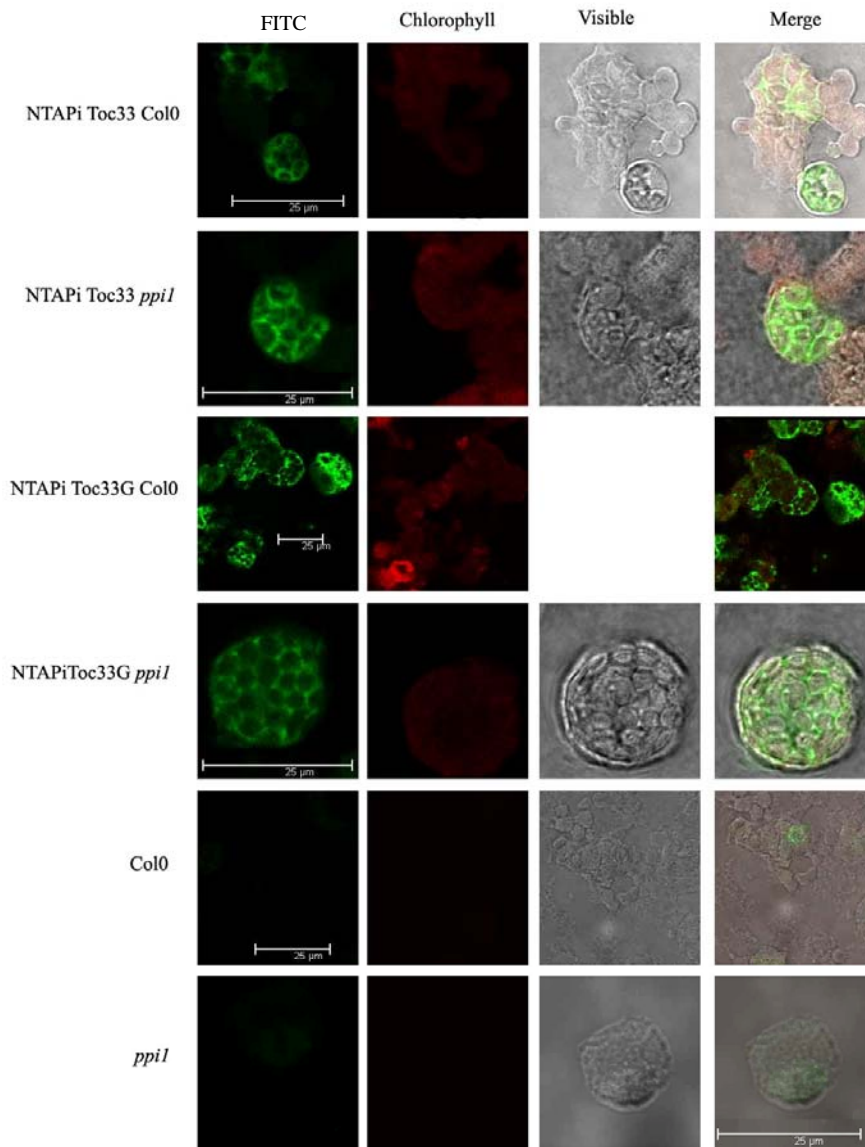


Fig.4: Immunolocalisation of TAP-tagged Toc33 protein using antibodies raised against atToc33. Protoplasts were fixed and probed with anti-toc33 antibodies. Secondary FITC-coupled antibodies were used for visualisation. The detection of the chromophore fluorescence (FITC) by confocal microscopy, chlorophyll auto-fluorescence, bright-field microscopic images (visible) and merge (from bright-field and fluorescent images) are shown.

3.2 Localisation of the fusion proteins

The *ppi1* phenotype is complemented by the NTAPi-Toc33 fusion protein but not by NTAPi-Toc33G. This could be explained by the fact that NTAPi-Toc33 unlike NTAPi-Toc33G is inserted to the chloroplast outer membrane. To test whether NTAPi-Toc33 is localized in the chloroplast outer membrane, fluorescence immunolocalisation of the fusion proteins NTAPi-Toc33 and NTAPi-Toc33G was carried out. Protoplasts were isolated, fixed and probed with α -atToc33 (Fig.4) and α -IgG antibodies (data not shown). Fluorescence (FITC) was detected by confocal microscopy. Probing with anti-atToc33 gave an identical cytosolic fluorescence pattern for both NTAPi-Toc33 and NTAPi-Toc33G with no apparent difference between the two.

In parallel, high speed (100'000xg) centrifugation of total plant protein extracts was done to separate soluble (SN) and membrane fractions (P) (Fig.5). Both fractions were analysed by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with antibodies raised against components of the Toc-complex: α -atToc75, α -atToc159G, α -atToc159A and α -atToc33.

Toc75, the Toc protein channel, was indeed located in the membrane fraction in all samples tested. Toc159 was mainly present in the insoluble fraction. Some Toc159 was also detected in the soluble fraction and seemed to be more abundant in untransformed *ppi1* plants and of plants expressing Col0 NTAPi-Toc33G. Surprisingly, in Col0 plants expressing NTAPi-Toc33 or NTAPi-Toc33G (Fig.5A left), both NTAPi-Toc33 and NTAPi-Toc33G were detected in the soluble and insoluble fractions. In *ppi1* plants expressing NTAPi-Toc33 (Fig.5 right), NTAPi-Toc33 was detected in the membrane fraction only. In *ppi1* NTAPi-Toc33G plants, NTAPi-Toc33G was present in both soluble and pellet fractions, but mainly in the soluble one.

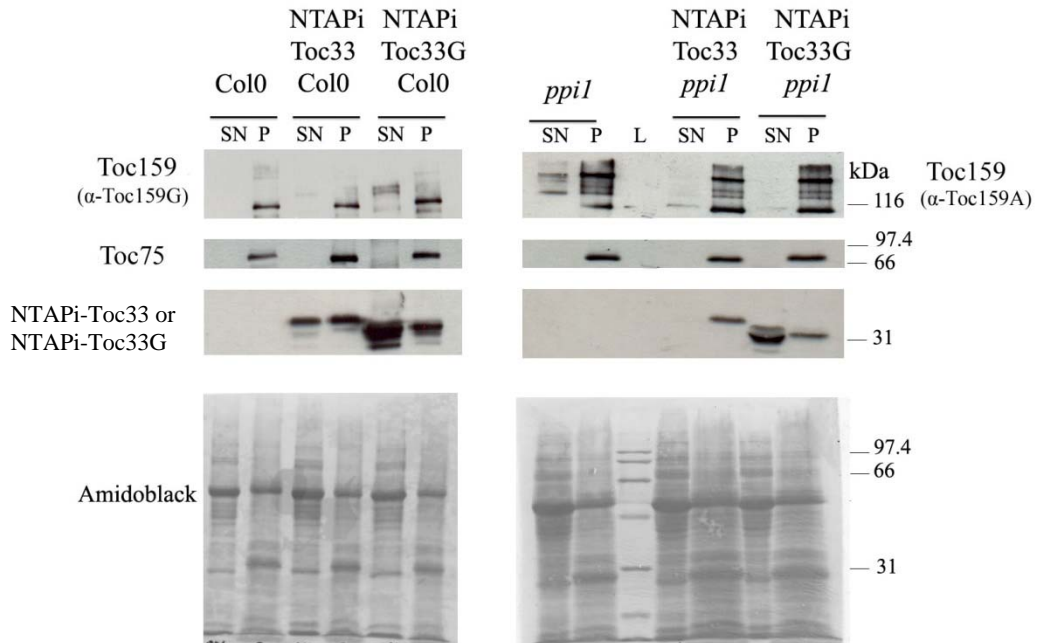


Fig.5: Localisation of Tap-Tagged Toc33 proteins and complexes by ultracentrifugation

Plant extracts were centrifuged at 100'000g for 1h. Supernatant (SN) and pellet (P) were collected separately and analysed by SDS-PAGE followed by Western blotting with antibodies raised against atToc75, atToc132 and atToc159A or atToc159G. Protein sizes are indicated in kDa (L: protein molecular weight marker).

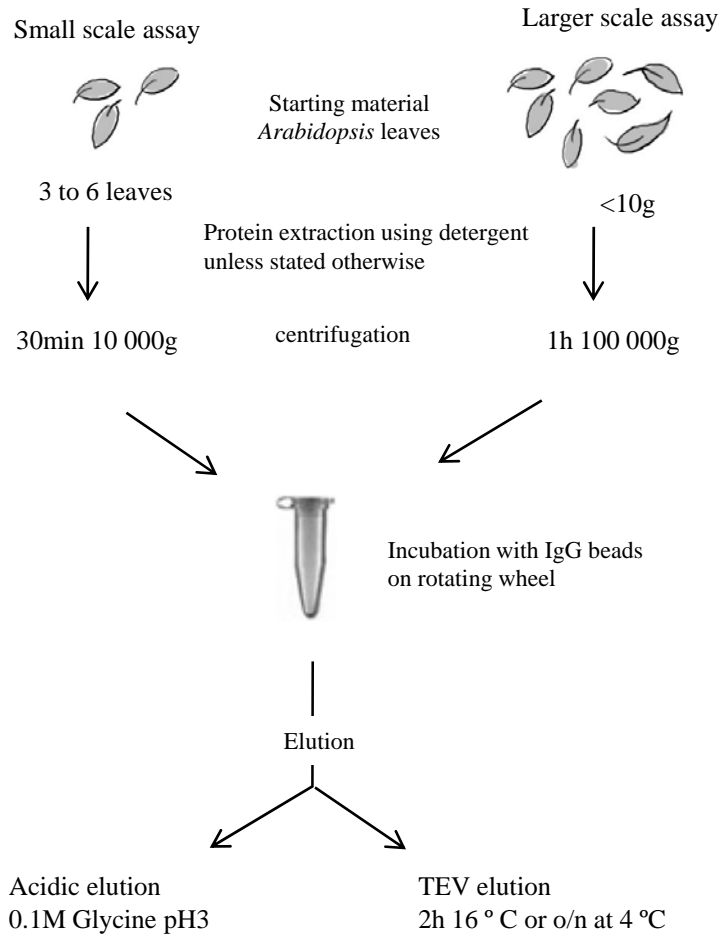


Fig.6: Overview of the TAP procedure.

