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The role of a putative arginine finger and guanine nucleotides in homo- and heterodimerization of two GTPases involved in protein import into chloroplasts

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Abstract

The vast majority of the chloroplast proteins is synthesized in the cytosol with an N-terminal transit peptide and has to be posttranslationally imported into chloroplasts. This import is facilitated by import machineries at the outer and inner chloroplast membrane, termed the Toc- (translocon at the outer chloroplast membrane) and the Tic- (translocon at the inner chloroplast membrane) complex, respectively. The trimeric Toc-complex at the outer chloroplast membrane consists of Toc34 and Toc159, two integral membrane GTP binding proteins involved in preprotein recognition and binding, and Toc75, forming part of the protein-conducting channel through the outer chloroplast membrane.

The three-dimensional structure of Toc34 from pea, psToc34, has been recently solved, revealing new insights into the function of psToc34. In the crystal, psToc34 formed dimers with bound GDP, resembling a GTPase with its corresponding GAP (GTPase activating protein). It has been hypothesized that one monomer functions as GAP for the other monomer in the pea Toc34 dimer, suggesting that dimerization and GTP hydrolysis are functionally related. Furthermore, the location of Arg133 at the interface of the pea Toc34 dimer suggests that this residue functions as arginine finger. An arginine finger is inserted by many GAPs into the active site of their corresponding GTPase, thereby activating GTP hydrolysis.

To examine the arginine finger hypothesis, we mutated the putative arginine finger (Arg130) of the *Arabidopsis* ortholog of psToc34, atToc33, to alanine (atToc33 R130A). As Arg130 was found not to be required for GTP hydrolysis, Arg130 does not appear to be an arginine finger. Moreover, GTP hydrolysis by atToc33 wt is slow in comparison to the rate of GTP hydrolysis by activated GTPases, suggesting that one monomer does not function as GAP for the other monomer in the atToc33 dimer. Instead, Arg130 appears to be essential for atToc33 homodimerization. Moreover, it was shown that Arg130 is involved in heterodimerization of atToc33 and atToc159.

The crystal structure of the psToc34 homodimer suggests that psToc34 homodimerizes preferentially in the GDP bound form. Homodimerization of atToc33, the *Arabidopsis* ortholog of psToc34, was indeed found to be favoured in presence of GDP.

Toc159 does not only exist in an integral membrane form, but also in a soluble, cytosolic form. Similar to SRα forming the receptor for SRP54 at the ER membrane, Toc34 appears to function as receptor for soluble Toc159. Furthermore, the interaction between Toc34 and Toc159 is essential for chloroplast biogenesis *in vivo* and therefore likely plays a crucial role during protein import into chloroplasts.

It is still under debate which guanine nucleotide is bound to the two GTPases when they interact. We provide evidence that the heterodimerization between atToc33 and atToc159 is favoured in presence of GDP, suggesting that GTP hydrolysis is a prerequisite for this interaction.

Zusammenfassung

Die grosse Mehrheit der Chloroplasten-Proteine wird im Cytosol mit einem N-terminalen Transitpeptid synthetisiert und anschliessend posttranslational in die Chloroplasten importiert. Dieser Import wird durch Import-Maschinerien in der äusseren und inneren Chloroplasten-Membran bewerkstelligt, die Toc-, respektive Tic-Komplex genannt werden. Der trimere Toc-Komplex besteht aus drei Komponenten: Toc34 und Toc159, zwei GTP-bindende Membran-Proteine, die an Erkennung und Bindung der Vorläuferproteine beteiligt sind, und Toc75, ein Protein das einen Teil des Kanals in der äusseren Chloroplasten-Membran bildet durch den die Vorläuferproteine transportiert werden.

Die kürzlich publizierte Kristall-Struktur von Toc34 aus Erbsen lieferte neue Erkenntnisse bezüglich der Funktion von psToc34. PsToc34 liegt in den Kristallen als Dimer vor, das GDP gebunden hat. Weil dieses psToc34 Dimer einem GTPase/GAP (GTPase aktivierendes Protein)-Komplex sehr ähnlich ist, wurde vermutet, dass im psToc34 Dimer ein Monomer als GAP für das andere Monomer dient. Das impliziert, dass Dimerisierung und GTPase Aktivität miteinander verknüpft sind. Des Weiteren hat die Position eines Arginin-Restes (Arg133) im psToc34 Dimer zur Spekulation geführt, dass dieser Arginin-Rest ein Arginin-Finger sein könnte. Solche Arginin-Finger finden sich in vielen GAPs. Sie ragen ins aktive Zentrum der dazugehörigen GTPase und tragen so zur Beschleunigung der GTP-Hydrolyse bei.

Um diese Arginin-Finger Hypothese zu überprüfen, haben wir den vermeintlichen Arginin-Finger (Arg130) von atToc33, dem zu psToc34 orthologen Protein in *Arabidopsis*, zu einem Alanin mutiert. Es wurde nachgewiesen, dass Arg130 nicht für die GTP-Hydrolyse benötigt wird. Deshalb scheint Arg130 kein Arginin-Finger zu sein. Zusätzlich war die GTP-Hydrolyse von atToc33 wt zu langsam im Vergleich zu anderen aktivierten GTPasen, was gezeigt hat, dass im atToc33 Dimer nicht ein Monomer als GAP für das andere Monomer dient.

Im Gegensatz dazu scheint der mutmassliche Arginin-Finger für die Dimerisierung von atToc33 essentiell zu sein. Ebenso konnte nachgewiesen werden, dass Arg130 an der Heterodimerisierung zwischen atToc33 und atToc159 beteiligt ist.

Wie die Kristall-Struktur gezeigt hat, homodimerisiert psToc34 bevorzugt in GDP-gebundener Form. AtToc33, das orthologe *Arabidopsis* Protein zu psToc34, bildet Homodimere ebenfalls bevorzugt in GDP-gebundener Form.

Toc159 kommt nicht nur in Membran-gebundener Form, sondern auch in löslicher Form im Cytosol vor. Es wurde gezeigt, dass Toc34 der Rezeptor für die lösliche Form von Toc159 ist. Eine analoge Situation findet sich an der ER-Membran, wo SRα als Rezeptor für SRP (Signalerkennungs-Partikel) dient. Die Interaktion zwischen Toc34 und Toc159 ist essentiell für die Chloroplastenbiogenese *in vivo* und scheint deshalb eine sehr wichtige Rolle im Import von Vorläuferproteinen in Chloroplasten zu spielen. Wir zeigen, dass atToc33 und atToc159 bevorzugt in GDP-gebundener Form interagieren. Dies weist darauf hin, dass vermutlich die GTP-Hydrolyse eine Voraussetzung für eine stabile Interaktion zwischen atToc33 und atToc159 ist.

Abbreviations

αatToc33	antibodies recognizing atToc33
atToc159G	GTP binding domain of atToc159
atToc159GM	combined G- and M-domains of atToc159
A-domain	acidic-domain
αHis_6	antibodies specific for His_6
APS	ammonium persulfate
at	*Arabidopsis thaliana*
BSA	Bovine serum albumin
BN-PAGE	blue native polyacrylamide gel electrophoresis
CAB	light-harvesting chlorophyll a/b binding protein of Photosystem II
cop	constitutively photomorphogenic
cue	CAB underexpressed
D1	dimerization motif
DTT	1,4-dithio-DL-threitol
det	de-etiolated
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
FNR	ferredoxin-$NAD(P)^+$ oxidoreductase
fus	fusca
GAP	GTPase activating protein
G-domain	GTP binding domain
GEF	guanine nucleotide exchange factor
GMP-PMP	5'-guanylyl-imidodiphosphate
GST	gluathione S-transferase
gun	genome uncoupled
GUS	β-Glucuronidase
H_6	hexahistidinyl-tag
H-bond	hydrogen bond
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
His_6	hexahistidinyl-tag

hy	hypocotyl elongated
IgG	immunoglobuline G
IPTG	isopropyl-β-D-1-thiogalactopyranoside
KOAc	potassium acetate
M-domain	membrane domain
MeOH	Methanol
$Mg(OAc)_2$	magnesium acetate
NC	negative control
N.D.	not detectable
Ni-NTA	nickel-nitrilotriacetic acid
ORF	open reading frame
PCR	polymerase chain reaction
PEI-cellulose	polyethyleneimine-cellulose
ppi	plastid protein import
preSSU	precursor of the small subunit of RubisCO
ps	*Pisum sativum*
RubisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR	signal recognition particle receptor
SRP	signal recognition particle
SSU	small subunit of RubisCO
sw I and II	switch I and II
T-DNA	transfer DNA
TEMED	N,N,N’,N’,-tetramethyl-ethylenediamine
Tic	translocon at the inner chloroplast membrane
TM	transmembrane helix
Toc	translocon at the outer chloroplast membrane
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume per volume
wt	wildtype
w/v	weight per volume

atToc…, atTic…	indicates the Toc-, Tic-protein of *A. thaliana*
psToc…, psTic…	designates the Toc-, Tic-protein of *P. sativum*
Toc…, Tic…	refers to Toc- and Tic-proteins of both *A. thaliana* and *P. sativum*, unless specified otherwise

upper case, italic	gene, wild type allele (e.g. *TOC159*)
lower case, italic	mutant allele(s) (e.g. *ppi2*)
regular case	protein (e.g. atToc159)

1. Introduction

1.1. Chloroplasts, the photosynthetic plastids

The uptake of a cyanobacterium by a eukaryotic cell marked the evolutionary birth of the plant cell. Since this event the relationship between the eukaryotic host and the cyanobacterial endosymbiont has become closer and closer. The endosymbiont lost its autonomy and most of its genome (Martin et al., 1998, 2002), giving rise to a group of organelles, called plastids. Plastids are organelles that are characteristic for plant and algal cells. They are one of the primary features that distinguish plant cells from other eukaryotic cells (Pyke, 1999). In higher plants different types of plastids exist that vary in size, shape, content and function (Thomson and Whatley, 1980). But they all originate from proplastids, which can be found in dividing cells in meristems. These proplastids develop into plastid types adapted to different functions (Thomson and Whatley, 1980; Surpin and Chory, 1997; Bauer et al., 2001). Amyloplasts, elaioplasts and proteinoplasts are specialized in the storage of starch, lipids and proteins, respectively. Chromoplasts accumulate carotenoids and provide the red, orange and yellow colors of many flowers, old leaves and fruits. Chloroplasts, the photosynthetic plastids, are, due to the presence of chlorophyll, characteristic for green plant tissues. Proplastids develop into chloroplasts when leaf cells differentiate in the presence of light or into etioplasts in the absence of light (Chory, 1991, 1993; Surpin and Chory, 1997).

Chloroplasts, like mitochondria, have two envelope membranes separated by an intermembrane space. Within the chloroplast stroma, a third membrane system, the thylakoids, exists. The photosynthetic apparatus is located at the thylakoid membrane (Allen and Forsberg, 2001) which surrounds the thylakoid lumen.

The fact that unique reactions, such as light-induced production of ATP and NADPH and carbon assimilation, take place in chloroplasts, turns chloroplast research into an important topic in plant research.

1.1.1. The evolutionary origin of chloroplasts

The endosymbiotic theory for the formation of eukaryotic cells containing chloroplasts and mitochondria is now widely accepted (Surpin and Chory, 1997). This theory states that these organelles arose from endosymbiontic events involving a eubacterial ancestor and a eukaryotic host cell (Margulis, 1970). It is thought that cyanobacteria and α-proteobacteria are the eubacterial ancestors of chloroplasts and mitochondria, respectively (Gray, 1999).

Several facts support the prokaryotic origin of chloroplasts and mitochondria. They contain their own genome which consists of several copies of a circular chromosome (Surpin and Chory, 1997). Chloroplasts proliferate by simple fission involving cell division proteins with high sequence identity to bacterial and cyanobacterial proteins (Osteryoung and Vierling, 1995). Chloroplast ribosomes are 70S in size, i.e. of the prokaryotic type, and chloroplast ribosomal-proteins show structural similarities to those of cyanobacteria (Meng et al., 1989).

Furthermore, sequence comparison of genes encoding ribosomal proteins revealed a gene organization in *Synechococcus* sp. similar to that of chloroplasts of red and brown algal species (Sugita et al., 1997). This sisterhood of green plants and red algae was also affirmed by sequence comparison of marker proteins, underlining that they have the same ancestor and that endosymbiosis was established before the separation of green plants and red algae (Moreira et al., 2000).

A critical step in the transition from the autonomous endosymbiont to the organelle was the reduction of the genome. Many endosymbiont genes were lost and most of those retained were transferred to the nucleus (Douglas, 1998; Martin et al., 1998). Recent data suggest that around 18% of the coding genes of *Arabidopsis* were acquired from the cyanobacterial ancestor of chloroplasts (Martin et al., 2002). Transfer of genes to the nucleus necessitated targeting of cytosolically synthesized gene products to the chloroplast as well as their subsequent import. This resulted in the establishment of an import complex in the outer and inner membrane of chloroplasts, called Toc- and Tic-complex, respectively (discussed in detail later). For various proteins of this import machinery homologues, or at least similar ORF sequences, were detected in the *Synechocystis* sp. genome (Bölter et al., 1998b; Reumann et al., 1999; Reumann and Keegstra, 1999; Fulda et al., 2002), supporting again the hypothesis for a cyanobacterial origin of chloroplasts and suggesting that, at least in part, the import machinery evolved from ancient cyanobacterial proteins. Moreover, also the Sec-, Tat- and SRP-translocons for protein import into or across the thylakoid membrane, show clear signs of having a cyanobacterial origin (Robinson et al., 2000; Schnell, 2000; Dyall et al., 2004). The present conception is that many transport proteins of the outer membrane are of eukaryotic origin and those in the inner membrane are of mixed origin (Dyall et al., 2004).

1.1.2. The coordination of three different genomes in plant cells

The presence of three different genomes, namely in the nucleus, in mitochondria and in chloroplasts, led to the evolution of various mechanisms to coordinate gene expression in plant and algal cells.

Much of the regulatory traffic between the nucleus and the chloroplast occurs from nucleus to chloroplast (anterograde signaling). But the flow of information is not entirely unidirectional, as signals from chloroplasts influence gene expression in the nucleus (retrograde signaling) (Jarvis, 2001; Rodermel, 2001).

1.1.2.1. Nuclear control of chloroplast development

The development of proplastids to chloroplasts is triggered by light. When a seedling germinates in the dark, proplastids differentiate into etioplasts and the seedling has a phenotype which allows the young plant to penetrate the soil and reach the light, namely an elongated hypocotyl, an apical hook and unopened cotyledons (Fankhauser and Chory, 1997). When exposed to light, etioplasts develop into chloroplasts, the rate of hypocotyl elongation is reduced and the hook and the cotyledons are opened. This process is called de-etiolation.

Based on unusual morphologies of light- and dark-grown plants, respectively, different types of *Arabidopsis* mutants have been identified. Mutations in a positive regulator of photomorphogenesis result in a *hy* (hypocotyl elongated) phenotype (Koornneef et al., 1980) when plants are grown in the light, whereas mutations in negative regulators result in *cop* (constitutively photomorphogenic), *det* (de-etiolated) and *fus* (fusca) mutants (Wei and Deng, 1996), which exhibit features of light-grown plants in the dark.