A small scale TAP purification was carried out with 1mg of total protein (3-6 leaves). A medium scale TAP purification was carried out with 50 mg of total proteins (10g of leaves). The protein extract was cleared by centrifugation and incubated with IgG coated beads. The protein complexes bound to the beads were washed and eluted with 0.1M glycine pH3 or with TEV protease. Unless specified, all steps were done at 4 °C.

4 Purification of NTAPi-Toc33 and associated proteins

4.1 Small scale purification assays

A small scale purification experiment from a few leaves of Col0 and *ppi1* plants expressing either NTAPi-Toc33G or NTAPi-Toc33 was performed as schematized in Fig.6. Proteins were extracted, according to Rensink *et al*, 1998, in a buffer that contained 0.5% v/v Triton X-100 to extract membrane proteins. Proteins were incubated with IgG-coated beads, washed with buffer, and eluted with glycine pH3. In order to determine the presence of TAP-tagged proteins at each purification step, protein extracts, flow-through, eluates and beads were subjected to Western blot analysis using α -atToc33 antibodies (Fig.7). Both NTAPi-Toc33G and NTAPi-Toc33 proteins showed binding to IgG beads. Protein recovery from IgG beads by acidic elution appeared to be efficient for both TAP fusion proteins.

The same experiment was also subjected to immunoblot analysis using antibodies raised against atToc75, atToc132 and atToc159G to detect whether these Toc proteins co-purified with the TAP fusion proteins (Fig.7).

The NTAPi-Toc33 protein co-purified with atToc75, atToc132 and atToc159G both from Col0 and *ppi1* backgrounds. atToc159 was also detected in Col0 NTAPi-Toc33G eluates (Fig.7). No other Toc protein tested co-purified with Col0/*ppi1* NTAPi-Toc33G eluates.

Col0 NTAPi-Toc33G immunoblots always gave strong signals in the eluates fraction using α -atToc75 and α -atToc132. These were considered unspecific because these signals were present in all blotting experiments, no matter what antibodies were used. This is probably due to the high expression level of NTAPi-Toc33G and the high reactivity of ProtA tag with the antibodies.

4.2 Elution of NTAPi-Toc33 and NTAPi-Toc33G by TEV protease

The same small scale experiment was repeated. This time proteins were incubated with IgG-coated beads, washed and then treated with TEV protease (Fig.6). Aliquots of each purification step were subjected to Western blot analysis.

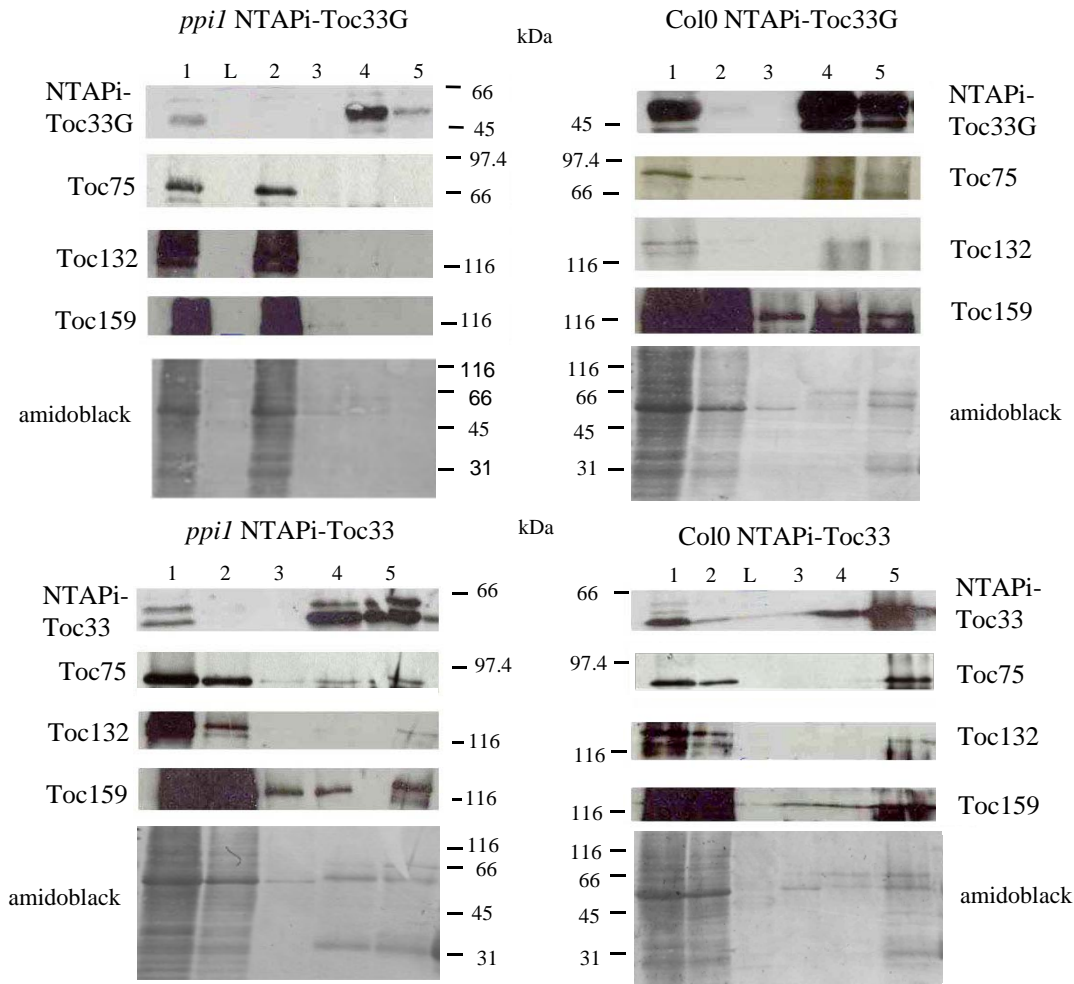


Fig.7 Western blot analysis of the Toc proteins associated with NTAPI-Toc33

Total protein (900 μ g) (1) of 6 weeks-old plants was incubated with IgG coated beads during 2 1/2 hours, the unbound proteins (2) were discarded. The beads were then washed 3 times (3) before elution. The protein bound to the beads were eluted with glycine pH3 (4), the beads and remaining proteins after elution were extracted in SDS-PAGE sample buffer (5). Samples were analysed by SDS-PAGE followed by Western blotting with antibodies raised against atToc33, atToc75, atToc132 and atToc159G. Protein sizes are indicated in kDa. (L: empty lane).

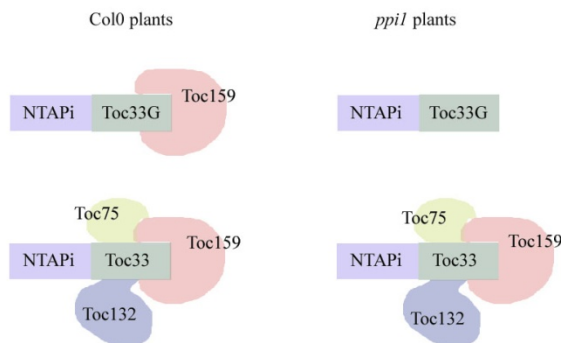


Fig.8: NTAPI-Toc33/Toc33G and associated proteins purified from Col0 or ppil plants.

Proteins were extracted and purified as described before. Western blots were performed with antibodies raised against Toc33, Toc159, Toc132 and Toc75 (Fig.7). The results are resumed in this figure.