The *hy* mutants include mutants deficient in one of the phytochromes (Somers et al., 1991), mutants affected in phytochromobilin biosynthesis and therefore depleted of all photoactive phytochromes (Parks and Quail, 1991), as well as factors that activate gene expression in response to light (Ang et al., 1998). Hy5 is a bZIP type transcription factor and directly interacts with the promoter of genes the expression of which is regulated by light (Ang et al., 1998). Hy5 is essential for light activation of the chalcone synthase gene which encodes an enzyme involved in flavonoid biosynthesis (Ang et al., 1998). Cop1, a RING-finger protein with a putative coiled-coil domain and WD-40 repeats, directly interacts with Hy5 in the nucleus (Ang et al., 1998). In the dark, this interaction may directly target Hy5 for proteasome mediated degradation (Osterlund et al., 2000). In the light however, Cop1 is directed to the cytosol (von Arnim and Deng, 1994), leading to the accumulation of Hy5 in the nucleus and resulting in photomorphogenic development. Because multiple pleiotropic Cop/Det/Fus proteins are also required for the light-regulated degradation of Hy5 and belong to the Cop9 signalosome, which is conserved from plants to animals and shows similarity to the lid of the 26S proteasome (Li and Deng, 2003; Smalle and Vierstra, 2004), it has been suggested that these proteins play a role in proteasome-mediated degradation (Osterlund et al., 2000; Schwechheimer and Deng, 2000; Serino and Deng, 2003). The fact that Cop1 possesses different motifs that function in protein-protein interaction makes it a valuable candidate as a central player in interacting with multiple upstream and downstream factors of the light regulatory cascade (Wei and Deng, 1996).

1.1.2.2. Chloroplast control of nuclear gene expression

As mentioned above, the exchange of information between nucleus and chloroplast is not unidirectional: The state of the choroplast affects the expression of many nuclear encoded proteins destined for the chloroplast (Surpin and Chory, 1997; Jarvis, 2001).

Mutants that fail to accumulate carotenoids show severe photooxidation of chlorophyll in white light. This leads to the arrest of chloroplast development at a very early stage (Oelmüller, 1989). Furthermore it is possible to artificially induce photooxidative damage by application of the herbicide Norfluorazon. This is an inhibitor of phytoene desaturase, which catalyzes a key step in the carotenoid biosynthetic pathway (Susek and Chory, 1992; Surpin and Chory, 1997).

In both carotenoid-deficient maize mutants or Norfluorazon treated maize plants it was shown that the abundance of mRNAs for some nuclear encoded chloroplast proteins is dramatically reduced under white light conditions, including the mRNAs coding for CAB (light-harvesting chlorophyll a/b protein of Photosystem II) and SSU (small subunit of RubisCO) (Mayfield and Taylor, 1984, 1987). But when either mutant seedlings or Norfluorazon treated plants were grown in dim white light, photooxidation was minimized and CAB and SSU mRNA levels were normal. This finding suggests that the failure to accumulate CAB and SSU mRNAs is not a direct result of carotenoid-deficiency or Norfluorazon treatment, but rather of the photooxidative conditions within the plastid (Mayfield and Taylor, 1987). The fact that the photooxidative damage of chloroplasts affects the accumulation of nuclear encoded mRNAs the protein products of which are destined for the chloroplast, has been confirmed in other plant species (Oelmüller and Mohr, 1986). This leads to the hypothesis that a signal from the intact chloroplast itself is needed for accumulation of CAB and SSU mRNAs, and, furthermore, that this signal is no longer present when the chloroplast is photooxidatively damaged (Oelmüller and Mohr, 1986; Mayfield and Taylor, 1987).

Interestingly, it has been demonstrated in yeast that mitochondria communicate with the nucleus by signals that regulate the expression of nuclear encoded mitochondrial proteins (Forsburg and Guarente, 1989). Heme was shown to be involved in this signaling (Forsburg and Guarente, 1989).

A genetic screen for *Arabidopsis* mutants still expressing CAB under Norfluorazon treatment gave insight into the possible nature of the plastid signal in plants. For this screen plants were transformed with a fusion construct between the *CAB* promoter and the *GUS* (β-Glucuronidase) gene. The *CAB* promoter was chosen because it is very responsive to photooxidative damage (Oelmüller, 1989). The mutants found were named *gun* (genome uncoupled) mutants and express nuclear-encoded photosynthetic genes in the absence of normal chloroplast development and function (Susek et al., 1993). Up to now five different *GUN* loci have been identified (Mochizuki et al., 2001). One of them, *GUN5*, codes for the H subunit of the plastid

Mg-chelatase (Mochizuki et al., 2001), an enzyme that is located at the branchpoint of chlorophyll and phytochromobilin synthesis respectively, catalyzing the conversion of Protoporphyrin IX to Mg-Protoporphyrin IX (Rodermel, 2001). It has been shown that Mg-Protoporphyrin IX acts as a signaling molecule between the chloroplast and the nucleus (Strand et al., 2003). For this signaling pathway, the following model was suggested: Accumulation of Mg-Protoporphyrin IX under photooxidative conditions leads to inhibition of the expression of nuclear-encoded photosynthetic genes. In contrast less Mg-Protoporphyrin IX is accumulated in *gun5* mutants due to the reduced flux in the photosynthetic pathway, leading to the reduced inhibition of photosynthetic genes (Strand et al., 2003).
In addition, other porphyrins, such as Protoporphyrin IX (Møller et al., 2001), as well as other molecules such as carotenoids (Rodermel, 2001) and even RNA (Bradbeer et al., 1979), may function as plastid signals.

1.1.3. Chloroplast biogenesis

Light plays a very important role throughout the entire life cycle of plants, from germination to senescence. Phytochromes, cryptochromes and UV-A and -B receptors are photoreceptors that absorb the light and induce the signal cascade leading to the expression of a set of photosynthetic genes in the plastid and in the nucleus (Fankhauser and Chory, 1997), including the genes coding for chlorophyll a/b binding protein (CAB) (Li et al., 1995).

1.1.3.1. The *cue* (CAB underexpressed) mutants

To identify positive factors acting on the signaling pathway to nuclear light-induced genes, a screen for *Arabidopsis* mutants expressing lower levels of CAB mRNA under light conditions was performed. Leading to the identification of different *cue* (CAB underexpressed) mutants (Li et al., 1995; Lopez-Juez et al., 1998). The genes affected in *cue* mutants appear to code for proteins involved in different processes, ranging from phytochrome signaling to the greening process of photosynthetic organs (Lopez-Juez et al., 1998).

1.1.3.2. Tight linkage between chloroplast protein import and chloroplast biogenesis

Recently, a new *Arabidopsis cue* mutant, called *ppi1* (for plastid protein import mutant 1), was identified due to its pale green phenotype during the early developmental stages (Jarvis et al., 1998). This mutant lacks atToc33, a GTPase that is involved in recognition and binding of preproteins during protein import into chloroplasts (Jarvis et al., 1998), pointing to a possible connection between protein import into chloroplasts and chloroplast biogenesis *in vivo*.
The importance of some translocon components of the chloroplast protein import machinery for chloroplast biogenesis was confirmed by analysis of the *Arabidopsis ppi2* mutant. The *ppi2* mutant was isolated using reverse genetic techniques. The mutant has an albino phenotype and is seedling lethal (Bauer et al., 2000). It lacks atToc159, a GTPase that functions as a receptor for preproteins during import into chloroplasts (Hirsch et al., 1994; Ma et al., 1996) and is thus essential for chloroplast biogenesis (Bauer et al., 2000). Technically, the *ppi2* mutant also belongs to the *cue* group of mutants, because CAB mRNA is strongly downregulated in this mutant (Bauer et al., 2000).

1.2. Protein import into chloroplasts

1.2.1. General overview

The vast majority of the approximately 3000 chloroplast proteins are encoded by nuclear genes and synthesized on cytosolic ribosomes as preproteins with N-terminal transit peptides which are recognized by components of the import machinery in the chloroplast membrane. After their synthesis these proteins have to be imported into the chloroplasts across the outer and inner chloroplast envelope membranes (Chen and Schnell, 1999; Keegstra and Cline, 1999; Cline, 2000; Jarvis and Soll, 2001; Soll and Schleiff, 2004). This import is facilitated by translocon machineries in the outer and inner chloroplast envelope, called Toc- (translocon at the outer chloroplast envelope) and Tic- (translocon at the inner chloroplast envelope) complex, respectively (Hiltbrunner et al., 2001a; Jarvis and Soll, 2001). The components of these two import machineries are designated Toc and Tic, respectively, followed by the respective calculated molecular mass in kilodaltons (Schnell et al., 1997).

The N-terminal transit sequence is both necessary and sufficient for translocation of the preprotein across both chloroplast membranes and is cleaved off by a stromal processing peptidase once the protein reaches the chloroplast stroma (Richter and Lamppa, 1998, 1999). If the protein is destined for the thylakoid lumen, the transit peptide may be bipartite and contains additional targeting information for the thylakoid (Cline and Henry, 1996).

There exist at least two different pathways for the targeting of precursor proteins to the outer chloroplast membrane. Small integral membrane proteins like Toc34 are synthesized without an N-terminal transit peptide and the targeting information is therefore contained inside the mature protein. In the case of Toc34, the chloroplast-targeting signal is located at the C-terminus of the protein (Chen and Schnell, 1997; Li and Chen, 1997).

In contrast, Toc75, which is part of the protein-conducting channel in the outer chloroplast membrane, is synthesized as precursor protein with a bipartite N-terminal targeting sequence. The N-terminal part of the targeting sequence functions as a transit sequence, engaging the general import pathway, and is cleaved off by the stromal processing peptidase (Tranel et al., 1995; Tranel and Keegstra, 1996). The C-terminal part of the targeting sequence, however, prevents translocation of Toc75 into the stroma, allowing Toc75 to assemble in the outer envelope membrane (Tranel and Keegstra, 1996).

To date neither a consensus sequence nor a common secondary structure motif for chloroplast transit peptides have been identified (Keegstra et al., 1989; von Heijne et al., 1989; von Heijne and Nishikawa, 1991). In general, these transit peptides vary in length from 20-150 amino acids (Soll and Schleiff, 2004). Generally, they are rich in serine and threonine residues and their overall charge is positive, because they largely lack acidic amino acids (Keegstra et al., 1989). Phosphorylation may play a regulatory role in protein import into chloroplast because several stromal targeting transit peptides can be phosphorylated at serine or threonine residues and also a loosely defined common sequence motif for this phosphorylation could be assigned (Waegemann and Soll, 1996). The phosphorylated precursor proteins reportedly bind to 14-3-3 proteins and Hsp70 chaperones, forming a heterooligomeric complex of around 200 kDa called cytosolic guidance complex, which renders the preprotein more import-competent compared to the free precursor protein (May and Soll, 2000). But it appears that phosphorylation of transit peptides does not affect targeting *in vivo*, because it has been demonstrated that mutations of the phosphorylation site neither affected its ability to target to the chloroplast nor did they result in mistargeting (Nakrieko et al., 2004). Moreover, also a non-phosphorylatable form of preSSU (precursor of the small subunit of RubisCO) is imported into chloroplasts as efficiently as wildtype preSSU (Waegemann and Soll, 1996).

1.2.2. The three stages of import characterized by their energy requirement

The import into chloroplasts can be divided into three stages, distinct from each other in terms of their energy requirement. In the first energy independent stage, the precursors associate reversibly with plastids (Perry and Keegstra, 1994; Ma et al., 1996).

In the second stage, the transit peptide binds irreversibly to the chloroplast import machinery, leading to the formation of the early-import-intermediate. At this point, the C-terminal portion of the preprotein is still in the cytosol, where it can be degraded by exogenous proteases (Schnell and Blobel, 1993). At this stage the transit peptide is already found in contact with components of the import complex at the inner membrane, most likely at contact sites between outer and inner membrane (Perry and Keegstra, 1994; Wu et al., 1994). This second step requires energy in the form of low concentrations of either hydrolyzable GTP or ATP (100 µM) (Olsen et al., 1989; Olsen and Keegstra, 1992; Schnell and Blobel, 1993). It was shown that the ATP is required in the intermembrane space (Olsen and Keegstra, 1992). The hydrolysis of ATP and GTP appears to be a prerequisite for the binding of precursor proteins, because non-hydrolyzable analogs of ATP and GTP do not support this binding step (Olsen et al., 1989; Olsen and Keegstra, 1992).

For the complete translocation of the preprotein through the outer and inner chloroplast envelope, a higher concentration of ATP is needed in the stroma (1-3 mM) (Pain and Blobel, 1987; Theg et al., 1989). Furthermore, it could be shown that nucleotides other than ATP can not support translocation across the outer envelope membrane (Scott and Theg, 1996). During translocation, the preprotein is in contact with both the outer and the inner chloroplast envelope, respectively. This concurrent translocation of preproteins is facilitated by the formation of contact zones in which the outer and inner chloroplast envelope are in close contact (Schnell and Blobel, 1993). Nevertheless it could be shown that the two import machineries can function independently (Scott and Theg, 1996). Therefore it is unlikely that the import machineries in the outer and inner chloroplast membrane are constantly linked.

Unlike in mitochondria, a membrane potential is not involved in translocation across the chloroplast envelope (Olsen et al., 1989; Theg et al., 1989). But it seems that GTP is involved in the early phase of translocation, because two members of the Toc-complex, namely Toc159 and Toc34, are GTP binding proteins (Kessler et al., 1994; Seedorf et al., 1995). The observation that slowly-hydrolyzable or non-hydrolyzable analogs of GTP inhibit binding of preproteins corroborates this hypothesis (Olsen and Keegstra, 1992; Kessler et al., 1994). Moreover, not even an excess of ATP could restore the binding of preproteins (Kessler et al., 1994).

1.2.3. The sites of GTP and ATP consumption during protein import into chloroplasts

As mentioned above, the requirement for GTP in the early stages of protein import into chloroplasts is thought to be the consequence of the involvement of two GTP binding Toc-proteins at these stages, namely Toc34 and Toc159 (Kessler et al., 1994; Seedorf et al., 1995). However, no known components of the Toc- and Tic-complexes bind and hydrolyze ATP.

Only the chaperones that are reported to be involved in protein import into chloroplasts (Jackson-Constan et al., 2001) are known sites of ATP consumption. It has been suggested that not less than 3 ATPases mediate protein import into chloroplasts (Scott and Theg, 1996). At least two Hsp70-chaperones are in close contact with the outer envelope membrane. Com70 faces the cytosol (Kourtz and Ko, 1997), and a second Hsp70-protein protrudes into the intermembrane space between outer and inner membrane (Schnell et al., 1994). Com70 could be crosslinked to preproteins at an early stage of import, leading to the hypothesis that it is involved in keeping the preprotein unfolded during protein import (Kourtz and Ko, 1997). The second member of the Hsp70-family faces the intermembrane space (Schnell et al., 1994) and is thought to interact with preproteins that appear in the intermembrane space, preventing their backward movement. Moreover it has been shown that this Hsp70-protein interacts with a recently identified DnaJ-like protein, Toc12, of the Toc-complex during the translocation process (Becker et al., 2004a). The finding that ~83% of all chloroplast transit peptides investigated in a recent study contain a predicted Hsp70-binding site, corroborates the involvement of Hsp70-chaperones in protein import into chloroplasts (Zhang and Glaser, 2002). Furthermore, the binding of Hsp70-chaperones to chloroplast transit peptides was demonstrated (Ivey et al., 2000; Rial et al., 2000).

Two other chaperones, ClpC and Cpn60, are located in the stroma and were shown to interact with Tic110, consistent with its role as a docking site for chaperones (Kessler and Blobel, 1996; Nielsen et al., 1997). Whereas ClpC possibly prevents misfolding of preproteins, Cpn60 is thought to support the folding process of newly imported proteins (Nielsen et al., 1997; Kouranov et al., 1998; Inaba et al., 2003).

1.3. The chloroplast translocation machinery

1.3.1. Chronology of the research on the chloroplast translocation machinery

Initially, pea was used as a model organism to study protein import into chloroplasts. Isolated pea chloroplasts allowed the identification of the components of the protein import machineries in the outer and inner chloroplast membrane. This led to the identification of the trimeric pea Toc-complex, including Toc34, Toc75 and Toc159 (Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995). The components of the trimeric pea Toc-complex will be described in detail in the subchapters below. Additionally, components of the pea Tic-complex were identified (Kessler and Blobel, 1996; Lübeck et al., 1996; Kouranov and Schnell, 1997), which are also discussed below.

Primarily, the insight into protein import into chloroplasts stemmed mainly from *in vitro* experiments with isolated pea chloroplasts (Chen and Schnell, 1999). This has changed since *Arabidopsis thaliana* replaced pea as the main model plant to study protein import into chloroplasts, because *Arabidopsis* has severals advantages as a model organism which render it suitable for *in vivo* mutant studies: The complete genome sequence is known (The Arabidopsis Genome Initiative, 2000) and the size of the genome is rather small in comparison to other flowering plants. Furthermore, *Arabidopsis* has a short life cycle, it can be easily transformed and progeny can be produced in high numbers from a single plant (Somerville and Koornneef, 2002). Therefore some results of *Arabidopsis* mutant studies are already included in the subchapters below, even though the genetic analyses of the *Arabidopsis* Toc-proteins will be discussed in detail in the following chapter.

1.3.2. The Toc-complex

The Toc-complex is located at the outer chloroplast envelope and fulfills several functions: It specifically recognizes the transit peptide of preproteins destined for the chloroplast, it mediates translocation once binding is accomplished, and it participates in the formation of contact zones between outer and inner membrane (Hiltbrunner et al., 2001a; Jarvis and Soll, 2001; Soll and Schleiff, 2004). Transport across the inner chloroplast envelope is effected by the Tic-complex (Jarvis and Soll, 2001; Soll and Schleiff, 2004).

During the import, both complexes are in close contact, allowing the simultaneous translocation across both membranes. It has been shown that outer and inner chloroplast membrane can form contact zones, most likely where Toc- and Tic-complexes are located (Schnell and Blobel, 1993). Furthermore, components of the Toc- and Tic-complex could even be co-immunoprecipitated in the absence of a precursor protein (Nielsen et al., 1997).

The subunits of the Toc-complex in pea were initially identified by different co-isolation experiments as well as studies on crosslinking to precursor proteins, showing their involvement in protein import into chloroplasts (Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995). These studies revealed a trimeric Toc-complex, consisting of Toc159, Toc34, which both are GTP binding proteins, and Toc75, part of the protein-conducting channel in the outer chloroplast envelope (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995). The components of this trimeric Toc-complex will be discussed in detail below.

By co-immunoprecipitation with antibodies raised against Toc34 it could be shown that the trimeric Toc-complex forms a stable complex in the outer chloroplast envelope. This complex is formed even in the absence of a precursor protein (Ma et al., 1996). It was proposed that this core complex has a molecular weight of ~500 kDa and a stochiometric ratio of 4:4:1 between Toc34:Toc75:Toc159 (Schleiff et al., 2003b). The notion that Toc159 and Toc75 are components of the import machinery of chloroplasts, was supported by the finding that antibodies raised against either Toc159 or Toc75 inhibit precursor protein import (Hirsch et al., 1994; Tranel et al., 1995). Furthermore, it has been shown by reconstitution experiments in liposomes that Toc159 and Toc75 form the minimal translocon unit required for preprotein import *in vitro* (Schleiff et al., 2003a).

A new component of the Toc-complex, Toc64, was characterized by co-fractionation with Toc159, Toc34 and Toc75 (Sohrt and Soll, 2000). However, in a more recent study, Toc64 was not present in isolated Toc-complexes of pea chloroplasts (Schleiff et al., 2003b). Nonetheless, it is hypothesized that Toc64 could function early in the binding of preproteins by specifically recognizing the guidance complex formed by the preprotein, 14-3-3 proteins and Hsp70 (Sohrt and Soll, 2000). In addition, it has been suggested, that the association of Toc64 with the core complex may only be transient (Schleiff et al., 2003b).

Another component of the Toc-complex, Toc12, has been identified using a proteomics approach and shown to co-immunoprecipitate with Toc34, Toc75, Toc64 and Toc159 (Becker et al., 2004a). Toc12 is located in the outer chloroplast membrane and its C-terminal part, facing the intermembrane space, consists of a characteristic J-domain (Becker et al., 2004a). DnaJ-like proteins share a J-domain, which is required for interaction with Hsp70 proteins, resulting in increased ATP hydrolysis at Hsp70 and subsequent high affinity substrate binding (Bukau and Horwich, 1998). Furthermore, DnaJ-like proteins are known to recruit Hsp70 chaperones to sites of protein translocation (Corsi and Schekman, 1997). In agreement with this and based on their own results, the authors propose that Toc12 recruits Hsp70 to the Toc-complex during preprotein translocation, causing stimulation of ATP hydrolysis and thereby increased affinity of Hsp70 for the preprotein in the intermembrane space (Becker et al., 2004a).

1.3.2.1. Toc159

Toc159 was initially characterized as a protein with a calculated mass of 86 kDa, and was therefore named Toc86 (Hirsch et al., 1994; Kessler et al., 1994). But two different groups could show that Toc86 is only a proteolytic fragment of a larger protein with a calculated mass of 159 kDa, i.e. Toc159 (Bölter et al., 1998a; Chen et al., 2000). Crosslinking of Toc159 to preproteins and the demonstration that antibodies against Toc159 inhibit precursor binding led to the conclusion that Toc159 functions as the primary receptor for transit peptides at the outer chloroplast membrane (Hirsch et al., 1994; Perry and Keegstra, 1994).

Full-length Toc159 consists of three domains: An N-terminal acidic-domain (A-domain), a GTP binding domain (G-domain) and a C-terminal protease-resistant membrane-anchor (M-domain) (Bauer et al., 2000; Chen et al., 2000; Hiltbrunner et al., 2001a).

The A-domain is lacking from the originally characterized Toc86 fragment. This domain is predicted to be soluble and is not predicted to contain a membrane-spanning region. The A- and G-domains of Toc159 are accessible to thermolysin digestion, resulting in a 52-kDa protected fragment that corresponds to the M-domain. These results indicate that both the A- and G-domains are exposed to the cytosol (Hirsch et al., 1994; Chen et al., 2000). The function of the A-domain remains elusive. It was shown, however, that a truncated version of atToc159, lacking the A-domain, behaves like intact atToc159 in *in vivo* targeting experiments with isolated *Arabidopsis* protoplasts (Bauer et al., 2002). Furthermore, atToc159 lacking the A-domain is sufficient to support the import of preproteins into chloroplasts (Lee et al., 2003). Finally, it was possible to complement the atToc159 knock-out mutant of *Arabidopsis* (*ppi2*) with a construct of atToc159 lacking the A-domain (Lee et al., 2003). These data provide evidence for the functionality of the truncated Toc159, lacking the A-domain and suggest that Toc86, as it was characterized initially, is a functional protein.

The central domain, the G-domain, contains consensus motifs for a GTP binding site (Bourne et al., 1991), and specifically binds GTP (Kessler et al., 1994). The G-domain targets atToc159 to the outer chloroplast membrane of isolated *Arabidopsis* chloroplasts in a GTP-dependent way (Bauer et al., 2002; Smith et al., 2002; Lee et al., 2003). In addition to the G-motifs, the Toc-GTPases share a dimerization motif in the G-domain (Sun et al., 2002), suggesting that the G-domains act as interaction domains.

The M-domain is the C-terminal part of the protein, anchoring Toc159 in the outer chloroplast envelope. The importance of this region was shown recently by demonstrating that this is the minimal region required for protein import into chloroplasts and that it is even capable to partially complement the *ppi2* mutant which lacks Toc159 (Lee et al., 2003). But nonetheless it was shown that the functional G-domain is necessary for the proper targeting of atToc159 to the outer *Arabidopsis* chloroplast envelope, because mutations in the G-domain resulted in a dominant negative effect on targeting of atToc159 to chloroplasts (Lee et al., 2003). Thus, the hypothesis was put forward that the M-domain contains the necessary targeting information, but that the G-domain plays, in addition, a regulatory role in the targeting process (Lee et al., 2003).

While it has long been known that Toc159 is a protein of the outer chloroplast membrane (Hirsch et al., 1994; Kessler et al., 1994), it was only recently demonstrated that a part of Toc159 exists in a soluble, cytosolic form (Hiltbrunner et al., 2001b; Lee et al., 2003). Furthermore, the direct interaction *in vitro* between atToc159 and a soluble form of atToc33, the *Arabidopsis* ortholog of pea Toc34, was substantiated (Hiltbrunner et al., 2001b) and it was shown later that the respective G-domains are sufficient for this binding reaction (Smith et al., 2002). This has led to the hypothesis that Toc34 could serve as the receptor for soluble Toc159 at the outer chloroplast envelope (Hiltbrunner et al., 2001b). Support for this hypothesis came also from earlier targeting experiments, in which it was shown that targeting and insertion of Toc159 is dependent on protease-sensitive receptor components at the outer envelope (Muckel and Soll, 1996). It was therefore suggested that Toc159 not only functions as a receptor at the outer chloroplast membrane, but also as a soluble receptor that recognizes the preprotein in the cytosol and, by association with Toc34, transfers it to the Toc-complex for membrane translocation (discussed later in detail in the targeting hypothesis) (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002; Wallas et al., 2003).

1.3.2.2. Toc34

Toc34 is a GTP binding integral membrane protein. It has strong sequence similarity with Toc159 in the G-domain, including also the regions not directly participating in GTP binding (Kessler et al., 1994). Toc34 has a short C-terminal membrane-spanning tail, while the majority of its hydrophilic domain, including the G-domain, is exposed to the cytosol (Seedorf et al., 1995).

Crosslinking studies have shown that Toc34 is in direct contact with preproteins during the early translocation process and that this interaction is dependent on GTP binding but not hydrolysis (Kouranov and Schnell, 1997). Moreover, also the GTP dependent binding between recombinant, purified Toc34, lacking the C-terminal tail, and preproteins has been shown (Sveshnikova et al., 2000b; Schleiff et al., 2002), implying a role for Toc34 in precursor recognition and binding. Besides GTP binding, phosphorylation may affect precursor binding to Toc34. GTP bound Toc34 binds to phosphorylated preproteins with high affinity (Sveshnikova et al., 2000b; Jelic et al., 2002; Schleiff et al., 2002). The phosphorylated preprotein enhances GTP hydrolysis by Toc34 up to 30-fold, thereby releasing the preprotein (Jelic et al., 2002, 2003). However, the requirement for precursor phosphorylation before binding to Toc34 is still under debate, because proper preprotein targeting is not affected when the phosphorylation site is abolished by mutation (Nakrieko et al., 2004).

Nonetheless, phosphorylation of Toc34 could play a minor regulatory role in import, because it was demonstrated that phosphorylation of Toc34 inhibits binding of preproteins, suggesting that Toc34 has to be dephosphorylated prior to the interaction with the preprotein (Sveshnikova et al., 2000b; Fulgosi and Soll, 2002).

1.3.2.3. Toc75

Toc75 constitutes at least part of the protein-conducting channel in the outer envelope of chloroplasts (Schnell et al., 1994; Tranel et al., 1995). Structural analysis of Toc75 predicts that it forms a β-barrel-type channel such as those typically found in porin-like channel proteins. Moreover, it has been proposed that Toc75 traverses the outer envelope with 16 β-sheets (Hinnah et al., 1997; Sveshnikova et al., 2000a) and that both the N-terminus and the C-terminus of Toc75 face the intermembrane space (Sveshnikova et al., 2000a).

During preprotein import into chloroplasts, Toc75 is in contact with the transit peptide of the precursor protein (Ma et al., 1996). Furthermore, Toc75 and Toc159 could be crosslinked to preproteins at the early import intermediate stage (Perry and Keegstra, 1994; Kouranov and Schnell, 1997). It has even been demonstrated that Toc75 can discriminate between preprotein and mature protein (Hinnah et al., 2002).

Reconstitution of overexpressed and purified Toc75 into liposomes results in voltage-gated ion channels (Hinnah et al., 1997). A homolog of Toc75, termed syn75, was identified in the cyanobacterium *Synechocystis* PCC6803 (Bölter et al., 1998b; Reumann et al., 1999), with electrophysiological properties similar to those of Toc75 (Bölter et al., 1998b), thus providing support for the prokaryotic nature of the outer chloroplast membrane. The finding that Toc75 shows sequence similarity with outer envelope proteins of gram-negative bacteria that function in insertion of β-barrel proteins (Voulhoux et al., 2003) and are involved in secretion (Bölter et al., 1998b), supports the hypothesis that Toc75 functions at least as part of the translocation channel in the outer envelope membrane.

1.3.3. The Tic-complex

The Tic-complex facilitates the import of preproteins across the inner chloroplast envelope. This step requires ATP hydrolysis in the stroma (Pain and Blobel, 1987; Theg et al., 1989).

In comparison to the Toc-complex, much less is known about the function of the Tic-complex. The following components have been assigned to the Tic-complex: Tic110 (Kessler and Blobel, 1996; Lübeck et al., 1996), Tic62 (Küchler et al., 2002), Tic55 (Caliebe et al., 1997), Tic40 (Stahl et al., 1999), Tic22 (Kouranov et al., 1998) , Tic20 (Kouranov et al., 1998) and just recently also Tic32 (Hörmann et al., 2004).

1.3.3.1. Tic110

The component of the Tic-complex to be identified first and presently being best characterized is Tic110. In a recent publication it was demonstrated that an *Arabidopsis* mutant carrying a homozygous T-DNA insertion in Tic110 is embryo-lethal. Furthermore it was shown that Tic110 has an essential role in embryo development (Kovacheva et al., 2005).

Tic110 is anchored by its N-terminal domain in the inner membrane (Kessler and Blobel, 1996; Lübeck et al., 1996). Regarding its overall topology and function, three different models exist. It has been suggested that Tic110 spans the membrane once and that the hydrophilic domain of Tic110 is oriented towards the intermembrane space, thereby possibly mediating the interactions between Toc- and Tic-complex (Lübeck et al., 1996). Other topology studies did not support this model (Kessler and Blobel, 1996; Jackson et al., 1998). These studies propose that Tic110 spans the membrane twice and that its hydrophilic C-terminal domain faces the stroma, leading to the model that Tic110 serves as docking site for stromal chaperones that assist in translocation and folding of imported proteins (Kessler and Blobel, 1996; Jackson et al., 1998). Recently, a third model has been proposed, according to which Tic110 is a β-barrel protein and functions as the central part of the protein-conducting channel in the Tic-translocon (Heins et al., 2002). But the fact that Tic110, lacking its two predicted N-terminal transmembrane domains, when expressed both in *Escherichia coli* or *Arabidopsis thaliana*, is a soluble protein with a high α-helical content, renders this third model rather unlikely (Inaba et al., 2003).

The model proposing a hydrophylic, stromal domain has been supported by further studies. First, it was shown that Tic110 can be co-immunoprecipitated with ClpC and that it specifically interacts with Cpn60, providing evidence for its role as docking site for chaperones (Kessler and Blobel, 1996; Nielsen et al., 1997). Crosslinking of preproteins to Tic110 during preprotein translocation provoked the extended hypothesis that Tic110 additionally serves as transit peptide docking site for preproteins that emerge in the stroma (Inaba et al., 2003). Including the chaperone data in this model, ClpC may prevent misfolding of the preprotein and provide the driving force for the complete translocation, whereas Cpn60 assists in the folding of the newly imported protein before diffusion into the stroma (Kessler and Blobel, 1996; Nielsen et al., 1997; Inaba et al., 2003).