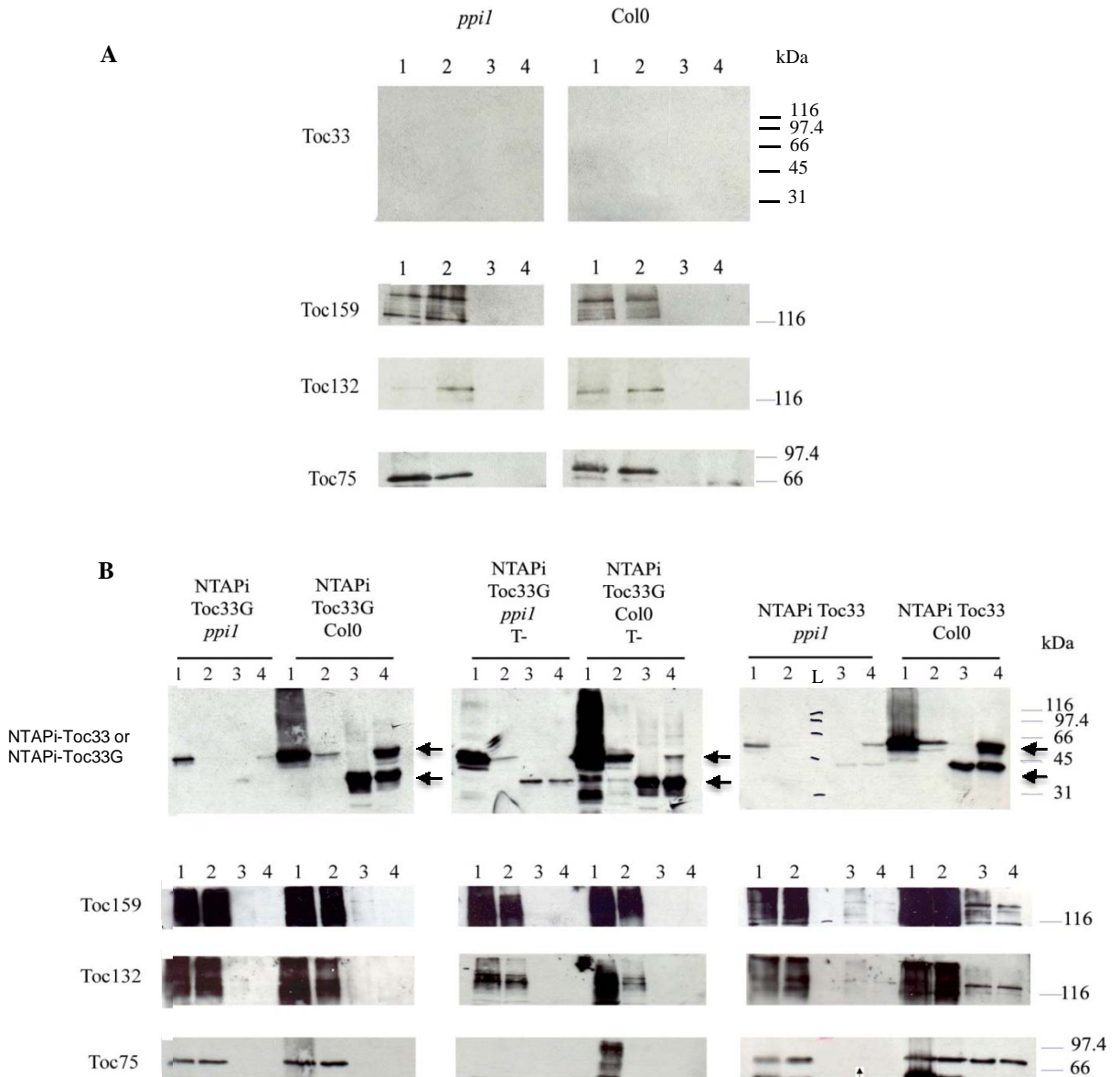


Fig.9: Purification of Tap-Tagged Toc33 protein complexes and elution by TEV cleavage.

Non transformed plants (A) and plants expressing the TAP-tag fusion protein (B) were used for this experiment. 3g of plants were ground resulting in 6mg of total protein that were bound to the beads (1). Different steps of the purification were loaded on a 12% SDS gel (Of each fraction 1/5 of total volume were applied per lane): unbound proteins (2). TEV eluates (3). Boiled beads after TEV elution (4). Presence of Tap-tagged Toc33 was monitored using antibodies raised against Toc33. The purification experiment was performed with Triton or without any detergent (T-). Presence of the different Toc proteins in the eluate were checked with antibodies raised against atToc75, atToc132 and atToc159G. Protein sizes are indicated in kDa. (L: protein molecular weight marker. Upper arrows correspond to NTAPi-Toc33 or NTAPi-Toc33G, Lower arrow correspond to CBP-Toc33 or CBP-Toc33G).

Immunoblot using α -atToc33 antibodies showed shift in molecular mass of 15kDa in the TEV eluates of NTAPi-Toc33G and NTAPi-Toc33 fusion proteins when compared to the acidic elution. This demonstrated that elution from IgG beads using TEV protease is working (Fig.9B). atToc75, atToc132 and atToc159 were also present in NTAPi-Toc33 eluates although the signals corresponding of these three Toc proteins were much weaker in *ppil* NTAPi-Toc33 samples (with a very faint signal for Toc75, Fig.9B arrow). In this experiment, atToc75, atToc132 and atToc159 did not co-purify with Col0/*ppil* NTAPi-Toc33G proteins.

In these “small scale” assays, proteins were extracted in a buffer that contained Triton X-100 detergent to solubilise membrane proteins. NTAPi-Toc33G was expected to be cytosolic, but we previously showed, by ultracentrifugation, that NTAPi-Toc33G was present in both soluble and insoluble fractions (Fig.5). Col0 and *ppil* plants expressing NTAPi-Toc33G were subjected to protein extraction using a Triton-free (T-) extraction buffer. Proteins were isolated as described above. Immunoblot analysis using α -atToc33 antibodies showed that the TAP-fusion protein was bound to and recovered from the IgG beads in the absence of detergent (Fig.9B NTAPi-Toc33G Col0/*ppil* T-).

As a negative control, total proteins from untransformed plants, Col0 and *ppil*, were extracted and subjected to TAP tag purification and Western blot analysis (Fig.9A). As expected neither Toc75, nor Toc132 or Toc159 were present in the eluates.

5 Mass spectrometric protein identification

To attempt identification of NTAPi-Toc33 associated proteins, a larger scale TAP purification was performed (Fig.6). Proteins were solubilised from 10g of leaves using the Triton X-100 buffer as described above. IgG affinity chromatography was carried out and the TEV protease eluates were separated by SDS-PAGE and visualized using SYPRO Ruby staining (Fig.10A). Two bands corresponding to the IgG heavy and light chains, at around 50 and 25kda respectively, appeared in the eluates. In addition to these two bands, several other bands were observed (Fig.10A). These bands were excised (Fig.10B) and analysed by mass spectrometry after in gel digestion of the proteins with trypsin. In Col0 and *ppil* plants bands of the same mass as the ones chosen in TAP tag eluates were selected as controls (Fig.10B).

Data received from peptide analysis from each band are presented at the end of this manuscript (see Appendix). The peptide profile showed not many differences whether bands were cut from Col0/*ppil* NTAPi-Toc33 or NTAPi-Toc33G plants (Appendix). A selection of these results is presented in Table1.

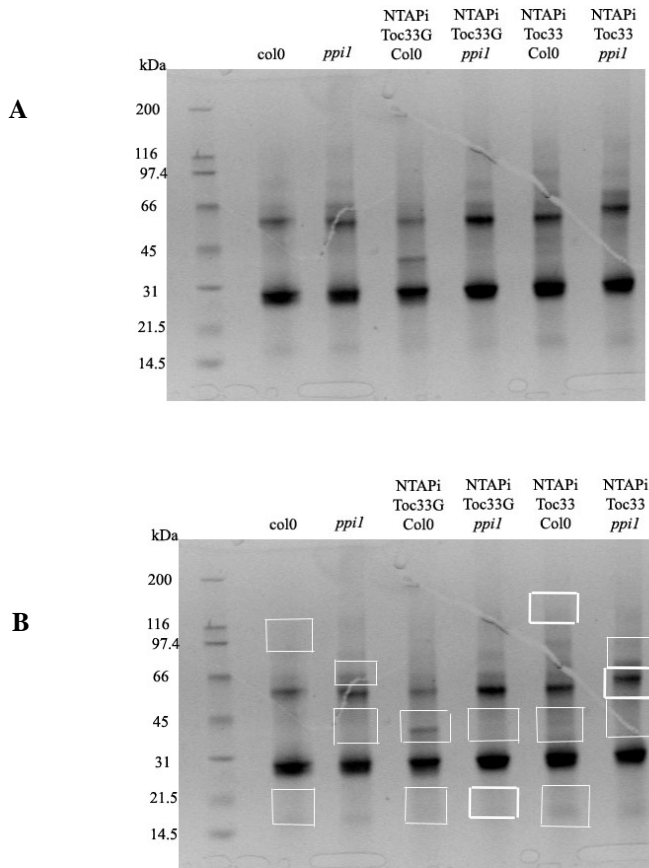


Fig.10 Visualisation of SYPRO Ruby colored gel before trypsin digest

The TEV eluates were analysed by SDS-PAGE followed by SYPRO Ruby staining. Staining is visualized by UV light (A).

Control eluates from Col0 and *ppil* were used as negative controls. Bands exclusively present in plants expressing TAP tag constructs were selected to be trypsin digested (B). The white rectangles indicate areas of the gel excised for mass spectrometric analysis.

Protein description	Predicted localisation	Accession number	Number of peptides
atToc33	Outer envelope membrane	AT1G02280	114
atTic110	Inner envelope membrane	AT1G06950	53
Actin	Cytoskeleton	AT1G49240, AT2G37620, AT5G09810	45
Cpn60A (CHAPERONIN60 ALPHA)	Chloroplast	AT2G28000	40
Cpn60B (CHAPERONIN 60 BETA)	Chloroplast	AT1G55490	29
Hsp93-V	Chloroplast stroma	AT5G50920	50
Ptac16 (PLASTID TRANSCRIPTIONALLY ACTIVE18); similar to catalytic/ coenzyme binding [Arabidopsis thaliana] similar to Tic62 protein [Pisum sativum]	Chloroplast thylakoid membrane	AT3G18890	4
Tubulin	Various compartments, cytoskeleton	AT1G04820	7
EF1 α Elongation factor	Cytoskeleton	AT1G07920	43
Pat1 (PATELLIN 1) Protein targeting and vesicular trafficking	Cytosolic	AT1G72150	13
DnaJ heat shock family protein	Endomembrane	AT3G62600	3
cpHSC70-2	Chloroplast stroma	AT5G49910	46
(Calmodulin 4); calcium ion binding	?	AT1G66410	5
calcium-binding protein, putative	?	AT1G18210	6

Table 1: Mass spectrometry analysis of TEV eluted proteins from NTAPi-Toc33 purification experiments.

Eluates were loaded on a commercial Biorad 4-12% Bis-Tris gel coloured with SYPRO Ruby. Bands were excised and digested in gel with trypsin. Peptides eluted from the gel piece were subjected to mass spectrometric identification.