1.3.3.2. Tic20 and Tic22

Tic20 and Tic22 were both identified in crosslinking experiments with recombinant preproteins. They both crosslink to preproteins at early and late stages in protein import, providing support for them being part of the translocon at the inner chloroplast envelope (Kouranov and Schnell, 1997). Tic20 is an integral inner membrane protein whereas Tic22 seems to be peripherally associated with the outer surface of the inner membrane (Kouranov et al., 1998). The fact that crosslinking of preproteins to Tic20 increases at later stages of preprotein import and that Tic20 is an integral membrane protein (Kouranov et al., 1998), led to the hypothesis that Tic20 may constitute part of the protein-conducting channel at the inner membrane (Chen et al., 2002). This hypothesis was corroborated by the analysis of plants expressing an antisense construct of Tic20. They were specifically affected in protein translocation across the inner membrane and consequently also in chloroplast biogenesis, resulting in a pale phenotype (Chen et al., 2002).
The role of Tic22 is not clear yet, but its localization at the outer surface of the inner membrane and its interaction with preproteins during translocation suggests that it may helps to coordinate the interaction of the Toc- and Tic-complexes during import (Kouranov et al., 1998).

1.3.3.3. Tic40

Tic40 is located in the inner chloroplast membrane (Stahl et al., 1999; Chou et al., 2003), protruding with its hydrophylic domain into the stroma (Chou et al., 2003). Tic110 has been shown to co-immunoprecipitate with Tic40 from isolated inner membrane vesicles and from isolated chloroplasts in presence of crosslinkers, suggesting that Tic40 is in close proximity to Tic110 (Stahl et al., 1999; Chou et al., 2003). Tic40 has also been shown to interact with Toc75 and a stromal chaperone (Chou et al., 2003). Additionally, the hydrophylic, C-terminal part of Tic40 shows a sequence homology with the C-terminal globular domain of certain co-chaperones (Chou et al., 2003), allowing the suggestion that Tic40 possibly recruits chaperones to the inner envelope membrane (Stahl et al., 1999; Chou et al., 2003).

1.3.3.4. Tic55 and Tic62

Tic55 and Tic62 have been identified by their interaction with Tic110 using BN-PAGE analysis (Caliebe et al., 1997; Küchler et al., 2002). Tic55 is an integral membrane protein and contains a predicted Rieske-type iron-sulfur cluster (Caliebe et al., 1997). Tic62 is also an integral protein of the inner chloroplast membrane. Its C-terminal part faces the stroma and interacts with a ferredoxin-NAD(P)$^+$ oxidoreductase (FNR) (Küchler et al., 2002). The findings that a Rieske-type iron-modifying reagent inhibited preprotein import into chloroplasts (Caliebe et al., 1997) and that Tic62 interacts with FNR (Küchler et al., 2002), led to the hypothesis that Tic55 and Tic62 may function as redox sensors during preprotein import into chloroplasts (Küchler et al., 2002).

1.3.3.5. Tic32

Tic32 has been identified by its interaction with Tic110 using a soluble-phase binding assay and behaves like an integral membrane protein (Hörmann et al., 2004). But even though it has been shown that Tic32 is involved in protein import into chloroplasts and that the *Arabidopsis* Tic32 null mutant is embryo lethal, the function of Tic32 remains elusive (Hörmann et al., 2004).

1.4. Genetic analyses of the *Arabidopsis* Toc-proteins

1.4.1. Reverse genetics

The sequence of the *Arabidopsis* genome was the first plant genome to be completed (The Arabidopsis Genome Initiative, 2000). But by then, the function of only a small part of these ~25’000 genes was known and the enormous undertaking to assign functions to all of these genes started, the ultimate goal being to understand the function of all genes in this model organism (Chory et al., 2000). With availability of the first fully sequenced genomes of various organisms a set of new

techniques emerged, referred to as reverse genetics. Whereas the mutant leads to the gene of interest in forward genetics, the gene of interest leads to the corresponding mutant in reverse genetics and thereby possibly also to the function of the gene (Østergaard and Yanofsky, 2004).
T-DNA insertional mutagenesis turned out to be a very useful tool for the creation of loss-of-function mutants of *Arabidopsis*. Several collections of genome-wide T-DNA insertional mutants of *Arabidopsis* exist today, for example the SIGnAL collection (Alonso et al., 2003). The databases, generated from these collections by sequencing the flanking regions of the T-DNA insertions, allow the identification of mutants *in silico*. Once a gene of interest has been found, the respective seeds, with the T-DNA mutation in the gene of interest, can be ordered from the *Arabidopsis* Stock Center and the mutant analysis can start.

1.4.2. The Toc-translocon in *Arabidopsis thaliana*

Unlike in pea, the two GTPases of the Toc-complex in *Arabidopsis*, Toc159 and Toc34, are encoded by small gene families. Four genes encode homologues of pea Toc159 (atToc159, atToc120, atToc132 and atToc90) (Bauer et al., 2000; Jackson-Constan and Keegstra, 2001; Hiltbrunner et al., 2004), and two genes encode homologues of pea Toc34, which are atToc33 and atToc34 (Jarvis et al., 1998; Jackson-Constan and Keegstra, 2001).
All the six members of the *Arabidopsis* homologues of psToc159 and psToc34 share a conserved GTP binding domain (G-domain) (Hiltbrunner et al., 2001a). Whereas atToc33 and atToc34 consist only of this G-domain and an additional C-terminal hydrophobic tail, the members of the Toc159 family share the tripartite structure already described for Toc159 (Fig. 1). The only exception is atToc90, lacking the N-terminal A-domain (Hiltbrunner et al., 2001a; Hiltbrunner et al., 2004) (Fig. 1).

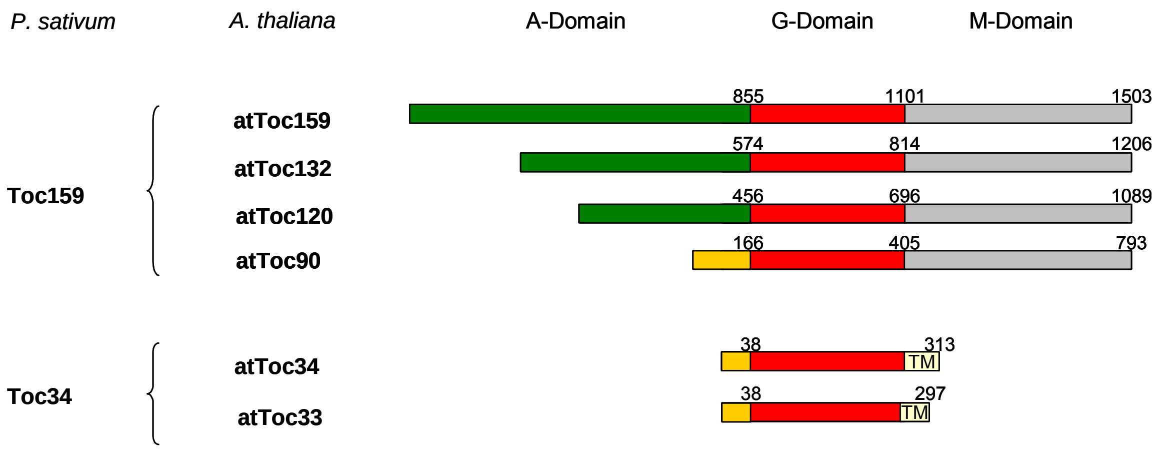


Fig. 1. The family of GTP binding proteins of the Toc-complex in *A. thaliana*: The members of the family of GTP binding proteins of *Arabidopsis* fall into two subgroups, the homologues of pea Toc159 (atToc159, atToc132, atToc120 and atToc90) and the homologues of pea Toc34 (atToc34 and atToc33). They all share a G-domain (given in red). The M-domains are depicted in grey and the variable A-domain in green. The transmembrane helix (TM) of the Toc34 homologues is given in a pale yellow and domains other than those already mentioned are colored orange. The numbers indicate amino acids.

1.4.3. The *ppi1* mutant

In the first *Arabidopsis* T-DNA insertion mutant to be identified in which a member of the Toc-complex was affected, the gene coding for atToc33, the *Arabidopsis* ortholog of pea Toc34, was disrupted. This loss-of-function mutant was named *ppi1*, for plastid protein import 1 (Jarvis et al., 1998). The *ppi1* mutant has a pale green seedling phenotype. After three weeks this phenotype disappears, suggesting that atToc33 has a function early in development (Jarvis et al., 1998). This was substantiated by showing that the mRNA levels of atToc33 were increased in young plants and decreased rapidly in older plants (Jarvis et al., 1998). The *ppi1* mutant is specifically impaired in expression and plastid import of photosynthetic proteins, whereas non-photosynthetic proteins are not affected (Kubis et al., 2003). However, it has been demonstrated recently in another atToc33 T-DNA insertion mutant that the accumulation of photosynthetic as well as of non-photosynthetic proteins respectively is affected, suggesting that the assumption of Kubis and colleagues that atToc33 is preferentially involved in the import of photosynthetic proteins is maybe too simple to describe the whole range of atToc33 substrates (Gutensohn et al., 2004).

A second homolog of pea Toc34 exists in *Arabidopsis*, i.e. atToc34, and the weak *ppi1* phenotype suggests that atToc33 and atToc34 are functionally partially redundant. Indeed it was possible to complement the *ppi1* mutant with both atToc33 and atToc34, confirming a functional redundance of the two proteins (Jarvis et al., 1998).

Recently, the production of antisense *Arabidopsis* plants with reduced expression of either atToc33 or atToc34 was reported (Gutensohn et al., 2000). As expected, the phenotypes of the atToc33 antisense plants and the *ppi1* mutant were identical (Jarvis et al., 1998; Gutensohn et al., 2000). Similar to the *ppi1* phenotype, the atToc34 antisense plants also have a slightly pale-green phenotype at the seedling stage, whereas the older plant look like wildtype (Gutensohn et al., 2000).

Furthermore, two independent *Arabidopsis* mutants with T-DNA insertions in the *TOC34* gene were identified. According to the nomenclature previously used, they were named *ppi3* (Constan et al., 2004). Unlike the atToc34 antisense plants, *ppi3* mutants did not have a visible phenotype, reflecting that atToc33 can substitute for the function of atToc34 (Gutensohn et al., 2000; Constan et al., 2004). But when *ppi3* mutants were crossed with *ppi1* mutants, the seedlings homozygous for both T-DNA insertions were not viable, indicating that the combined function of atToc33 and atToc34 is essential and that functional redundancy between atToc33, atToc34 and other Toc-components does not exist (Constan et al., 2004), even though the exact functions of both atToc33 and atToc34 are not known yet.

1.4.4. The *ppi2* mutant

The *Arabidopsis thaliana* mutant *ppi2* has a T-DNA insertion in the gene coding for atToc159 (Bauer et al., 2000; Bauer, 2001). This mutant has a striking albino phenotype and is seedling lethal. As expected in an albino seedling, *ppi2* plastids fail to develop and resemble undifferentiated proplastids, revealing that atToc159 is essential for chloroplast biogenesis (Bauer et al., 2000). Surprisingly, the expression and import of non-photosynthetic proteins does not seem to be affected, whereas the expression of photosynthetic proteins is strongly downregulated. Nevertheless, reduced import of these photosynthetic proteins is still possible (Bauer et al., 2000). Therefore, it has been suggested, that the homologues of atToc159, namely atToc120, atToc132 and atToc90, that are expressed in *ppi2*, may partly compensate for the function of atToc159 (Bauer et al., 2000; Hiltbrunner et al., 2004). The fact that only the expression and import of a distinct group of proteins was affected, led to the hypothesis that atToc159, atToc120, atToc32 and atToc90 could have certain specificities for different classes of preproteins. (Cline, 2000). Therefore, atToc159 could be a receptor with a certain specificity for photosynthetic preproteins (Bauer et al., 2000). Further evidence for this model came recently from two groups reporting the identification of the *Arabidopsis* mutants disrupted in either *TOC120* (*attoc120*) or *TOC132* (*attoc132*) (Ivanova et al., 2004; Kubis et al., 2004). Both homozygous single mutants have the wildtype phenotype, suggesting that atToc120 and atToc132 are functionally redundant (Ivanova et al., 2004; Kubis et al., 2004). Regarding the viability of the double knock out mutant, the two groups obtained contradictory results. One reports that a mutant homozygous for the T-DNA insertion in both *TOC120* and *TOC132* is embryo lethal (Ivanova et al., 2004), whereas the other group reports that the double knock out mutant has a phenotype similar to *ppi2* (Kubis et al., 2004). Nonetheless, both groups suggest that atToc120 and atToc132 may be receptors specific for non-photosynthetic proteins (Ivanova et al., 2004; Kubis et al., 2004). In support of this hypothesis it has been shown that atToc132 binds preferentially to the transit peptide of a preprotein that is non-photosynthetic and constitutively expressed (Ivanova et al., 2004).

Recently, the *Arabidopsis* mutant disrupted in *TOC90*, named *ppi4*, has also been identified and characterized (Hiltbrunner, 2003; Hiltbrunner et al., 2004). In contrast to *ppi1* and *ppi2*, which both display pale phenotypes reflecting a defect in chloroplast biogenesis, *ppi4*, like *attoc120* and *attoc132*, does not show a comparable phenotype (Hiltbrunner et al., 2004; Kubis et al., 2004). This suggests that atToc90 has a non-essential function and that atToc159, atToc120 and atToc132 probably fully substitute for the absence of atToc90 in the *ppi4* mutant (Hiltbrunner et al., 2004). Furthermore, it has been shown that *Arabidopsis* double knock out mutants deficient in either atToc90 and atToc132, or atToc90 and atToc120, do not have a visible phenotype either, suggesting that functional redundancy does not exist between atToc90 and atToc120 or atToc132 (Kubis et al., 2004).

Only a double knock out plant, i.e. *ppi2ppi4*, homozygous for T-DNA insertions in both *TOC159* and *TOC90*, showed an even paler phenotype than the *ppi2* mutant (Hiltbrunner et al., 2004). Whereas atToc75 and atTic110 were found to be present at equal levels in double knock out plants and in wildtype plants, the *ppi2ppi4* mutants, in contrast to *ppi2* seedlings, did not import detectable levels of CAB (Hiltbrunner et al., 2004), suggesting that atToc90 may be involved in the import of CAB and other photosynthetic proteins. But, at least in case if atToc159 is not functional (Hiltbrunner et al., 2004).

1.5. The superfamily of GTP binding proteins

1.5.1. The characteristics of small GTPases and the function of GAPs and GEFs

The small GTP binding proteins of the Ras-like superfamily control a wide variety of functions in the cell, including nucleocytoplasmic and vesicular trafficking, cytoskeletal orientation and assembly, cell proliferation and differentiation processes (Bourne et al., 1990; Scheffzek et al., 1998; Paduch et al., 2001). The superfamily of small Ras-like GTPases contains at least five subfamilies of small GTPases, including Ras, Rho, Rab, Arf and Ran (Kahn et al., 1992; Bischoff et al., 1999; Paduch et al., 2001; Yang, 2002; Vernoud et al., 2003). These GTPases share a conserved GTP binding domain, composed of the characteristic G1 to G5 motifs (Bourne et al., 1991; Kjeldgaard et al., 1996; Paduch et al., 2001), which are essential for GTP binding, GTP hydrolysis and exchange of GDP for GTP (Bourne et al., 1991).

Furthermore, they all have in common that they cycle between a GTP bound active form and a GDP bound inactive form (Bourne et al., 1990, 1991). The slow intrinsic GTP hydrolysis rate of these GTPases is increased by GAPs, i.e. GTPase activating proteins (Wittinghofer et al., 1997; Scheffzek et al., 1998), by several orders of magnitude (Gideon et al., 1992), leading to the inactivation of the GTPase. The switch-on process involves the exchange of GDP for GTP. This exchange of the bound GDP is facilitated by GEFs (guanine nucleotide exchange factors), which catalyze the dissociation of GDP from the inactive GTPase (Cherfils and Chardin, 1999). Furthermore, they stabilize the nucleotide-free form of the GTPase and allow the binding of GTP to regenerate the active GTPase (Cherfils and Chardin, 1999).

1.5.2. The arginine finger

Many GAPs insert a catalytic arginine residue, called arginine finger, into the catalytic site of their corresponding GTPase (Ahmadian et al., 1997; Scheffzek et al., 1997, 1998; Li and Zhang, 2004). The positive charge of this arginine most likely neutralizes the negative charge occurring during the transition from the active GTP bound form to the inactive GDP bound form (Ahmadian et al., 1997; Scheffzek et al., 1998; Li and Zhang, 2004). In this way, the arginine finger of the GAP contributes to the increase of the GTPase activity of the respective GTPase, resulting in its inactivation. The importance of the arginine finger has been substantiated by the demonstration that arginine finger mutants of RasGAPs were not able to accelerate the slow intrinsic GTP hydrolysis of Ras *in vitro* (Ahmadian et al., 1997; Sermon et al., 1998). Furthermore, the mutation of the putative arginine finger of a RasGAP (neurofibromin) to proline results in the complete loss of GAP function *in vivo* and can actually be found in malignant human tumors (Klose et al., 1998).

In the recently solved crystal structure of pea Toc34 homodimer, an arginine (Arg133) of one monomer extends into the GDP binding region of the other monomer, provoking the hypothesis that this arginine, Arg133, possibly functions as arginine finger in the Toc34 homodimer (Sun et al., 2002).

Nonetheless, the arginine finger does not seem to be a prerequisite for GAP function on every small GTP binding protein. Thus, RanGAP accelerates GTP hydrolysis of Ran without an arginine finger (Seewald et al., 2002).

1.5.3. The SRP family of GTPases

1.5.3.1. The characteristics of SRP GTPases

SRP54, the 54 kDa subunit of the signal recognition particle, and SRα (signal recognition particle receptor) and their bacterial homologues Ffh and FtsY, are

GTPases that are involved in cotranslational targeting of nascent proteins to the endoplasmic reticulum (ER) membrane and to the bacterial plasma membrane, respectively (Keenan et al., 2001). Even though the members of the SRP family have a G-domain similar to that of small Ras-like GTPases, they additionally contain an unique N-domain, which forms a characteristic NG-domain together with the G-domain and defines them as a separate subfamily of GTP binding proteins (Freymann et al., 1997; Montoya et al., 1997). No GAPs and GEFs are known for SRP54, SRα and their bacterial homologues up to now (Shan et al., 2004), indicating that they are regulated in a different way. Ffh and FtsY have a very low affinity for nucleotides in comparison to Ras-like GTPases. Furthermore, nucleotide dissociation and exchange are very fast, suggesting that no GEFs are needed for the regeneration of the active GTPase (Jagath et al., 1998; Peluso et al., 2001). Additionally, Ffh and FtsY reciprocally stimulate each other's GTPase activity (Powers and Walter, 1995; Shan et al., 2004). Therefore, no accessory GAP is needed to negatively regulate the GTP bound state.

1.5.3.2. Cotranslational targeting to the ER

Proteins destined for the ER are synthesized with an N-terminal hydrophobic signal sequence on ribosomes in the cytosol. The signal sequence emerging from the ribosome is recognized by the SRP54 subunit of SRP, leading to a pause in translation of the nascent protein (elongation arrest) (Walter and Johnson, 1994). Subsequently, the complex consisting of ribosome, nascent chain and SRP54 is targeted to the ER membrane via interaction with the SRP receptor (SR). The SRP receptor consists of a heterodimer including SRα, interacting with SRP54, and SRβ providing a membrane binding site for SRα (Young et al., 1995). Both SRP54 and SRα have a low intrinsically affinity for GTP (Miller et al., 1995; Bacher et al., 1996; Rapiejko and Gilmore, 1997). The affinity of SRP54 for GTP is increased by interaction with the ribosome (Bacher et al., 1996), whereas the binding of SRP54 including the RNC (ribosome-nascent chain-complex) to SRα leads to an enhanced GTP affinity of SRα and to a stable complex between SRP54 and SRα in their GTP bound state (Rapiejko and Gilmore, 1997). GTP binding to SRβ is then required for the release of the signal sequence from SRP54, leading to the presentation of the signal sequence to the translocon, Sec61 (Fulga et al., 2001). SRP54 and SRα reciprocally activate their GTP hydrolysis (Powers and Walter, 1995; Shan et al., 2004), which is a prerequisite for the dissociation of SRP54 from SRα, allowing SRP54 to initiate a new round of nascent protein targeting. Upon dissociation of SRP54 from SRα, the elongation of the polypeptide chain resumes, leading to an integration of the growing polypeptide into the ER membrane or its translocation across the ER membrane.

1.5.4. The family of small GTPases in plants

An analysis of the *Arabidopsis* genome revealed 93 genes coding for homologues of small GTP binding proteins. These genes code for Rab, Rho, Arf and Ran GTPases, but not for Ras GTPases (Vernoud et al., 2003). The fact that plants contain no Ras GTPases suggests unique mechanisms for the control of cell signaling during plant development (Meyerowitz, 2002).

Nevertheless, many plant small GTPases appear to fulfill functions similar to those of the respective small GTPases in animals and yeast (Yang, 2002; Vernoud et al., 2003). Furthermore, plant small GTPases, like their animal and yeast counterparts, switch between a GTP bound active and a GDP bound inactive state. And this switch is likewise controlled by the action of GAPs and GEFs. In *Arabidopsis*, already some of these interacting GAPs (Wu et al., 2000; Pay et al., 2002; Vernoud et al., 2003) and GEFs (Geldner et al., 2003; Vernoud et al., 2003) for many subfamilies of small GTPases could be identified.

1.5.5. The Toc-GTPases, a new family of plant GTPases

The two GTPases of the pea Toc-complex, psToc34 and psToc159, including their *Arabidopsis* homologues, belong to a new family of GTPases (Sun et al., 2002). They share a very similar GTP binding domain (Hiltbrunner et al., 2001a). But in comparison to the small GTPases, only the G1 and the G3 motif are quite conserved, whereas the other motifs, G2, G4 and G5, are quite dissimilar (Fig. 2). It has been hypothesized that the Toc-GTPases may have evolved novel mechanisms for GTP binding and hydrolysis different from those of small GTPases (Kessler and Schnell, 2002; Sun et al., 2002). This was corroborated by the recently solved crystal structure of pea Toc34 (Sun et al., 2002) (Fig. 3). The structure of psToc34, in comparison to the structure of Ras, contains 6 inserts, which are, with one exception, involved in dimerization and GTPase activity (Sun et al., 2002).

```
                                                                     G1
atToc33      1 MGSLVREWVGFQQFPAATQEKLIEFFGKLKQKDMNSMTVLVLGKGGVGKSSTVNSLIGEQ
atToc159G  855 -----------------------------------SLNILVLGKAGVGKSATINSILGNQ
h-ras        1 ---------------------------------MTEYKLVVVGAGGVGKSALTIQLIQNH

                        G2                    G3
atToc33     61 VVRVSPFQAEGLRPVMVSRTMGGFTINIIDTPGLVEAGYVN---HQALELIKGFLVNRTI
atToc159G  880 IASIDAFGLSTTSVREISGTVNGVKITFIDTPGLKSAAMDQSTNAKMLSSVKKVMKKCPP
h-ras       28 FVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNT

                   D1                   G4
atToc33    118 DVLLYVDRLDVYRVDELDKQVVIAITQTFGKEIWCKTLLVLTHAQFSPPD-----ELSYE
atToc159G  940 DIVLYVDRLDTQTRDLNNLPLLRTITASLGTSIWKNAIVTLTHAASAPPDGPSGTPLSYD
h-ras       88 KSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARSYG-----IPYI

                 G5
atToc33    173 TFSSKRSDSLLKTIRAGSKMRKQEFEDSAIAVVYAENSGRCSKNDKDEKALPNGEAWIPN
atToc159G 1000 VFVAQCSHIVQQSIGQAVGDLRLMNPSLMNPVSLVENHPLCRKNREGVKVLPNGQTWRS-
h-ras      143 ETSAKTRQGVEDAFYTLVREIRQHKLRKLNPP--DESGPGCMSCKCVLS-----------

atToc33    233 LVKAITDVATNQRKAIHVDKKMVDGSYSDDKGKKLIPLIIGAQYLIVKMIQGAIRNDIKT
atToc159G 1060 --------------------QLLLLCYSLKVLSETNSLLRPQEPLDHRKVFGFR------
h-ras          ------------------------------------------------------------

atToc33    293 SGKPL
atToc159G      -----
h-ras          -----
```

Fig. 2 Sequence alignment of atToc33, atToc159G and human ras (h-ras) to visualize the G-motifs characteristic for the GTPase superfamily: Amino acids identical in all three sequences are colored red, whereas amino acids identical in two sequences are given in blue. The motifs are indicated above the three sequences (Bourne et al., 1991; Paduch et al., 2001). G-motifs quite conserved in Toc-GTPases are given in black (G1 and G3), whereupon G-motifs not conserved in Toc-GTPases are colored grey (G2, G4 and G5). The dimerization-motif (D1) specific for Toc-GTPases is also indicated above the sequences (Sun et al., 2002). The multiple sequence alignment was done using the BCM search launcher, followed by edition employing the Boxshade programme version 3.21. Accession numbers: atToc33 (U89959), atToc159 (AF069298), h-ras (AF493916). atToc159G includes amino acids 855-1092 of full-length atToc159.

Pea Toc34 is found in the crystal exclusively as homodimer (Fig. 3). One GDP as well as one Mg^{2+} ion are bound to each monomer in the dimer (Sun et al., 2002) (Fig. 3). The GDP binding to the pea Toc34 dimer is observed in the absence of additional nucleotides during the purification step, thus psToc34 has sufficient affinity for guanine nucleotides to allow stable binding under the conditions used (Sun et al., 2002). The Mg^{2+} ion which was found in the pea Toc34 dimer is in agreement with the essential role of Mg^{2+} for GTP binding and/or hydrolysis of many GTPases (Sprang and Coleman, 1998), suggesting that Mg^{2+} also contributes to guanine nucleotide binding in Toc-GTPases.

The fact that the crystal structure of pea Toc34 is comprised of homodimers resembling a small GTPase with its bound GAP led to the hypothesis that one monomer may function as the GAP for the other monomer in the pea Toc34 dimer (Sun et al., 2002), similar to the reciprocal activation of the members of the SRP family of GTPases, i.e. *E. coli* Ffh and FtsY and their eukaryotic homologues SRP54 and SRα, which are thought to act as GAPs for one another (Powers and Walter, 1995; Rapiejko and Gilmore, 1997; Shan et al., 2004).

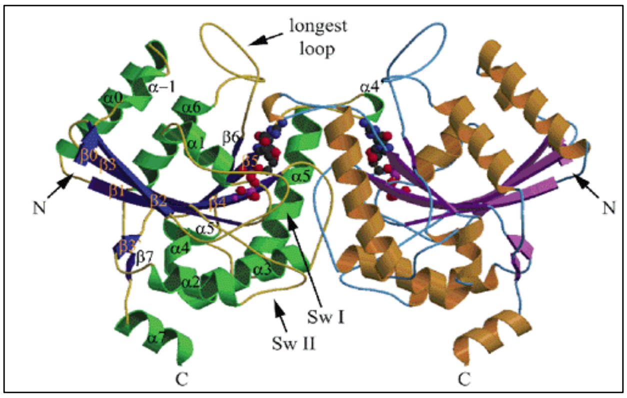


Fig. 3 The crystal structure of pea Toc34: A ribbon drawing of the psToc34 dimer (Sun et al., 2002). The α-helices of monomer 1 are depicted in green, the β-sheets in dark blue and the longest loop in yellow. Monomer 2 is colored orange, violet and light blue, respectively. GDP is shown as a ball-and-stick model and Mg^{2+} is colored magenta. SwI and SwII (switch I and II) indicate two regions of the active site that adopt different conformations in the active and inactive state of the GTPase.

1.6. Two current models of preprotein import across the outer pea chloroplast membrane

It has long been known that hydrolysis of GTP plays a role in preprotein import into chloroplasts (Kessler et al., 1994; Young et al., 1999). But the precise function of the two GTPases, Toc159 and Toc34, is still under intense debate. To date there exist two different models with regard to the function of these GTPases at the outer pea chloroplast membrane (Kessler and Schnell, 2004).

The two current models for preprotein import into chloroplasts are discussed here for pea, due to the fact that the situation at the outer chloroplast membrane of *Arabidopsis* appears to be more complex than in pea, and similar models do not exist yet.

1.6.1. The motor hypothesis

The recent motor hypothesis (Becker et al., 2004b; Kessler and Schnell, 2004) (Fig. 4) suggests that the C-terminal, phosphorylated part of the transit peptide of a preprotein binds to the primary preprotein receptor, Toc34-GTP, at the chloroplast outer membrane, whereas the N-terminal part of the transit peptide contacts Toc159-GTP (Becker et al., 2004b) which results in a strong interaction between Toc34-GTP, Toc159-GTP and the preprotein (Becker et al., 2004b). The binding of the preprotein to Toc34 activates the GTP hydrolysis of Toc34 (Jelic et al., 2002), whereupon the precursor protein is released from Toc34. The dephosphorylation of the transit peptide by a yet unidentified phosphatase causes its tight binding to Toc159-GTP (Becker et al., 2004b). Thereafter, Toc34-GDP dissociates from Toc159-GTP which acts as a motor protein and translocates the preprotein through the protein-conducting channel via repeated cycles of GTP hydrolysis and subsequent exchange of GDP for GTP (Becker et al., 2004b). Once the import is completed, both Toc34 and Toc159 are regenerated for a new round of preprotein import by exchange of GDP for GTP.

In summary the GTP hydrolysis in this model serves to pass the preprotein from Toc34 to Toc159 as well as to accomplish the motor function at Toc159 (Becker et al., 2004b; Kessler and Schnell, 2004) (Fig. 4).