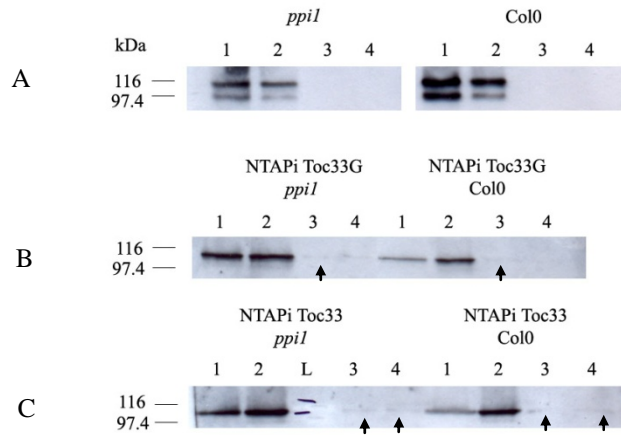


Fig.11: Presence of Tic110 in the Tap-tagged Toc33 complex.

Presence of Tic110 in protein eluates was checked by Western blot using antibodies raised against atTic110 on untransformed Col0 and *ppil* plants (A), plants expressing NTAPi-Toc33G (B) and NTAPi-Toc33 (C). (1: starting material, 2: unbound protein, 3: TEV eluates, 4: remaining proteins left on IgG beads). Protein sizes are indicated in kDa. (L: protein molecular weight marker).

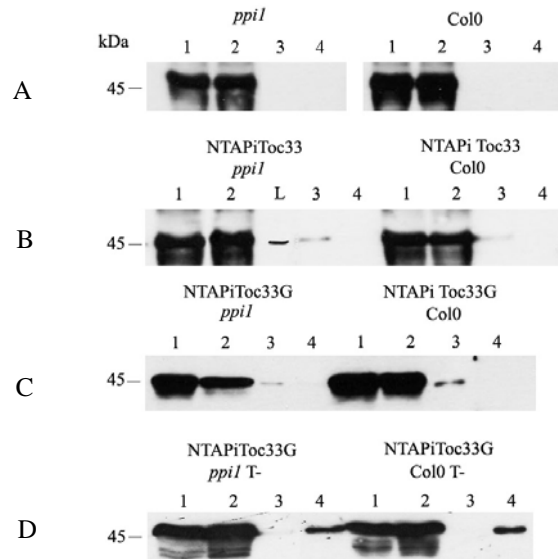


Fig.12: Presence of actin in the Tap-tagged Toc33 complex.

Presence of actin in protein eluates was checked by Western blot using antibodies raised against actin on untransformed Col0 and *ppil* plants (A), plants expressing NTAPi-Toc33 (B) and NTAPi-Toc33G (C) and on plants expressing NTAPi-Toc33G that were extracted without TritonX-100 (T-) (D). (1: starting material, 2: unbound proteins, 3: TEV eluates, 4: remaining proteins left on IgG beads). Protein sizes are indicated in kDa. (L: protein molecular weight marker).

atToc33, atTic110, a protein similar to psTic62, heat shock proteins and chaperones were identified as well as actin, tubulin, elongation factors and calmodulin binding proteins (Table1).

For confirmation of the mass spectrometry results, immunoblots on existing membranes were performed with antibodies raised against atTic110 and actin (Fig.11 and 12) (these membranes correspond to the small scale TAP tag experiments presented in Fig.9).

Immunoblotting using α -atTic110 (Fig.11) failed to detect atTic110 in eluates of control experiment using extracts from Col0 and *ppi1* plants (Fig.11A). A very faint band corresponding to atTic110 was present in NTAPi-Toc33G (Fig.11B) and NTAPi-Toc33 (Fig.11C) eluates.

Immunoblotting using α -actin (Fig.12) showed that actin was not present in the eluates of control experiment using extracts from Col0 and *ppi1* plants (Fig.12A). On the other hand, actin co-purified with NTAPi-Toc33G (Fig.12B) and NTAPi-Toc33 (Fig.12C). In plants expressing either NTAPi-Toc33 or NTAPi-Toc33G that were extracted without detergent prior to IgG affinity chromatography (Fig.12D), actin was not present in the eluates. However, actin could be detected after extraction of the IgG beads with a SDS-containing buffer.

6 Discussion

6.1 Interaction of Toc33 with Toc159 and Toc75 depends on its membrane-association

6.1.1 Complementation of the *ppi1* phenotype and chlorophyll accumulation defect by NTAPi-Toc33

ppi1 NTAPi-Toc33G plants were much paler than *ppi1* NTAPi-Toc33 plants. Chlorophyll measurements (Fig.3) showed that chlorophyll accumulation was defective and as low in *ppi1* NTAPi-Toc33G plants as it is in *ppi1* plants (Jarvis *et al.*, 1998). This result suggests that Toc33 needs to be inserted to chloroplast outer membrane to play its role during import, and therefore only the full length Toc33 having the C-terminal transmembrane sequence can complement the *ppi1* phenotype.

We therefore tested whether NTAPi-Toc33 and NTAPi-Toc33G were inserted into the chloroplast outer membrane. We could not visualise the insertion of the fusion proteins directly by immunolocalisation (Fig.4). This could be explained by the fact that much of NTAPi-Toc33 and NTAPi-Toc33G were soluble and that we could not distinguish the membrane-bound fusion proteins from the cytosolic ones by immunofluorescence. However, after ultracentrifugation of total plant protein extracts, we could discriminate

soluble and membrane-associated fractions of NTAPi-Toc33 and NTAPi-Toc33G (Fig.5). In *ppi1* plants, NTAPi-Toc33 was only in the membrane fraction. In Col0 plants however, a smaller fraction of NTAPi-Toc33 appeared soluble. As NTAPi-Toc33 was expressed under the 35S promoter, it seems possible that Toc33 insertion sites were saturated and that not all of the molecules were membrane-inserted. Particularly in Col0 plants where endogenous atToc33 is present, such insertion “sites” for Toc33 may be more easily saturated. In the same experiment, part of the NTAPi-Toc33G fusion protein was present in the pellet fraction; this may be due to the over expression of the fusion protein that leads to assembly of a non-physiological complex or alternatively NTAPi-Toc33G may have aggregated.

6.1.2 Differences in Toc proteins association with NTAPi-Toc33 and NTAPi-Toc33G

Our TAP tag purification experiments suggest that the C-terminal transmembrane region of Toc33 is required for interaction with the other members of the Toc core complex. Indeed, by Western blotting, we showed that atToc159 and atToc75 co-purified with NTAPi-Toc33. When NTAPi-Toc33G, lacking the C-terminal transmembrane region, was isolated, neither Toc159 nor Toc75 were detected. The notable exception was Toc159, that was detected with Col0 NTAPi-Toc33G glycine eluates. This suggests that a small part of Toc159 may have been trapped in the cytosol by the soluble G-domain of Toc33. atToc132 also co-purified with NTAPi-Toc33. Since not Toc33 but Toc34 was proposed to interact preferentially with atToc132 and/or atToc120 (Ivanova *et al.*, 2004), it will be interesting to test for the presence of atToc120 or atToc34 in NTAPi-Toc33 eluates.

The crystal structure of pea Toc34 (Sun *et al.*, 2002) and its homologue atToc33 (Koenig *et al.*, 2008), show homodimers of the two proteins. We therefore expected the presence of endogenous Toc33 within the NTAPi-Toc33 purified complex. Unfortunately, we were unable to detect endogenous Toc33 using our α -Toc33 antibodies. In the future, the homodimerisation hypothesis could be addressed by co-expressing two different tagged proteins *in planta*; for example Toc33-TAP tagged and Toc33-His tagged proteins.

6.2 Hypothetic partners revealed by mass spectrometry

6.2.1 Tic110 and chaperones

Mass spectrometry analysis revealed the presence of atTic110 peptides (Table1). We then confirmed by Western blot (Fig.11) the presence of atTic110 in the eluted TAP-tagged Toc33 protein complexes. Signals were faint but atTic110 was detected in both NTAPi-Toc33G and NTAPi-Toc33 TEV eluates. However, atToc75 and atToc75 were not detected by Western blot in NTAPi-Toc33G eluates, which raise the question whether atTic110 is a contaminant, or an interacting partner. Mass spectrometry also revealed the presence of several chaperones in our TEV eluates: cpn60A (Hsp60), Hsp70, Hsp93 and co-chaperones DnaJ proteins (Table1). Chaperones are involved during protein import: Hsp70, for example, is involved in chloroplast import (Schnell *et al.*, 1994) and plays a role in the guidance complex (May and Soll, 2000). Hsp70-protein interacts with a recently identified the DnaJ-like protein, Toc12, during translocation of preproteins (Becker *et al.*, 2004a). Moreover, it was demonstrated that Hsp70 binds to the transit peptides of preproteins (Ivey *et al.*, 2000; Rial *et al.*, 2000).

Cpn60 helps the folding process of newly imported proteins (Kouranov *et al.*, 1998; Inaba *et al.*, 2003). Tic110 is known to recruit chaperones Hsp93 and Cpn60 with the help of Tic40 (Kessler and Blobel, 1996; Kessler and Schnell, 2006; Kovacheva *et al.*, 2005, Kovacheva *et al.*, 2007) to complete precursor translocation across the inner membrane and aid in subsequent protein folding.

The presence of Tic110 in NTAPi-Toc33 complexes suggests a role for Toc33 in establishing contacts between the Toc and Tic complexes.

6.2.2 Actin

Mass spectrometry analysis revealed the presence of actin in the eluted Tap-Tagged Toc33 protein complexes which was confirmed by Western blot (Fig.12). Actin was detected in both NTAPi-Toc33G and NTAPi-Toc33 TEV eluates. However, when NTAPi-Toc33G proteins were extracted in a Triton-free buffer, actin was not detected, in the TEV eluates.

A possible link between Toc proteins and actin is interesting. Actin takes part in several “chloroplastic processes“. Actin plays a role in chloroplast movement (Kandasamy and Meagher, 1999). Actin interacts with a protein involved in chloroplast movement in response to high or low light exposure: CHUP1 (for chloroplast unusual positioning phenotype) (Oikawa *et al.*, 2003; Schmidt von Braun and Schleiff, 2008). Actin (actin cytoskeleton) is also involved in stromules movement. Stromules are stroma-filled

tubules that extend from the chloroplast surface and permit the exchange of material between interconnected chloroplasts (Gray *et al.*, 2001).

6.2.3 α -tubulin and EF1 α proteins

α -tubulin protein peptides were found in our eluates. α - and β -tubulins are known to compose microtubules that are involved in different protein processes including vesicular transport. In plant cells, recent evidence suggests that some detergent-resistant membranes called lipid rafts may play an important role in protein targeting (as introduced in Borner *et al.*, 2005). Proteomics analysis on these lipid rafts in *Arabidopsis* detected the presence of atToc75 (Borner *et al.*, 2005). Moreover, proteomics analysis on these lipid rafts in tobacco cells detected α - and β -tubulin and 14-3-3 like proteins (Mongrand *et al.*, 2004). We could then imagine that some proteins, including components of the Toc core complex, could be targeted to the chloroplast membrane by these lipid rafts with the help of microtubules.

Elongation factor 1 protein peptides were also detected by mass spectrometry. EF1 α is known to be an abundant protein that interacts with the cytoskeleton by binding and bundling actin filaments and microtubules, and it participates in actin polymerization (Murray *et al.*, 1996). EF1 α was also found by proteomics in *Arabidopsis* in lipid rafts (Borner *et al.*, 2005).

6.2.4 Calmodulin

Mass spectrometry also identified two calmodulin isoforms. Recent data showed that calcium and calmodulin inhibitors affected protein import, which suggests that the import of chloroplast proteins is regulated by calcium and calmodulin (Chigri *et al.*, 2005). Tic32 was found to interact with calmodulin (Chigri *et al.*, 2006). As the calmodulin isoforms involved in protein import have not been identified yet, the two proteins identified in our complexes may be interesting candidates.

6.2.5 Missing interaction partners

According to the Western blots performed on our TAP tag purification assays, we were strongly expecting to identify peptides of atToc159, atToc75 and atToc132. Surprisingly, apart from Toc33 no component of the Toc core complex was detected by mass spectrometry in our TEV eluates. We may need to scale up the experiment to

detect these proteins by mass spectrometry. On one hand, we could imagine that atToc33 may have role in the formation of Toc-Tic supercomplexes and indirectly in the recruitment of atTic110 and chaperones. On the other hand, due to the high expression level of the fusion proteins, many NTAPi-Toc33 and NTAPi-Toc33G molecules may have aggregated which could prevent the association to atToc159, atToc75 or atToc132 and could explain why these Toc proteins could not be identified by mass spectrometry.

7 Future work to improve isolation and purification of Toc proteins associated complexes

Our first data show that the TAP tag method is suitable to purify Toc33. We will have now to improve this system to find interacting partners, primary by scaling up our experiment and by testing the second purification step on calmodulin-beads. The use of crosslinker chemicals could also be interesting to enlarge Toc33 protein interaction network.

Overexpression of a protein of interest can be problematic as protein-protein interactions are known to take place in a crowded cellular environment and heterogeneous; interaction pairs may exist at low relative concentrations.

Several modelling studies predict that there are many more risks for unspecific interactions compared with specific interaction possibilities (Deeds *et al.*, 2007). For example, in the TAP tag method paper of Puig *et al.*, 2001, the authors cite that overexpression of protein induces association with non natural partners such as heat shock proteins. The TAP tag method is successfully working with proteins expressed at their natural levels (Rigaut *et al.*, 1999), and the next step of this project is to repeat work with plants expressing NTAPi-Toc33/Toc33G under endogenous promoter and terminator.

We also have to consider the fact that proteins may interact with the N-terminal of Toc33 proteins. These studies will have to be carried out with plants expressing Toc33 fused to the TAP-tag in C-terminal (Toc33G-CTAPi and Toc33-CTAPi), although using a C-terminal tag for Toc33 will certainly prevent the protein from being inserted to the membrane.

Materials and Methods

1 Material

1.1 Plants

In Chapter 1, wild type (wt) or Col2 *Arabidopsis* plant always refers to *Arabidopsis thaliana* (L.) Heynh. var. Columbia 2. Tobacco plants refer to *Nicotiana tabacum* cv Petit Havana SR1. In Chapter 2, Wild type (wt) or Col0 *Arabidopsis* plant always refers to *Arabidopsis thaliana* (L.) Heynh. var. Columbia O. *ppi1* plants are a gift from Dr. Paul Jarvis, University of Leicester. UK.

1.2 Bacteria and yeast strains

Escherichia coli DH5 α and *Agrobacterium tumefaciens* strain GV3101 are from Invitrogen. Yeast strain used in split-ubiquitin assays are JD53 (Dohmen *et al.* 1995).

1.3 Chemicals

Unless stated otherwise, the chemicals were purchased from Fluka Chemie GmbH.

1.4 Oligonucleotides and Plasmids

Oligonucleotides were synthesized at Microsynth (Switzerland). Plasmids containing yeast split-ubiquitin cassettes *STE14-Cub-RURA3*, *PEX11-Cub-RURA3* and *Nub-PEX11* were a gift from prof. Nils Johnsson (University of Münster, Germany). Nub (amino acids 1-37 of ubiquitin) fusions are expressed from a pRS314 plasmid under control of the P_{CUP1} promoter, Cub-RUra3p (amino acids 37-76 of ubiquitin) fusions from a pRS313 vector under control of the P_{MET17}-promoter (Eckert and Johnsson, 2003).

pCL60 (described in Bauer *et al.*, 2000) is a pBluescript SK-(Stratagene) derivative with a CaMV 35S promoter and a nopaline synthase (nos) terminator cassette containing the coding sequence for enhanced green fluorescent protein (GFP).

The NTAPi cassette was cloned by Sibylle Infanger into the pCHF8 vector. pCHF8 was kindly provided by Dr. C.Frankhauser (Center for Integrative Genomics, University of Lausanne, Switzerland). pCHF8 is a pCAMBIA3300 derivative with the cauliflower mosaic virus (CaMV) 35S promoter and the RuBisCO small subunit terminator flanking the multiple cloning site.

1.5 Antibodies

Polyclonal antibodies specific to atToc159, atToc132, atToc75 have been described (Bauer *et al.*, 2000; Hiltbrunner *et al.*, 2001b; Ivanova *et al.*, 2004). Anti-Toc159G antibodies were obtained and purified by Dr Birgit Agne. Anti-Toc159A antibodies were done by Dr Birgit Agne and purified by Anouk Sester (master thesis 2008). Antibodies raised against atTic110 were obtained from Dr. Mayte Alvarez-Huerta (University of Utrecht, The Netherlands). Antibodies specific to atToc33 were done and purified with the help of Sylvain Bischof (master thesis 2006).

Monoclonal actin antibodies (clone 10B3 recognizing all eight *Arabidopsis* actins isoforms (ACT1, 2, 3, 4, 7, 8, 11 and 12)) and IgG antibodies (Human IgG Purified Immunoglobulin Reagent Grade) were purchased at Sigma-Aldrich.

α -HA monoclonal antibodies were bought from Roche, α -Myc monoclonal antibodies from Santa Cruz Biotechnology, and antibodies against phosphoribulokinase (PRK) were a gift from Dr. Pia Stieger, University of Neuchâtel.

2 Methods

2.1 Methods used for cloning

2.1.1 PCR, restriction digest and ligation

Standard protocols were used for cloning (Sambrook and Russell, 2001). DNA fragments were amplified using a proof-reading DNA polymerase (Pwo, Roche) and oligonucleotides including appropriate restriction sites. Pre-existing plasmid-constructs were used as templates for PCR. PCR products and plasmids were digested (restriction enzymes from New England Biolabs or Promega) and purified from agarose gels using

the QIAquick kit (Quiagen). Vectors were dephosphorylated using shrimp alkaline phosphatase (Roche) according to the manufacturer's recommendations. T4 DNA ligase (New England Biolabs) was used to ligate vectors and inserts as described (Sambrook and Russell, 2001). PCR reactions and purifications steps were analysed by agarose gel electrophoresis according to standard protocols.

2.1.2 Transformation of chemically competent *E. coli* cells and plasmid isolation

Ligation reactions were subsequently transformed by heat shock into competent *E. coli* DH5 α cells (Sambrook and Russell, 2001). Competent *E. coli* cells were prepared as described (Inoue et al., 1990). Selection was done on LB medium [25g/l LB Broth Miller (Becton Dickinson Diagnostic Systems), 1.2% (w/v) Agar Bacteriological Grade (ICN Biomedicals)] supplemented with appropriate antibiotics. Clones were selected by PCR and restriction digestion and DNA sequences were verified by sequencing (Microsynth).

For plasmid isolation, the GenElute Plasmid Miniprep Kit (Sigma) was used according to the suppliers' instructions.

2.2 DNA constructs

2.2.1 Yeast split ubiquitin cloning

The yeast split-ubiquitin constructs were derived from the *STE14-Cub-RURA3* (Wittke et al., 1999), *PEX11-Cub-RURA3* and *Nub-PEX11* constructs (Eckert and Johnson 2003). Two hemagglutinin epitopes (HA) were added to the Cub constructs by annealing the primers 5'-TCG ACC TAC CCA TAC GAC GTA CCA GAT TAC GCT GCT TAC CCA TAC GAC GTA CCA GAT TAC GCT-3' and 5'-TCG AAG CGT AAT CTG GTA CGT CGT ATG GGT AAG CAG CGT AAT CTG GTA CGT CGT ATG GGT AGG-3' and ligation into the unique *SalI* restriction site in front of the Cub coding sequence.