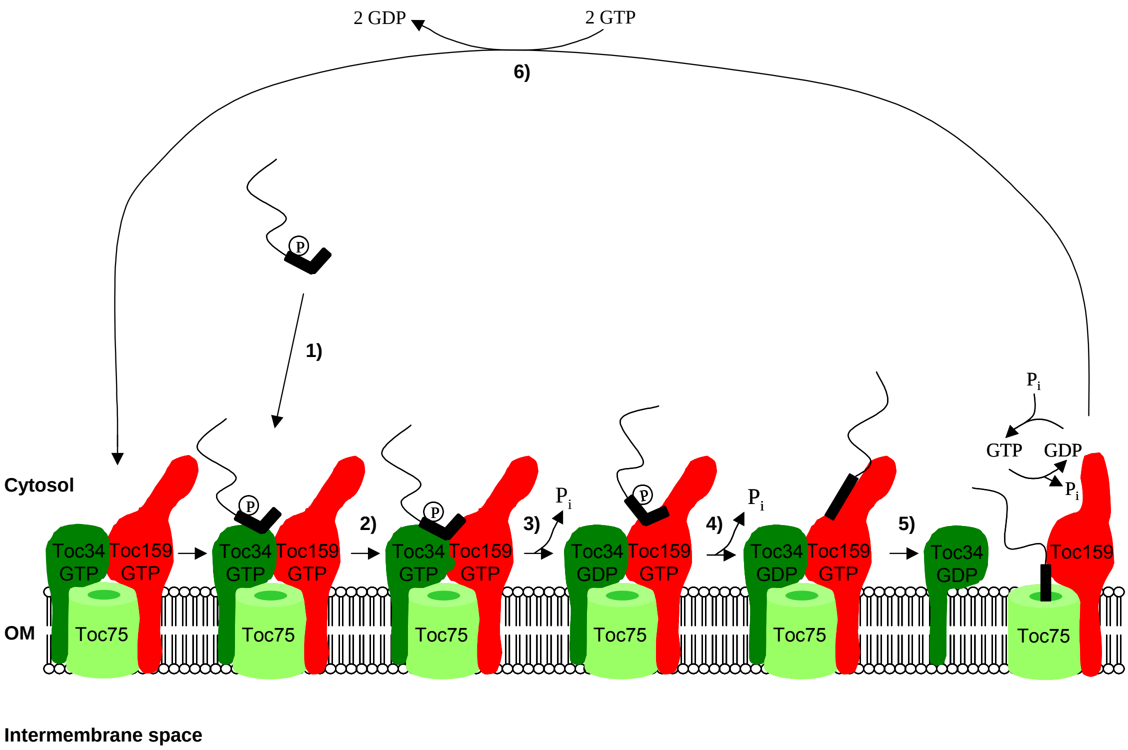


Fig. 4 The motor hypothesis for the preprotein import across the outer chloroplast membrane (OM) of pea: Toc34-GTP acts as the primary receptor at the outer chloroplast membrane and binds to the C-terminal part of the phosphorylated transit peptide (1), whereas Toc159-GTP contacts the N-terminal part of the transit peptide, resulting in a strong interaction between Toc34-GTP, Toc-159-GTP and the preprotein (2). The interaction with the transit peptide increases the GTPase activity of Toc34, which decreases the affinity of the transit peptide for Toc34 (3). Dephosphorylation of the transit peptide is prerequisite for the binding of the C-terminal part to Toc159-GTP (4). The GDP bound Toc34 dissociates from the Toc-complex, allowing Toc159 to thread the preprotein through the Toc75 channel in the outer membrane via repeated cycles of GTP hydrolysis (5). Exchange of GDP for GTP at both Toc34 and Toc159 regenerates the trimeric Toc-complex for a new cycle of preprotein targeting and translocation (6).

1.6.2. The targeting hypothesis

1.6.2.1. Protein import across the outer chloroplast membrane according to the targeting hypothesis

Initially, Toc159 was proposed to act as the primary receptor for the preproteins at the chloroplast membrane. The recent identification of a cytosolic form of Toc159, suggests that Toc159 not only acts as a preprotein receptor at the chloroplast membrane but also as a soluble receptor in the cytosol (Hiltbrunner et al., 2001b). The cytosolic Toc159 binds to the preprotein mainly through its G-domain, a process requiring guanine nucleotides (Smith et al., 2004). Because the direct interaction between atToc159 and atToc33, the *Arabidopsis* ortholog of pea Toc34, was shown (Hiltbrunner et al., 2001b; Smith et al., 2002; Weibel et al., 2003), it was proposed that Toc34 serves as the docking site for the cytosolic Toc159 carrying the preprotein (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002; Wallas et al., 2003). Thereby Toc159-GTP and Toc34-GTP may associate with low-affinity through their G-domains. The association of Toc34, Toc159, Toc75 and the preprotein stimulates GTP hydrolysis at both GTPases. GTP hydrolysis by Toc159 results in the insertion of its membrane domain into the outer chloroplast membrane (Smith et al., 2002). Furthermore, Toc159-GDP interacts with Toc34-GDP with high affinity. Support for this interaction between the two GTPases in their GDP bound form comes from *in vitro* experiments, showing that the binding between atToc159 and atToc33, the ortholog of pea Toc34, is stabilized by GDP (Smith et al., 2002). Moreover, the high similarity of the G-domains of Toc159 and Toc34 makes the occurrence of not only GDP bound homodimers of Toc34, as seen in the crystal (Sun et al., 2002), but also of GDP bound heterodimers of Toc159 and Toc34 highly likely (Kessler and Schnell, 2002).

The tight interaction between Toc159-GDP and Toc34-GDP initiates the translocation of the preprotein through the protein-conducting channel at the outer chloroplast membrane, Toc75, and subsequently also across the inner membrane. After the disengagement of the preprotein from Toc159, an exchange of GDP for GTP may take place at Toc159. Thereafter Toc159 is released into the membrane or the cytosol to undergo a new round of preprotein import.

In this model, GTP hydrolysis serves to coordinate the action of Toc159, Toc34 and Toc75 during the import of preproteins into chloroplasts, ensuring the unidirectionality of the process (Weibel et al., 2003; Kessler and Schnell, 2004) (Fig. 5).

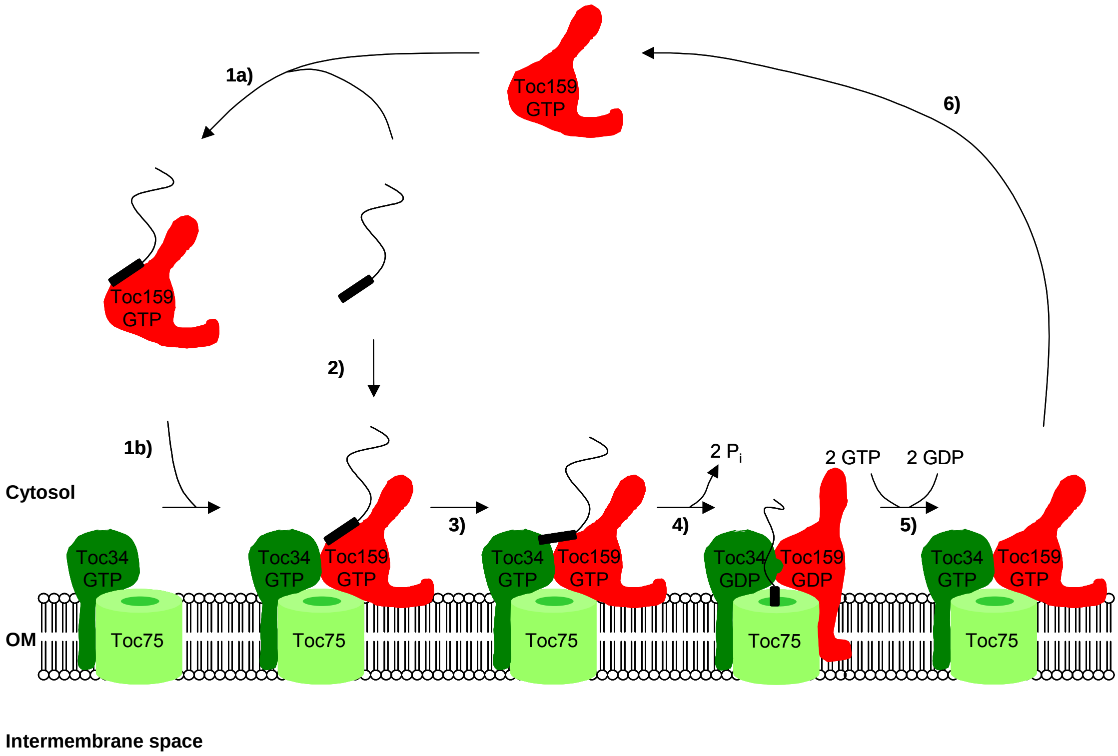


Fig. 5 The targeting hypothesis of protein transport across the outer pea chloroplast membrane (OM): The soluble Toc159-GTP acts as the primary receptor and binds to the transit peptide of the preprotein in the cytosol (1a). Subsequently Toc159-GTP, with the preprotein, is targeted to the outer chloroplast membrane via a low affinity interaction with Toc34-GTP (1b). Alternatively, the preprotein may interact directly with the membrane-bound Toc159-GTP (2). This interaction between the preprotein and the trimeric Toc-complex (3) results in GTP-hydrolysis at both Toc34 and Toc159. Thereby a stable interaction between the two GTPases in their GDP bound state is formed, triggering the insertion of Toc159-GDP into the membrane. Thereafter, the preprotein is handed to Toc75 for further translocation (4). After completion of translocation, GDP at Toc34 and Toc159 is exchanged for GTP (5) and Toc159 may be released into the cytosol for a new cycle of preprotein targeting (6).

1.6.2.2. The main differences between targeting and motor hypothesis

The main differences between the targeting and the motor hypothesis concern the nature of the primary preprotein receptor, the function of GTP hydrolysis at both Toc-GTPases, and the existence of the soluble form of Toc159.

Because it has been demonstrated *in vitro* as well as *in vivo* that Toc159 is not only an integral membrane protein but does also exist in a soluble, cytosolic form (Hiltbrunner et al., 2001b; Lee et al., 2003), the targeting hypothesis suggests that

Toc159 acts as a soluble preprotein receptor in the cytosol. This was corroborated by the finding that soluble Toc159 interacts directly and specifically with the transit peptide of preproteins (Smith et al., 2004). In contrast to this, evidence has been provided by the supporters of the motor hypothesis, that Toc159 is exclusively found in the outer membrane and that the soluble form of Toc159 is due to membrane shreds derived from partial membrane disruption during the experimental membrane fractionation process (Becker et al., 2004b).

According to the targeting hypothesis, Toc159 is the primary preprotein receptor at the outer chloroplast membrane. This is based on preprotein binding (Chen et al., 2000) and crosslinking studies (Perry and Keegstra, 1994; Ma et al., 1996; Kouranov and Schnell, 1997) using isolated chloroplasts. Furthermore, it has been demonstrated that soluble Toc159 directly interacts with preproteins (Smith et al., 2004). In opposition to this, several findings led the authors of the motor hypothesis to propose that Toc34 serves as the primary receptor for preproteins at the outer chloroplast membrane. First, Toc34 binds to phosphorylated chloroplast preproteins with high affinity (Sveshnikova et al., 2000b; Schleiff et al., 2002), whereas Toc159 only binds to non-phosphorylated preproteins (Becker et al., 2004b). Since it was shown that dephosphorylation of precursor proteins is required for their complete translocation into isolated chloroplasts (Waegemann and Soll, 1996), Toc34 may function before Toc159 in precursor recognition and binding. Second, the preprotein binding to Toc34 was shown to enhance GTP hydrolysis of Toc34 (Jelic et al., 2002). Third, Toc34 was mainly crosslinked to preproteins at early import stages (Kouranov and Schnell, 1997).

Regarding the function of GTP hydrolysis at the two GTPases, Toc159 is, according the motor model, suggested to function as a GTP driven motor that pushes the preproteins across the membrane (Schleiff et al., 2003a). Reconstitution experiments into liposomes, in which Toc159 and Toc75 are the minimal translocon machinery allowing preprotein import dependent on GTP hydrolysis, corroborate this assumption (Schleiff et al., 2003a). Moreover, cryoelectron microscopy of purified Toc-complexes revealed a toroid structure with a dense ring at the outside and a less dense part in the center (Schleiff et al., 2003b). Isolated Toc-complexes revealed an approximative stoichiometry of 1:4:4 between Toc159:Toc75:Toc34, and it was suggested that Toc159, the motor molecule, is located in the center of the Toc-complex, surrounded by four molecules of Toc34 and four molecules of Toc75

(Schleiff et al., 2003b). In contrast to this, the targeting model proposes first, that the GTP hydrolysis at both GTPases is needed for the proper insertion of Toc159 into the outer chloroplast membrane (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003), leading to the assembly of the functional Toc-complex, and second, that GTP hydrolysis at Toc34 and Toc159 triggers the insertion of the preprotein into the protein-conducting channel (Young et al., 1999).

1.6.2.3. The homotypic interaction between Toc34 and Toc159

The homotypic interaction between Toc34 and soluble Toc159 plays a central role in the targeting model for preprotein import into pea described above. Similar to SRα functioning as a receptor for SRP54 including the RNC (ribosome-nascent chain complex) at the ER membrane (Keenan et al., 2001), it has been suggested that Toc34 acts as receptor for soluble Toc159 including the preprotein at the outer chloroplast membrane (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002; Wallas et al., 2003). This is supported by several findings. Toc34 and Toc159 contain a conserved dimerization-motif in their G-domain (Sun et al., 2002), suggesting that they both form homo- and/or heterodimers (Kessler and Schnell, 2002). This has been corroborated by *in vitro* pull-down assays showing that atToc33, the *Arabidopsis* ortholog of pea Toc34, interacts with atToc159 and that the G-domains are sufficient for this interaction (Hiltbrunner et al., 2001b; Bauer et al., 2002; Smith et al., 2002). In isolated *Arabidopsis* chloroplasts it has been demonstrated that soluble atToc33 inhibits the insertion of atToc159 into the chloroplast membrane, suggesting that soluble atToc33 and atToc159 interact with each other in the cytosol and prevent the interaction of cytosolic atToc159 with membrane bound atToc33 (Hiltbrunner et al., 2001b).
Furthermore it has been shown, that the GTPase activity of both GTPases is needed for proper insertion of Toc159 into chloroplasts (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003), suggesting that GTP hydrolysis plays an important role in receptor targeting at the outer chloroplast membrane (Wallas et al., 2003). In support of this, it has been demonstrated that the stable dimerization between the G-domains of atToc159 and atToc33 is favored by GDP *in vitro* (Smith et al., 2002).

1.7. Aims of this thesis

Transport machineries in the outer and inner chloroplast membrane, i.e. the Toc- and Tic-complex respectively, accomplish the import of cytosolically synthesized preproteins into chloroplasts. The crystal structure of pea Toc34, a core component of the Toc-complex, was solved (Sun et al., 2002). In the crystal, pea Toc34 existed exclusively as GDP bound dimers resembling a GTP binding protein with its corresponding GAP. Thus, it was suggested that one monomer may act as a GAP (GTPase activating protein) on the other monomer in the pea Toc34 dimer (Sun et al., 2002). Moreover, the position of Arg133 of one monomer, extending into the GDP binding site of the other monomer in the pea Toc34 dimer, resembled that of an arginine finger (Sun et al., 2002). The first aim of this thesis is the examination of the arginine finger hypothesis.

Toc159 and Toc34 are two integral membrane, GTP binding proteins (Kessler et al., 1994), involved in preprotein recognition and binding at the outer chloroplast membrane. They share a conserved GTP binding domain containing an unique motif shown to be involved in Toc34 homodimerization (Sun et al., 2002). This finding suggested that both GTPases may be able to form homo- as well as heterodimers. The crystal structure of psToc34 demonstrated homodimerization in the GDP bound form. The second aim of this work is the examination of the requirement for guanine nucleotide for the homodimerization of atToc33, the *Arabidopsis* ortholog of psToc34, in a soluble-phase pull-down assay to test whether dimerization is supported exclusively by GDP.

It has been demonstrated that Toc34 functions as the receptor for soluble Toc159 at the outer chloroplast membrane (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002; Wallas et al., 2003). Furthermore, it has been shown that the GTPase activity at both GTP binding proteins is essential for proper insertion of Toc159 into isolated chloroplasts (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003), suggesting that the GTPase activity at both GTPases is a prerequisite for the interaction between Toc159 and Toc34. Nonetheless, the requirement for guanine nucleotide for the heterodimerization between Toc159 and Toc34 is still under debate (Smith et al., 2002; Becker et al., 2004b). The third aim of this thesis is the examination of the guanine nucleotide dependency of the heterodimerization between atToc159 and atToc33 in an *in vitro* pull-down assay.

2. Materials and methods

2.1. Materials

2.1.1. Vectors

pET21d was purchased from Novagen, Inc., Madison, WI, USA. The vector pGEX-4T-1 was obtained from Amersham Biosciences AB, Uppsala, Sweden.

2.1.2. *E. coli* strains

E. coli DH5α was from Invitrogen AG, Basel, Switzerland. *E. coli* BL21(DE3) cells were obtained form Novagen, Inc., Madison WI, USA.

2.1.3. Oligonucleotides

The oligonucleotides were synthesized at Microsynth GmbH, Balgach, Switzerland.

2.1.4. Antibodies

Antibodies specific for the hexahistidinyl-tag were purchased from Qiagen AG, Basel, Switzerland. The antibody recognizing atToc33 (αatToc33) was a gift from Dr. D.J. Schnell (University of Massachusetts, Amherst MA, USA)

2.1.5. Chemicals

Unless noted otherwise, the chemicals were from Fluka Chemie GmbH, Buchs SG, Switzerland. [^{35}S]-methionine for the *in vitro* transcription/translation reactions was from Hartmann Analytic GmbH, Braunschweig, Germany. [^{32}P]-GTP was either from

ICN Biomedicals, Inc., Costa Mesa, USA or from Hartmann Analytic GmbH, Braunschweig, Germany.

2.2. Methods

2.2.1. Methods used for standard cloning

2.2.1.1. PCR, restriction digest and ligation

The required DNA-fragment was amplified in a PCR reaction including a plasmid template, two primers containing appropriate restriction sites and Taq-polymerase (Roche Diagnostics GmbH, Mannheim, Germany), according to the instructions of the supplier. Following the PCR reaction, the PCR products were digested with the respective restriction enzymes (New England BioLabs, Inc., Beverly MA, USA or Fermentas UAB, Vilnius, Lithuania), following a standard protocol (Sambrook and Russell, 2001). The vector was treated equally. The restriction fragments were separated on low melting agarose gels (Eurobio, Les Ulis Cedex B, France) and the fragments corresponding to the gene and the vector were cut out of these gels. The DNA in the gel-slices was then directly used for the ligation with T4 DNA ligase (Fermentas UAB), according to standard protocols (Sambrook and Russell, 2001).

2.2.1.2. Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells, DH5α and BL21(DE3), were prepared as described previously (Inoue et al., 1990).
The competent *E. coli* cells were transformed with the ligation products obtained (see 2.2.1.1.), according to standard protocols (Sambrook and Russell, 2001). Clones containing the transformed plasmid were selected on LB plates (25g/l LB Broth Miller (Beckton Dickinson Diagnostic Systems, Sparks MD, USA), 1.5% Agar Bacteriological Grade, ICN Biomedicals, Inc., Costa Mesa CA, USA) containing Ampicillin (100 µg/ml) (Eurobio, Les Ulis Cedex B, France).

2.2.1.3. Plasmid isolation

For small-scale isolation of plasmids from 5 ml cell cultures, the QIAprep Spin Miniprep Kit was used, following the manufacturer's insctructions (Qiagen AG, Basel, Switzerland). For the isolation of higher amounts of plasmids from 50 ml cell cultures the QIAprep Spin Midiprep Kit was used (Qiagen AG).

2.2.1.4. DNA-sequencing

DNA-sequencing was done at Microsynth GmbH, Balgach, Switzerland.

2.2.2. DNA constructs used for expression and in *vitro* transcription/ translation

2.2.2.1. pET21d constructs

pET21d-Toc33$_{1\text{-}265}$H$_6$ (Toc33 wt):

The cloning of pET21d-Toc33$_{1\text{-}265}$H$_6$ (Toc33 wt) has been described previously (Hiltbrunner et al., 2001b).

pET21d-Toc33$_{1\text{-}265}$H$_6$R130A (Toc33 R130A):

pET21d-Toc33$_{1\text{-}265}$H$_6$ was used to mutate R130 via site directed mutagenesis. Toc33$_{1\text{-}265}$H$_6$R130A (Toc33 R130A) was amplified in two pieces from pET21d-Toc33$_{1\text{-}265}$H$_6$ using PCR with the following primer pairs: forward primer 5'-GAA ATT AAT ACG ACT CAC TAT AGG GG-3' and reverse primer 5'-CCC AAG CTT GAG CTC ATC GAC TGC ATA CAC ATC CAA ACG ATC A-3', including a *Sac*I site and forward primer 5'-CAT GGA GCT CGA TAA GCA AGT TGT TAT AG-3', including a *Sac*I site, and reverse primer 5'-TTA TGC TAG TTA TTG CTC AG-3'. PCR fragments were then ligated into the *Eco*RI-*Bam*HI site of pET21d-Toc33$_{1\text{-}265}$H$_6$ in a triple ligation.

pET21d-Toc159 (Toc159):

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

pET21d-Toc159$_{727\text{-}1093}$ (Toc159G):

Toc159$_{727\text{-}1093}$ (Toc159G) was amplified from pET21d-Toc159 using primers including a *Bsp*HI (5'-CAT GTC ATG ACT AGT CAG GAT GGT ACG-3') and a *Not*I site (5'-ATA AGA ATG CGG CCG CTT AAA CTC GGA AAC CAA ATA CTT TAC G-3'), respectively. Ligation of the *Bsp*HI/*Not*I digested fragment into the *Nco*I/*Not*I site of pET21d resulted in pET21d-Toc159$_{727\text{-}1093}$ (Toc159G).

2.2.2.2. pGEX-4T-1 constructs

pGEX-4T-1-Toc33$_{1\text{-}265}$ (Toc33-GST):

Using primers including a *Bgl*II site (5'-GGA AGA TCT ACC ATG GGG TCT CTC GTT CG-3') and a *Sal*I site (5'-ACG CGT CGA CTT ACT TTC CTT TAT CAT CAG AG-3'), Toc33$_{1\text{-}265}$ was amplified from pET21d-Toc33$_{1\text{-}265}$H$_6$. Subsequently, the *Bgl*II/*Sal*I digested PCR-fragment was ligated into the *BamH*I/*Sal*I site of pGEX-4T-1, resulting in pGEX-4T-1-Toc33$_{1\text{-}265}$ (Toc33-GST).

pGEX-4T-1-Toc159$_{727\text{-}1093}$ (Toc159G-GST):

Toc159$_{727\text{-}1093}$ was amplified from pET21d-Toc159$_{727\text{-}1093}$ with primers introducing a *BamH*I site (5'-CGG GAT CCA TGA CTA GTC AGG ATG GTA CGA A-3') and a *Not*I site (5’-ATA AGA ATG CGG CCG CTT AAA CTC GGA AA-3’). The *BamH*I/*Not*I digested fragment was subcloned into the *BamH*I/*Not*I site of pGEX-4T-1 to generate pGEX-4T-1-Toc159$_{727\text{-}1093}$ (Toc159G-GST).

2.2.3. Bacterial expression and purification

2.2.3.1. pET21d constructs

For bacterial expression, the pET21d constructs were transformed into *E. coli* BL21(DE3) cells. Following expression, the protein was purified under non-denaturing conditions using Ni-NTA (QIAGEN AG, Basel, Switzerland) chromatography. The bound protein was eluted with an imidazole step gradient ranging from 50 mM to 250 mM. Fractions containing the protein were dialyzed against 50 mM Tris/HCl pH 8.0, 25 mM KOAc, 1 mM DTT and 1 mM $MgCl_2$. The dialysate was centrifuged at 15’000 x *g* to remove insoluble aggregates. The protein concentration of the purified recombinant protein was determined using the Bradford assay (Bradford, 1976).

2.2.3.2. pGEX-4T-1 constructs

The pGEX-4T-1 constructs were transformed into *E. coli* BL21(DE3) cells. After the bacterial expression, the protein was purified under non-denaturing conditions using GST-sepharose (Amersham Biosciences AB, Uppsala, Sweden) chromatography, according to the specifications of the supplier. After elution of the bound protein from the column with 10 mM reduced glutathione and 50 mM Tris/HCl pH 8.0, the protein fractions were dialyzed against 50 mM Tris/HCl pH 8.0, 25 mM KOAc, 1 mM DTT and 1 mM $MgCl_2$, followed by centrifugation of the dialysate at 15’000 x *g* and determination of the protein concentration of the purified protein according to Bradford (1976).

2.2.4. SDS-PAGE, Western blot analysis and immunodetection

2.2.4.1. SDS-PAGE and immunoblotting

An apparatus from Bio-Rad (Bio-Rad Laboratories, Hercules CA, USA) was used for protein analysis by SDS-PAGE (Laemmli, 1970). The percentage of the separating gel was chosen according to the mass of the proteins to be separated, ranging from 10 to 12%. The separating gel consisted of 10-12% (w/v) acrylamid/bisacrylamide (37.5:1, Bio-Rad), 0.4 M Tris/HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (v/v) TEMED (Bio-Rad) and 0.08% (w/v) APS (Bio-Rad). The stacking gel that was poured on top of the separating gel was composed of 4% (w/v) acrylamid/bisacrylamide (37.5:1, Bio-Rad), 0.06 M Tris/HCl pH 6.8, 15% (w/v) sucrose, 0.1% (w/v) SDS, 0.1% TEMED (Bio-Rad) and 0.08% APS (w/v). Prior to the separation of the proteins on the SDS-PAGE gel, the proteins, taken up in 1xSDS-PAGE sample buffer (0.04 M Tris/HCl pH 6.8, 0.1 M DTT, 2% (w/v) SDS, 0.1% (w/v) Bromphenol blue, 10% glycerol (v/v)), were heated to 65°C for 10 minutes.
Following the separation, the gels were either stained with Coomassie blue, according to standard protocols (Sambrook and Russell, 2001), and dried, or used for Western blot transfer to Protran® Nitrocellulose Transfer Membrane (Schleicher & Schuell, Dassel, Germany) according to standard protocols (Sambrook and Russell, 2001).

2.2.4.2. Immunodetection using antibodies recognizing atToc33

The western blot membrane (2.2.4.1.) was colored with Amido black according to a standard protocol (Sambrook and Russell, 2001), destained (in 40% (v/v) MeOH, 10% (v/v) acetic acid) and scanned. To block the unspecific binding of antibodies, the membrane was incubated for 1 hour in 5% (w/v) skim milk powder in 1xTBS (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris/HCl pH 7.4). Subsequently, the membrane was incubated for another hour with antibodies specific for atToc33, diluted 1:1’000 in 5% (w/v) skim milk powder in 1xTBS. After 3 washing steps with 1xTBS, the membrane was incubated for 30 minutes with horseradish peroxidase coupled goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules CA, USA) diluted 1:3’000 in 5% (w/v) skim milk powder in 1xTBS. After another three extensive washing steps, the signals were detected by enhanced chemoluminescence. The blot was incubated for 1 minute in 0.1 M Tris/HCl pH 8.5, 0.2 mM p-coumaric acid, 1.25 mM luminol (3-aminophthalhydrazide) and 0.009% H_2O_2, followed by exposure to X-ray films (Eastman Kodak Company, Rochester NY, USA).

2.2.4.3. Immunodetection with antibodies specific against the hexahistidinyl-tag

Following the staining and destaining of the western blot according to standard protocols (Sambrook and Russell, 2001), the immunodetection using antibodies specific for the hexahistidinyl-tag was performed pursuant to the protocol of the supplier (Qiagen AG, Basel, Switzerland), whereas the detection of the signals was done as described above (2.2.4.2.).

2.2.5. Blue native PAGE

The 5.5%-16% polyacrylamide gradient gels, as well as the electrophoresis buffers, were prepared according to previously described protocols (Schagger and von Jagow, 1991). Normally, 10 µg BSA (Eurobio, Les Ulis Cedex B, France) was used as a standard. Electrophoresis, performed at 4°C, was started at 80 V and increased to 200 V after the proteins had reached the separating gel. The gels were additionally stained with Coomassie blue, according to standard protocols (Sambrook and Russell, 2001), prior to their analysis.
For western blot analysis following blue native PAGE, the protein bands of interest were excised from the gel. The gel pieces were destained and the protein eluted from the gel with 200 µl 2% (w/v) SDS, 50 mM Tris/HCl pH 7.5 and 1 mM DTT and precipitated using the chloroform/methanol method (Wessel and Flügge, 1984). Following the precipitation, the eluted protein was used for SDS-PAGE gel electrophoresis and subsequent western blotting (see 2.2.4.1.). The blot was probed with antibodies recognizing atToc33 (αatToc33), and the signals were detected using enzyme-linked chemoluminescence (see 2.2.4.2.).

2.2.6. [α^{32}P]-GTP binding and hydrolysis assays

2.2.6.1. [α^{32}P]-GTP binding

[α^{32}P]-GTP binding to purified, recombinant proteins was determined as follows: A 1 µM aliquot of the recombinant protein was incubated on ice for 60 minutes in the presence of binding buffer (20 mM Tris/HCl pH 8.0, 50 µM $MgCl_2$, 0.3% (v/v) Tween 20, 1 mM ATP) containing 0.1 µM [α^{32}P]-GTP (3 Ci/µmol) in a final volume of 10 µl. A 2 µl aliquot of the reaction was spotted onto a nitrocellulose membrane, which had been preincubated in binding buffer and air-dried. The membrane was washed three times with 10 ml ice-cold wash buffer (20 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 0.3% (v/v) Tween 20) and air-dried. Bound [α^{32}P]-GTP was detected and quantified using a Phosphorimager (GS-250 Molecular ImagerTM) and Molecular Analyst software Mac Version 2.0.1 (Bio-Rad Laboratories, Hercules CA, USA).

To determine [α^{32}P]-GTP (50 nM) binding to the recombinant protein in the presence of non-labeled GTP or GDP, the binding buffer was supplemented with increasing concentrations (0-300 µM) of unlabeled GTP and GDP, respectively. The [α^{32}P]-GTP binding assay and the quantification was performed as described above. From this, the binding of [α^{32}P]-GTP to the recombinant proteins (expressed as the percentage of binding without competition) in presence of increasing concentrations of unlabeled GTP or GDP was calculated and plotted against the ratio of unlabeled nucleotide vs. [α^{32}P]-GTP.

2.2.6.2. Influence of Mg^{2+} on [α^{32}P]-GTP binding to the recombinant proteins

To study the influence of Mg^{2+} on [α^{32}P]-GTP binding to the recombinant GTPases, 2 µM of the recombinant protein was incubated during 30 minutes on ice with 0.2 µM [α^{32}P]-GTP (3 Ci/µmol) in binding buffer (20 mM Tris/HCl pH 8.0, 50 µM $MgCl_2$, 0.3% (v/v) Tween 20) and 1 mM ATP in a total volume of 40 µl. As a negative control, a reaction with 2 µM BSA (Eurobio, Les Ulis Cedex B, France) was performed. After this incubation, half of the reaction mixture (15 µl) was either incubated again with binding buffer or with binding buffer including 5 mM EDTA (final concentration) in a total volume of 30 µl. After 30 minutes incubation on ice, 10 µl of the reaction batch incubated in binding buffer was mixed with 10 µl binding buffer and half of the reaction batch that was treated with 5 mM EDTA (10 µl) was supplemented with the same volume of 5 mM EDTA and the other half with the same volume of $MgCl_2$ (10 mM final concentration) followed by an incubation on ice for 30 minutes. Finally, 2 µl of each reaction batch, corresponding to 500 nM recombinant protein, was spotted onto Protran® Nitrocellulose Transfer Membrane (Schleicher & Schuell, Dassel, Germany) which had been preincubated in binding buffer. Subsequently, the membrane was washed extensively in wash buffer (20 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 0.3% (v/v) Tween 20) and air-dried. Radioactivity in the spots was then quantified using a Phosporimager (GS-250 Molecular ImagerTM) and the Molecular Analyst software Mac Version 2.0.1 (Bio-Rad Laboratories, Hercules CA, USA).