The coding sequence of the G domain of Toc33 (amino acids 1-265) was amplified from existing plasmid pet21d-Toc33H6sol using a forward primer containing a *ClaI* restriction site 5'-CCA TCG ATC CAT GGG GTC TCT CG-3' and a reverse primer including a *SalI* site 5'-CAT ATG GTC GAC CCT ATC TTT CCT TTA TCA TC-3' and cloned into the *ClaI/SalI* digested *STE14-Cub-RURA3* construct (Wittke et al., 1999). The coding sequence of the G domain of Toc159 (amino acids 728-1093) was amplified from pet21d-Toc159G using the following forward primer containing the coding sequence for a single Myc epitope tag and a *BamHI* site 5'-CCC GGG ATC

CCT GGG GAT GAG GAG CAG AAG CTG-3' and a reverse primer with a *EcoRI* site 5'-CCA TCG ATC CAT GGG GTC TCT CG-3'. The resulting PCR product was ligated into the *BglIII* and *EcoRI* sites of the Nub containing plasmid *Nub-PEX11* thereby replacing *PEX11* (Eckert and Johnsson, 2003).

The coding sequence of preats1B (AT5G38430.1) was amplified from pET21d-preats1B using forward primer 5'-CAG GGA-TCC ATC GAT GAT ATC CTC TAT GCT C-3' containing a *ClaI* restriction site, and reverse primer 5'-CC GCT CGA GCC AGC ATC AGT GAA GCT TC-3' that contains a *XhoI* site. The coding sequence of ats1B (amino acids: 55-181) was amplified from pET21d-preats1B using forward primer 5'-G TAG GAT CCA TCG ATG AAG GTG TGG CCA C-3' that contains a *ClaI* restriction site and the same reverse primer used to amplify preats1B. The PCR products were then digested by *ClaI/XhoI* and cloned into the *ClaI/SalI* digested *STE14-Cub-RURA3* construct.

Mutations of Nub Ile13 to Gly (Nub^G) or Ile13 to Ala (Nub^A) were done by site-directed mutagenesis according to the manufacturer's manual (Quick change, Invitrogen). For Nub^G primers used were as follow: forward 5'-G ACT TTG ACC GGT AAA ACC **GGA** ACA TTG GAA GTT GAA TCT TC-3' and reverse 5'-GA AGA TTC AAC TTC CAA TGT **TGG** GGT TTT ACC GGT CAA AGT C-3'. For Nub^A we used: forward 5'-G ACT TTG ACC GGT AAA ACC **GCA** ACA TTG GAA GTT GAA TCT TC-3' and reverse 5'-GA AGA TTC AAC TTC CAA TGT **TGC** GGT TTT ACC GGT CAA AGT C-3'.

2.2.2 Plant split-ubiquitin constructs

The plant split-ubiquitin constructs were designed with the coding sequence of plant ubiquitin atUBQ11 (AT4G05050.1) (Callis *et al.* 1995). The sequence corresponding to the first 37 amino acids (Nub) was amplified using as a forward primer 5'-CGG GAT CCT CTA GAG TCG ACC ATG CAG ATC TTC G-3' including a *BamHI* site and a reverse primer containing a *NcoI* site 5'-TCA TGT CAT GAC ACC ACC GCG GAG ACG G-3'. A plasmid (BUGUS) containing the *atUBQ11* coding sequence, provided by Prof. Richard Vierstra (University of Wisconsin-Madison) served as template. The resulting PCR fragment was ligated into the vector pCL60 cut by *BamHI* and *NcoI* yielding pCL60-Nub. The sequence corresponding to the amino acids 35-76 of UBQ11 (Cub) was amplified using the forward primer 5'-CAT GCC ATG GGA TAC CCA TAC GAC GTA CCA GAT TAC GCT GGC ATT CCT CCG GAC C-3' including a *NcoI* site and the coding sequence for a single HA tag and the reverse primer 5'-TCA TGT CAT GAC ACC ACC GCG GAG ACG G-3' containing a *BspHI* site. The PCR product was ligated into pCL60 vector cut by *NcoI* yielding pCL60-Cub.

The following primers 5'-GTA CTC ATG AAG GAG CAG AAG CTG ATC-3' (forward), 5'-CTC AAG ACC CGT TTA GAGG-3' were used to amplify Toc159₇₂₈₋₁₀₉₃ (Toc159G) with the two-hybrid construct pGBKT7-Toc159G as DNA template. The amplified DNA was then cloned *NcoI* and *NotI* into pCL60-Nub. The complete sequence of Toc33 or Toc33G (Toc33₁₋₂₆₅) were amplified with the forward primer 5'-TGG GCC ATG GGG TCT CTC GTT CGT-3' and the reverse primers 5'-TGA ACT CAT GAG AAG TGG CTT TCC AC-3' or 5'-TGA ACT CAT GAG CTT TCC TTT ATC ATC-3', respectively. Ligation was done in pCL60-Cub vector cut by *NcoI*. The coding sequence of atPex11-2 (atPEX11e, AT3G61070.1) was amplified by the forward primer 5'-CAT GCC ATG GCA ACT ACA CTA GAT TTG ACC-3' containing a *NcoI* site and the reverse primer 5'-CTA TAG CGG CCG CTC ATG ATT TCT TCA AC-3' including a *NotI* site. The product was ligated into pCL60-Nub cut by *NcoI* and *NotI*. To clone into pCL60-Cub cut by *NcoI*, atPex11-2 was amplified with the same forward primer as above and reverse 5'-TGA ACT CAT GAG TGA TTT CTT CAA C-3' including a *BspHI* site. The template plasmid DNA pGEM-Teasy-PEX11.2 was kindly provided by the group of Alison Baker (University of Leeds, UK).

Mutation of Nub Ile13 to Gly (Nub^G) was done by site-directed mutagenesis according to the manufacturer's manual (Quick change, Invitrogen) using forward primer: 5'-CC GGA AAG ACC GGC ACT CTT GAA GTT GAG AGT TCC GAC ACC-3' and reverse primer: 5'-GGT GTC GGA ACT CTC AAC TTC AAG AGT GGG GGT CTT TCC GG-3'.

2.3 Stable transformation of *Arabidopsis* with TAP tag constructs.

cDNAs of Toc33 and Toc33G were amplified by PCR from pCL60-Toc33-Cub and pCL60-Toc33G-Cub (see split ubiquitin chapter) using as a forward primer 5'-GAA GAG GCC TTT ATG GGG TCT CTC GTT-3' including *StuI* site and reverse primers 5'-G CTC TAG AGC TTA AAG TGG CTT TCC-3' and 5'-G CTC TAG AGC TTA GAG CTT TCC TTT ATC-3' containing *XbaI* site to amplify Toc33 and Toc33G respectively. Toc33 and Toc33G amplified fragments were digested by *StuI/XbaI* and ligated in the corresponding sites of pCHF8-NTAPi leading to pCHF8-NTAPi-Toc33 and pCHF8-NTAPi-Toc33G.

Electrocompetent GV3101 cells were prepared as described (Weigel and Glazebrook, 2002). 30ng of plasmid DNA were used for electroporation in a MicroPulser (BioRad) device, according to the manufacturer's instructions. Transformants were selected on LB media supplemented with 1.2% (w/v) agar (bacteriological grade, ICN Biomedicals) and appropriate antibiotics.

A. thaliana plants were transformed using the floral dip method as described (Clough and Bent, 1998). Transformants were selected on plates or on soil by phosphinothricin (see below).

2.4 Plant growth

2.4.1 Growing plants in vitro

Seeds were surface-sterilised by liquid or vapour-phase methods as described (Clough and Bent, 1998). Col0 and *ppi1* seedlings were plated on 0.5x Murashige and Skoog medium (Duchefa) containing 0.8% Phyto Agar (Duchefa) and left for 2 days at 4°C in the dark. They were then grown under short-day conditions (8h light ($120\mu\text{mol m}^{-2} \text{s}^{-2}$) 16h dark, 20°C, 70% relative humidity). NTAPi-Toc33/Toc33G transformed seedlings were selected by adding in the Murashige and Skoog medium 30 $\mu\text{g/ml}$ phosphinothricin (BASTA; Duchefa).

Nicotiana tabacum cv Petit Havana SR1 were grown on 1x Murashige and Skoog medium containing 0.8% Phyto Agar under long-day conditions (16h light $120\mu\text{mol m}^{-2} \text{s}^{-2}$, 8h dark, 23°C, 60% relative humidity).

2.4.2 Growing *Arabidopsis* on soil

Seeds were planted on soil (Ricoter). Germination was synchronised as above. Plants were grown under short day conditions (8h light ($120\mu\text{mol m}^{-2} \text{s}^{-2}$) 16h dark, 20°C, 70% relative humidity) or long-day conditions (16 h light ($120\mu\text{mol m}^{-2} \text{s}^{-2}$), 8 h dark), 20°C, 70% relative humidity) to get seeds. NTAPi-Toc33/Toc33G transformed plants were selected by spraying on seedlings 30 $\mu\text{g/mL}$ phosphinothricin (BASTA; Duchefa) every 3 days.

2.5 Yeast split-ubiquitin assay

Yeast growth was performed as described (Johnsson and Varshavsky, 1994). Total protein extracts were prepared according to Kiel *et al.*, 2005.

2.6 Protoplasts transformation

Protoplasts were transiently transformed using the polyethylene glycol method according to (Jin *et al.* 2001) with 4 weeks-old *A. thaliana* or 6 weeks-old *N. tabacum* leaves.

Fluorescence in transformed protoplasts was monitored 24 to 48h after transformation using a Leica TCS 4D microscope. GFP was detected with the FITC (488 nm) laser line and TRITC (568 nm) was used for chlorophyll autofluorescence.

2.7 Chlorophyll measurements

Measurements were done according to Sundberg *et al.* 1997 using Col0, *ppi1*, Col0/*ppi1* NTAPi-Toc33/Toc33G seedlings. Experiment was repeated 10 times for each different seedlings.

2.8 Plant genomic DNA extraction and PCR analysis

Leaf tissue corresponding to 0.5cm² was ground in 0.2M Tris/HCl pH9, 0.4M LiCl, 25mM EDTA, 1% (w/v) SDS. The homogenate was cleared by centrifugation (5min, 16000xg, 4°C), supplemented with an equal volume of isopropanol and centrifuged again (45min, 16000xg, rt). The supernatant was discarded and the DNA pellet was washed with 80% (v/v) ethanol. Finally, DNA was dried and resuspended in 50µL TE (10mM Tris/HCl pH8, 1mM EDTA).

PCR reactions contained 2µL DNA solution, 0.5U GoTaq DNA polymerase (Promega), as well as 0.2mM dNTPs (Eurobio) in a total volume of 25µL. Toc33 gene presence was monitored using forward 5'-GGT CTC TCG TTC GTG AAT GG-3' and reverse 5'-TTG GAA AGG ACT GAC ACG GA-3' primers. *ppi1* genotype was checked using same forward primer as above and the following T-DNA left border reverse primer 5'-ATA ACG CTG CGG ACA TCT AC-3'. The PCR program was as follows: 5min, 94°C, 35x (30s, 94°C; 30s, 55°C; 30s, 72°C).

2.9 Plant proteins extraction and Western blot analysis

Transiently transformed protoplasts were centrifuged for 1 min at 100xg, or proteins were isolated from *Arabidopsis* leaves. Total proteins were extracted according to Rensink (100mM NaCl, 50mM TrisHCl pH7.5, 1mM PMSF, 0,5% v/v TritonX-100) (Rensink *et al.*,1998).To avoid proteolytic degradation, 0.5% (v/v) protease inhibitor cocktail for plant cell extracts (Sigma P9599) was added to the extraction buffer. Proteins were concentrated by chloroform-methanol precipitation (Wessel and Flügge, 1984) and resuspended in sample buffer [50mM Tris/HCl pH6.8, 0.1M DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol] and heated 10min at 65°C. Protein concentration was determined by the Bradford assay (Bradford 1976) and reagent purchased from BioRad using bovine serum albumine (BSA) as the standard.

Proteins were separated by SDS-PAGE and blotted onto Protran R nitrocellulose membrane (Schleicher & Schuell) using the Mini-PROTEAN System (BioRad). Western blot membranes were stained with Amido Black (Naphthol Blue Black) as described (Sambrook and Russell, 2001) and scanned.

To block unspecific binding of antibodies, membranes were incubated 1h or o/n in blocking buffer [PBS (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH7.4), TBS (25mM Tris/HCl pH7.4, 140mM NaCl, 2.7mM KCl) containing 5% (w/v) skim milk powder]. Membranes were then incubated for 1h or longer with adequate antibody dilutions in blocking buffer. (see below) and washed extensively with PBS, TBS or TBS-tween (TBST; TBS, 0.05% (v/v) Tween R 20). To reveal primary antibodies, membranes were incubated 1h with a 3'000x dilution of horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG (Bio-Rad Laboratories) in TBS-T buffer containing 1% (w/v) skim milk powder. After washing with PBS/TBS/TBS-T buffer, signals were detected by enhanced chemiluminescence.

The TAP tag experiments membranes were incubated 1 min in [0.1M Tris/HCl pH8.5, 1.25mM 3-aminophthalhydrazide (luminol), 0.2mM p-coumaric acid, 0.009% (v/v) H₂O₂] and exposed to high performance chemiluminescence films (Amersham Biosciences).

Split-ubiquitin experiments blots were developed using ECL and high performance films (GE Healthcare). Chemiluminescence signals were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>).

Antibody dilutions for immunoblotting, Incubation buffers and approximate incubation times for immunoblotting are indicated. Unless stated otherwise, antibodies were produced into rabbit organisms.

α -atToc159A (1/2000) PBS 5% Milk powder, 1h rt

α -atToc159G (1/200) PBS with no milk powder, o/n 4°C

α -atToc132 (1/2000) PBS 3% Milk powder, o/n 4°C

α -atToc75 (1/3000) PBS 3% Milk powder, 2h rt
 α -atToc33 (1/200) PBS 5% Milk powder, o/n 4°C
 α -atTic110 (1/3000) PBS 3% Milk powder, 2h rt
 α -actin (1/2000) PBS 1% Milk powder, 1h rt, mouse
 α -IgG (1/5000) PBS 5% Milk powder, 1-2h rt, human
 α -PRK (1/500) PBS 5% Milk powder, 1h rt
 α -HA (1/500 or 1/1000) TBS 1% Milk powder, 2h rt or o/n 4°C, mouse
 α -Myc (1/5000) TBS 1% Milk powder, 1h rt, mouse

[PBS (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH7.4), TBS (25mM Tris/HCl pH7.4, 140mM NaCl, 2.7mM KCl), TBST (TBS, 0.05% (v/v) Tween R 20)].

2.10 Separation of soluble and insoluble proteins

Transformed protoplasts were collected by centrifugation at 100xg for 1min and resuspended in lysis buffer (20mM Tris-HCl, pH 7.5, 50mM NaCl, 1mM DTT, 2mM MgCl₂, 0.5% w/v inhibitor cocktail for plant cell extracts) followed by freezing and thawing. The lysate was centrifuged at 100000xg for 1h at 4°C; the resulting supernatant was considered total soluble protein. Soluble protein was concentrated by chloroform-methanol precipitation. The pellet was resuspended with 50 mM Tris-HCl, pH7.5.

2.11 Immunolocalization on protoplasts

Protoplasts were prepared as described (Jin *et al.*, 2001), reducing cellulase and macerozyme (Serva) concentrations to 1% and 0.25% (w/v) respectively.

Immunolocalization in isolated protoplasts was performed as described (Matsui *et al.* 1995) Polysine slides (BDH Laboratories) were used. Protoplasts were fixed with 4% (v/v) formaldehyde in W5 buffer [154mM NaCl, 125mM CaCl₂, 5mM KCl, 5mM glucose, 1.5mM MES pH5.6] and permeabilized with 0.5% (v/v) NP-40 in W5. Primary antibodies were diluted 1:100 (v/v, anti-IgG) and to 1/20 (v/v, anti-atToc33). Secondary antibody was diluted at 1/100. Slides were mounted in SlowFade solution (Molecular Probes) and fluorescence of the fluorescein-coupled secondary antibody (Pierce) was monitored by confocal scanning microscopy using the FITC (488 nm) laser line from a LEICA TCS SP5 microscope.

2.12 TAP tag purification

Purification of TAP tag complexes from plants expressing tagged Toc33 was done as described (Rohila *et al.*, 2004, Rubio *et al.*, 2005, Witte *et al.*, 2005) using commercial IgG-sepharose beads (Amersham). Plant leaves were ground with liquid Nitrogen. Total plant proteins were extracted as described in section 2.9 (according to Rensink *et al.*, 1998) using or not using 0.5% v/v Triton X-100 in the extraction buffer depending on the experiment. Following this step, proteins were centrifuged at 10'000g for 30min. When experiment was done starting with 10g of plants, proteins were ultracentrifuged at 100'000g for 1h. This centrifugation step is necessary to separate solubilized and non-solubilized material. Protein concentration was determined by the Bradford assay. Proteins (e.g. centrifuged supernatant) were incubated to beads for 2h30min at 4°C on a rotating wheel in 1.5mL microtubes. Proteins were eluted with 0.1M Glycine pH3 (acidic elution) or with TEV protease (Promega). For acidic elution, beads were incubated 5min on ice with 100µL 0.1M Glycine pH3. This step was repeated 3 times. TEV elution was processed according to the manufacturer manual, adding 1µM protease inhibitor E64 Roche (Rohila *et al.*, 2004) to the cleavage buffer. Beads were incubated with TEV protease for 2h at 16°C or o/n at 4°C. Samples of the different steps of the experiment were concentrated and resuspended as described in section 6. Sample buffer was added to the eluted beads to load on SDS gel.

2.13 Protein identification by mass spectrometry

TEV eluates were subjected to SDS-PAGE and proteins were stained with SYPRO Ruby (Invitrogen, according to the manufacturer protocol except that methanol was replaced by ethanol). Visible protein bands were excised and subjected to in-gel tryptic digest. (Fig.11). In gel tryptic digest was performed as described (Shevchenko *et al.*, 1996) using for digestion 10ng/µL trypsin (Promega sequence-grade modified porcine trypsin, Cat. #V511A) over night at 30°C.

Mass spectrometry was performed by Sylvain Bischof, ETH, Zurich:

Mass spectrometry measurements were performed on an LTQ FT-ICR (Thermo Finnigan), coupled with a Probot (LC-Packings/Dionex) autosampler system and the UltiMate HPLC-system (LC-Packings/Dionex). Peptide mixtures were loaded onto laboratory made capillary columns (75µm inner diameter, 8 cm length, packed with Magic C18 AQ beads, 3µm, 100Å (Microm)). Peptides were eluted from the column by an increased acetonitrile concentration in the mobile phase from 5% acetonitrile, 0.2% formic acid to 40% acetonitrile, 0.2% formic acid over 75min, followed by a 10min wash step at 5% acetonitrile, 0.2% formic acid. Peptide ions were detected in a survey scan from 300 to 1'600 amu followed by 3 data-dependent MS/MS scans (isolation

width 2 amu, relative collision energy 35%, dynamic exclusion enabled, repeat count 1, followed by peak exclusion for 2min).

Interpretation of MS/MS spectra and data filtering:

MS/MS spectra were searched with TurboSequest and PeptideProphet by using the Trans-Proteomic Pipeline (TPP v2.9) against the *Arabidopsis thaliana* TAIR8 protein database (downloaded on December 14th 2007) supplemented with contaminants. The search parameters were: requirement for tryptic ends, one missed cleavage allowed, mass tolerance = ± 3 Da, variable modification of methionine (M, PSI-MOD name: oxidation, ModAccession: MOD:00412, mono $\Delta = 15.9949$) and static modification of cysteine (C, PSI-MOD name: iodoacetamide derivative, ModAccession: MOD:00397, mono $\Delta = 57.021464$).

For PeptideProphet, the cutoff was set to a minimum probability of 0.9

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Appendix

Mass spectrometry results

Mass spectrometry results for each band cut (Fig.10). Proteins identified with less than 3 peptides were not considered, as well as protein peptides presents in both control plants (Col0 and *ppi1*) and NTAPi-Toc33G/Toc33. In this work, a selection of these proteins was made (Table 1) as we discarded chloroplast metabolism proteins, plastid-lipid associated protein and thylakoids components.

Bands cuts between 200 and 80kDa

Col0 NTAPi Toc33				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	24	6	2
Actin	AT5G09810	17	4	1
tubulin TU A4	AT1G04820	8	3	0,8
EF1	AT1G07920	10	3	1
ATSCO1 translation EF	AT1G62750	10	7	2
CAB	AT1G29910	4	3	0,55
PATL1 (Patellin 1); transporter	AT1G72150	26,5	13	3,5

Bands between 90 and 66 kDa

<i>ppi1</i> NTAPi Toc33				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	8	3	0,42
atTic110	AT1G06950	5	3	0,67
actin	AT5G09810	11	3	0,5
EF1	AT1G07920	7,8	3	0,62
Arabidopsis Rab GTPase homolog E1b; translation elongation factor	AT4G20360	12	5	0,9
myrosinase-associated protein	AT1G54010	10,4	3	0,63
tubulin	AT1G04820	4	4	0,6
ESM1 carboxylic ester hydrolase	AT3G14210	17	8	1,9
Photosystem II subunit O-2	AT3G50820	8,5	3	0,63
ATMDAR1 (NaDH)	AT3G52880	14	5	0,9
fructose-1,6-bisphosphatase	AT3G54050	10,6	3	0,66
Glutamine synthase 2	AT5G35630	15	5	0,9
PetA cytF	ATCG00540	22	6	1,13

Bands 66kDa

<i>ppi1</i> NTAPi Toc33				
protein	accession number	percent coverage	number of peptides	percent share
atTic110	AT1G06950	12,6	10	1,5
Actin	AT5G09810	12	3	0,5
CPN60A chaperonine	AT2G28000	23	12	1,8
CPN60B chaperonine	AT1G55490	10,6	5	0,8
cpHSC70-2 (Heat shock protein 70-7)	AT5G49910	15	10	0,9
Heat shock protein (HSP) 93-V	AT5G50920	16	12	1,8

Bands between 66 and 35kDa

<i>col0</i> NTAPi Toc33G				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	47	52	11
atTic110	AT1G06950	19	15	3,1
Actin	AT5G09810	12	12	2
porin, putative \ voltage-dependent anion-selective channel protein,	AT5G15090	12	4	0,6
Elongation factor1 EF1	AT1G07920	18	20	3
CPN60A chaperonine	AT2G28000	20	17	2,5
CPN60B chaperonine	AT1G55490	16	7	0,9
cpHSC70-2	AT5G49910	17	20	1,8
HSP 93-V	AT5G50920	13,5	24	3,34
FIB (Fibrillin); structural molecule	AT4G04020	12	3	0,4
Pap fibrilin family	AT3G23400	8	3	0,2
plastid-lipid associated protein PAP	AT2G35490	9,6	3	0,4
PAP	AT4G22240	12	5	0,6
PUR ALPHA-1 (purin-rich alpha 1); nucleic acid binding	AT2G32080	19	5	1,1
FTSZ1-1 structural molecule	AT5G55280	7	3	0,75
PORB	AT4G27440	9,7	5	1
POR C	AT1G03630	6,8	6	0,75
hydrolase, alpha\beta fold family protein	AT1G52510	11	3	0,75
ATLFNR1 ATLFNR1 (LEAF FNR 1); NADPH dehydrogenase\ oxidoreductase	AT5G66190	14	3	0,74

Col0 NTAPi Toc33				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	40	27	5
atTic110	AT1G06950	12	9	1,4
Actin	AT5G09810	15	6	1,1
EF1	AT1G07920	13	4	0,6
CPN60A chaperonine	AT2G28000	15	8	1,3
CPN60B chaperonine	AT1G55490	16	8	1,2
chaperonine 20	AT5G20720	16	3	0,5
cpHSC70-2	AT5G49910	13	8	1,25
HSP 93-V	AT5G50920	8	6	0,96
PAP	AT4G22240	12	3	0,46
PRK	AT1G32060	6,3	3	0,4
PORB	AT4G27440	9,5	4	0,74
POR C	AT1G03630	5	3	0,5
PSBB (PSII)	ATCG00680	5	3	0,75

<i>ppl</i> NTAPi Toc33G				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	34	10	2
Actin	AT1G49240	11	5	0,5
EF1	AT1G07920	10	4	0,8
DNAJ heat shock family protein	AT3G62600	11	3	0,5
PSBB (PSII)	AT1G00680	5	3	0,6
plastid-lipid associated protein PAP	AT2G35490	9	3	0,6
oxidoreductase	AT1G23740	11	3	0,5
AGT (Alanine: glyoxylate aminotransferase)	AT2G13360	12	3	0,5
ADK1 (Adenosine kinase 1)	AT3G09820	15	4	0,85
CPN60B (Chaperonin 60 beta)	AT1G55490	6,4	3	0,6
PTAC16 (Plastid transcriptionally active 18)	AT3G46780	11	4	0,9

<i>ppi1</i> NTAPiToc33				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	8	3	0,5
atTic110	AT1G06950	5	3	0,7
Actin	AT5G09810	11	3	0,5
EF1	AT1G07920	8	3	0,6
myrosinase-associated protein	AT1G54010	10	3	0,6
PSBO2	AT3G50820, AT5G66570	8,5	3	0,6
ESM1 (Epithiospecifier modifier 1) carboxylic ester hydrolase	AT3G14210	17	8	1,7

Bands between 30 and 14 kDa

<i>col0</i> NTAPiToc33G				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	24	8	1,74
atTic110	AT1G06950	205	3	0,5
Actin11	AT3G12110	7,4	3	0,34
thylakoid lumen 18.3 kDa protein	AT1G54780	37	4	0,9
Bet v I allergen family protein	AT1G23130	31,5	7	1,34
LHCA1; chlorophyll binding	AT3G54890	10	4	0,9
PSBQ; calcium ion binding	AT4G21280	17,5	3	0,7
(Calmodulin 4); calcium ion binding	AT1G66410	23,5	5	1,2
calcium-binding protein, putative	AT1G18210	42	6	1,4

<i>ppi1</i> NTAPi Toc33G				
protein	accession number	percent coverage	number of peptides	percent share
atToc33	AT1G02280	20	5	1
atTic110	AT1G06950	2,5	3	0,41
Actin11	AT3G12110	7,4	3	0,4
EF1	AT1G07920	7,8	3	0,46
thylakoid lumen 18.3 kDa protein	AT1G54780	27	6	1,2

Col0 NTAPi Toc33				
protein	accession number	percent coverage	number of peptides	percent share
atTic110	AT1G06950	8,6	7	1,1
Actin	AT1G49240	7,4	3	0,3
EF1	AT1G07920	8	3	0,45
CPN60A chaperonine	AT2G28000	18	8	1,22
CPN60B chaperonine	AT1G55490	13,4	6	0,9
cpHSC70-2	AT5G49910	13	8	1,2
HSP 93-V	AT5G50920	10	8	1,2

Acknowledgments

First of all I would like to thank my thesis director, Prof. Felix Kessler, for giving me the opportunity to do my PhD thesis in his group at the Laboratory of Plant Physiology in Neuchâtel. I really appreciate your kindness, your motivating enthusiasm and encouragement and thank you for all suggestions and advices.

I would also like to acknowledge Prof. Jean-Marc Neuhaus, Dr. Sacha Baginsky and Dr. Norbert Rolland for accepting to serve in my thesis committee.

A special thank to Dr Birgit Agne for your help, advices and fruitful discussions.

I would like to thank Jana Smutny, Muamba Katambayi, and Sylvain Bischof for technical help.

Un grand merci à tous les membres passés ou présents du laboratoire LPV et en particulier à: Christiane Bettinelli, Claire Bréhélin, Michaël Ilegems, Sibylle Infanger, Christelle Joss, Didier Lochmatter, Meryll Martin, Marlyse Meylan, Joanne Schwaar, Jana Smutny, Pierre-Alexandre Vidi pour leur aide, leur bonne humeur, leur gentillesse et l'excellente atmosphère de travail qui en a découlé.

Je ne remercie pas du tout mais alors vraiment pas les phytotrons et chambres de culture qui ont pris un malin plaisir à tomber en panne très régulièrement, mais je dis un grand merci à M. Jean Pierre Duvoisin pour l'aide apportée dans ces moments là.

Je tiens à remercier également P.J Harvey, Interpol, William Sheller, DominiqueA, ma tablette graphique et les Basler Lækkerli qui m'ont accompagné lors de la rédaction de ce manuscrit ; ainsi que Paul-André Siegenthaler et Pia Stieger pour m'avoir fait une place dans leur grand bureau de chefs dès le début de ma rédaction.

Thanx Lalou and Javaid for your support, interest and everything!

Pour finir je souhaite remercier Guillaume : merci pour ton soutien, ton remontage de moral, et pour être là tout simplement (sans oublier de remercier le Dr Gouzerh pour l'aide scientifique !).