2.2.6.3. [α^{32}P]-GTP hydrolysis

GTP hydrolysis was measured using a method adapted from Liang et al. (2000). A 0.5 µM aliquot of the recombinant protein was incubated at 25°C in 20 mM Tris/HCl pH 8.0, 25 mM KOAc, 2 mM $MgCl_2$, 0.1 g/l BSA and 50 nM [α^{32}P]-GTP (3 Ci/µmol) in a final volume of 50 µl. After 0, 60, 120 and 240 minutes of incubation, 10 µl of the reaction was removed and stopped by the addition of 10 µl 0.4% (w/v) SDS, 20 mM EDTA, 8 mM GTP, 8 mM GDP and heated to 65°C for 5 min. Two µl of the samples were spotted onto PEI-cellulose F TLC plates (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and [α^{32}P]-GTP and [α^{32}P]-GDP were separated using 0.75 M KH_2PO_4, pH 3.5, as the solvent. The plates were air-dried and the spots corresponding to [α^{32}P]-GTP and [α^{32}P]-GDP, respectively, were quantified using a Phosphorimager (GS-250 Molecular ImagerTM) and Molecular Analyst software Mac Version 2.0.1 (Bio-Rad Laboratories, Hercules CA, USA).

To determine the catalytic constants (k_{cat}) for the GTP-hydrolysis of the recombinant GTPases, essentially the same approach was used. A 10 µM aliquot of the recombinant protein was incubated as described above, but additionally increasing concentrations of non-labeled GTP (0, 10, 20, 50, 100, 200, 500, 750 and 1000 µM) were added to the reaction. Samples were removed after 0, 30, 60 and 120 minutes and quantification of the signals was done as described above. From this, nanomoles of [α^{32}P]-GTP hydrolyzed per minute were calculated and plotted against the concentration of unlabeled GTP. The catalytic constant k_{cat} was calculated according to $k_{cat} = v_{max} / [E]$. Whereas [E] denotes the amount of the enzyme that was used (in nmoles) and v_{max} (nmol/s) is the highest reaction rate at a saturating substrate concentration.

2.2.7. Soluble phase binding assays

2.2.7.1. *In vitro* transcription/translation

[^{35}S]Toc159 and [^{35}S]Toc159G to be used in the pull-down assays were synthesized *in vitro* directly from the plasmids described above (pET21d-Toc159 and pET21d-Toc159$_{727\text{-}1093}$, respectively), using the reticulocyte-based TNT T7 Coupled Transcription/Translation System (Promega Corporation, Madison WI, USA), following the instructions of the supplier.

Templates for *in vitro* synthesis of Toc33 wt and Toc33 R130A lacking the hexahistidinyl-tag were obtained by PCR amplification from the respective constructs containing a hexahistidinyl-tag (pET21d-Toc33$_{1\text{-}265}$H$_6$ and pET21d-Toc33$_{1\text{-}265}$H$_6$R130A, respectively) using the primers 5'-GAA ATT AAT ACG ACT CAC TAT AGG GG-3'/5'-CCC AAG CTT GAC GTC TTA CTT TCC TTT ATC ATC AGA G-3'. Subsequently, the PCR fragment was used for the *in vitro* transcription/translation reaction with the reticulocyte-based TNT T7 Coupled Transcription/Translation System.

2.2.7.2. Pull-down assay using Ni-NTA chromatography

Recombinant hexahistidinyl-tagged proteins were purified as described above (see 2.2.3.) and incubated, at concentrations ranging from 0-40 µM, with 10 µl of the respective [^{35}S]-Met labeled protein in import buffer (final concentrations: 50 mM Hepes/KOH pH 7.5, 330 mM Sorbitol, 40 mM KOAc, 2 mM Mg(OAc)$_2$, 25 µM DTT, 0.1% (v/v) Triton X-100) supplemented with 0.4 mM GTP and 4 mM ATP, for 10 minutes on ice. A 10 µl aliquot of packed Ni-NTA agarose (Qiagen AG, Basel, Switzerland) equilibrated in import buffer was added, and the incubation continued for 30 minutes at 4°C under constant mixing to reisolate the hexahistidinyl-tagged proteins. The resin was washed three times with 0.5 ml import buffer, once with 40 mM imidazole and then the bound protein was eluted with 200 mM imidazole. Eluates were precipitated by the chloroform/methanol method (Wessel and Flügge, 1984) and analyzed by SDS-PAGE (see 2.2.4.1.) followed by Coomassie blue staining, according to standard protocols (Sambrook and Russell, 2001). Followed by autoradiography and quantification on a Phosphorimager (GS-250 Molecular ImagerTM) using the Molecular Analyst software Mac Version 2.0.1 (Bio-Rad Laboratories, Hercules CA, USA).

2.2.7.3. Pull-down assay utilizing GST-sepharose chromatography

The recombinant hexahistidinyl- or GST-tagged Toc-GTPases were expressed and purified as described above (see 2.2.3.). To remove the bound nucleotide, the protein fractions were dialyzed for two hours against 50 mM Tris/HCl pH 8.0, 25 mM KOAc, 5 mM EDTA and 1 mM DTT.

Following dialysis, 2 µM of the GST-tagged protein was incubated during 10 minutes on ice with increasing concentrations (0.5 and 1.5 µM) of the respective hexahistidinyl-tagged protein in import buffer (final concentrations: 50 mM Hepes/KOH pH 7.5, 330 mM sorbitol, 40 mM KOAc, 2 mM $Mg(OAc)_2$, 25 µM DTT, 0.1% (v/v) Triton X-100) supplemented with 4 mM ATP, 10 mM $MgCl_2$ and either 5 mM GTP, GDP, GMP-PNP or no guanine nucleotide. Then 10 µl packed GST-Sepharose equilibrated in import buffer was added, followed by an incubation for 30 minutes at 4°C under constant mixing. After extensive mixing, the resin was washed three times with import buffer and the bound protein was eluted with 200 µl 10 mM reduced glutathione in 50 mM Tris/HCl pH 8.0. The eluates were precipitated by the chloroform/methanol method (Wessel and Flügge, 1984), followed by SDS-PAGE and western blot analysis (see 2.2.4.). After staining with Amido black according to a standard protocol (Sambrook and Russell, 2001), the membrane was used for immunodetection with antibodies specific for the hexahistidinyl-tag (see 2.2.4.3.).

3. Results

3.1. Characterization of the putative arginine finger of atToc33

3.1.1. The crystal structure of pea Toc34 - the starting point

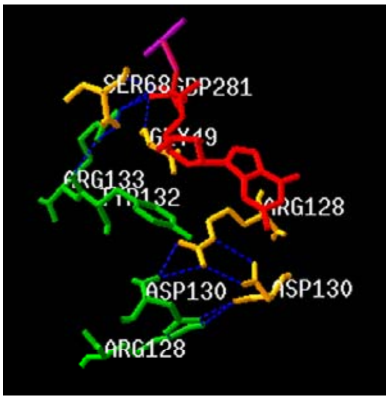
In 2002, the crystal structure of pea Toc34 was published. It is still the first and only known structure of a member of the Toc-complex (Sun et al., 2002). The fact that the Toc-GTPases share a conserved GTP binding domain (G domain) and, furthermore, that psToc34 mainly consists of this GTP binding domain plus small additional C- and N-terminal parts, makes the structure of psToc34 a paradigm for the unique Toc-GTPase family, allowing predictions about the structure of the GTP binding domain of Toc159.

Pea Toc34 forms dimers in the crystal, while it coexists in a monomeric and dimeric form in solution (Sun et al., 2002). Furthermore, the crystal structure revealed that every monomer contained a bound GDP as well as a Mg^{2+} ion, even in the absence of additional GDP during the purification and crystallization process, suggesting a sufficient affinity of pea Toc34 for guanine nucleotides to allow for stable binding under the conditions used to purify and crystallize the protein (Sun et al., 2002).

According to the crystal structure of pea Toc34, both GDP molecules and several amino acids, like Arg128 and Arg133, play an important role in dimerization (Fig. 6, A). Arg128 of one monomer forms hydrogen bonds with Asp130 of the other monomer, whereas Arg133 forms two hydrogen bonds with the GDP bound to the other monomer, as well as one hydrogen bond with Ser68 of the other monomer, pointing to an involvement of Arg128 and Arg133 in dimerization (Fig. 6, A) (Sun et al., 2002). The fact that a R128A mutant of pea Toc34 has reduced GTPase activity and is not able to form dimers, supported the hypothesis that Arg128 is involved in dimerization of psToc34. Moreover, the reduced rate of GTP hydrolysis of the R128A mutant suggested that dimerization and GTP hydrolysis may be interrelated (Sun et al., 2002).

The overall structure of the dimeric form of pea Toc34 resembles a small GTPase with its corresponding GTPase activating protein (GAP) (Sun et al., 2002). Many of these GAPs extend a catalytic arginine residue, called arginine finger, into the active site of their corresponding GTPase (Scheffzek et al., 1998). In the psToc34 structure, the position of Arg133, facing the GDP binding region of the other monomer (Fig. 6, B), resembles such an arginine finger. Based on these structural features it was proposed that one psToc34 monomer may function as a GAP for the other monomer in the dimer, employing an arginine finger, thus increasing the GTPase activity of the pea Toc34 dimer (Sun et al., 2002). Furthermore, the arginine finger hypothesis implies that dimerization and GTP hydrolysis are coupled. However, studies of mutants, with the exception of R128A (Sun et al., 2002), supporting the correlation between psToc34 dimerization and GTPase activity, are lacking.

A)



B)

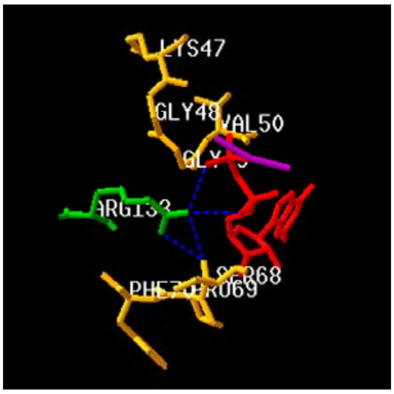


Fig. 6 Selected aspects of the psToc34 dimer: A) The interface in the psToc34 dimer. Selected amino acids of monomer 1 involved in dimerization are shown in green, while selected amino acids of monomer 2 are colored orange. GDP bound to monomer 2 is depicted in red and the Mg^{2+} in magenta. Hydrogen bonds are indicated by blue dashed lines. **B)** Visualization of the putative arginine finger. The proposed arginine finger (Arg133) of monomer 1 is depicted in green, whereas amino acids of monomer 2 are shown in orange. Amino acids of monomer 2 were mainly selected for the visualization of Arg133 of monomer 1 intruding into the GDP binding subregion of the other monomer. GDP and Mg^{2+} bound to monomer 2 are colored red and magenta, respectively. Hydrogen bonds between Arg133 and the α- and β-phosphates of GDP, as well as Ser68 are depicted in blue dashed lines. The structure of pea Toc34 for pictures A) and B) was downloaded from the Brookhaven Data Bank (1H65). The programme used for visualization of selected aspects of the pea Toc34 dimer was Swiss-PdbViewer 3.7.

3.1.2. Expression of Toc33 wt and Toc33 R130A

To examine the proposed interrelation between dimerization and GTPase activity, we used atToc33, the *Arabidopsis thaliana* ortholog of pea Toc34. As only *Arabidopsis* Toc-proteins were used in this study, the “at” prefix normally used for *Arabidopsis* proteins will be omitted.

The sequence comparison of pea Toc34 and *Arabidopsis* Toc33 shows that the proposed arginine finger (Arg133) of psToc34 aligns perfectly with Arg130 of atToc33 (Fig. 7, A, *). Furthermore, it can be seen that the putative arginine finger is in close proximity of a very conserved sequence, termed the dimerization motiv (D1) which is present in all Toc-GTPases (Sun et al., 2002) (Fig. 7, A, D1), including atToc159. AtToc159, in contrast to atToc33, does not contain a corresponding arginine matching the putative arginine finger of psToc34 (Fig. 7, A).

For the examination of the role of the proposed arginine finger, Arg130, in Toc33, we mutated Arg130 to alanine and expressed the hexahistidinyl-tagged wild type Toc33 (Toc33 wt) and mutant Toc33 (Toc33 R130A), respectively, in a soluble form lacking the C-terminal membrane domain, in *E. coli* BL21(DE3) cells, followed by purification under native conditions employing Ni-NTA chromatography.

Both proteins were expressed at high levels upon IPTG induction (Fig. 7, B, compare lanes 1 and 2 or 5 and 6, respectively). A large fraction of both proteins, around 20% of recombinant Toc33 wt and around 50% of recombinant Toc33 R130A, was expressed in a soluble form (Fig. 7, B, lanes 3 and 7), suggesting that they are probably correctly folded and functional. From the soluble fraction of the bacterial extract Toc33 wt and Toc33 R130A were purified in useful quantities to near homogeneity by Ni-NTA affinity chromatography (Fig. 7, B lanes 4 and 8). The purification process yielded ~40 mg of recombinant hexahistidinyl-tagged Toc33 wt and ~15 mg of recombinant hexahistidinyl-tagged Toc33 R130A.

A)

```
                                             D1       *
psToc34        1 MASQQQTVREWSGINTFAPATQTKLLELLGNLKQEDVNSLTILVMGKGGVGKSSTVNSII
atToc33        1 ---MGSLVREWVGFQQFPAATQEKLIEFFGKLKQKDMNSMTVLVLGKGGVGKSSTVNSLI
atToc159G    855 --------------------------------------SLNILVLGKAGVGKSATINSIL

psToc34       61 GERVVSISPFQSEGPRPVMVSRSRAGFTLNIIDTPGLIEGGYIN---DMALNIIKSFLLD
atToc33       58 GEQVVRVSPFQAEGLRPVMVSRTMGGFTINIIDTPGLVEAGYVN---HQALELIKGFLVN
atToc159G    877 GNQIASIDAFGLSTTSVREISGTVNGVKITFIDTPGLKSAAMDQSTNAKMLSSVKKVMKK

                       D1       *
psToc34      118 KTIDVLLYVDRLDAYRVDNLDKLVAKAITDSFGKGIWNKAIVALTHAQFSPPDG-----L
atToc33      115 RTIDVLLYVDRLDVYRVDELDKQVVIAITQTFGKEIWCKTLLVLTHAQFSPPDE-----L
atToc159G    937 CPPDIVLYVDRLDTQTRDLNNLPLLRTITASLGTSIWKNAIVTLTHAASAPPDGPSGTPL

psToc34      173 PYDEFFSKRSEALLQVVRSGASLKK-DAQASDIPVVLIENSGRCNKNDSDEKVLPNGIAW
atToc33      170 SYETFSSKRSDSLLKTIRAGSKMRKQEFEDSAIAVVYAENSGRCSKNDKDEKALPNGEAW
atToc159G    997 SYDVFVAQCSHIVQQSIGQAVGDLRLMNPSLMNPVSLVENHPLCRKNREGVKVLPNGQTW

                                                            ↓
psToc34      232 IPHLVQTITEVALNKSESIFVDKNLIDGPNPNQRGKLWIPLIFALQYLFLAKPIEALIRR
atToc33      230 IPNLVKAITDVATNQRKAIHVDKKMVDGSYSDDKGKKLIPLIIGAQYLIVK-MIQGAIRN
atToc159G   1057 RSQLLLLCYSLKVLS--ET--N-SLLRPQEPLDHRKVFGFRVR----------------

psToc34      292 DIATETKPAWETRDVGDRK
atToc33      289 DIKTSGKPL----------
atToc159G        -------------------
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B)

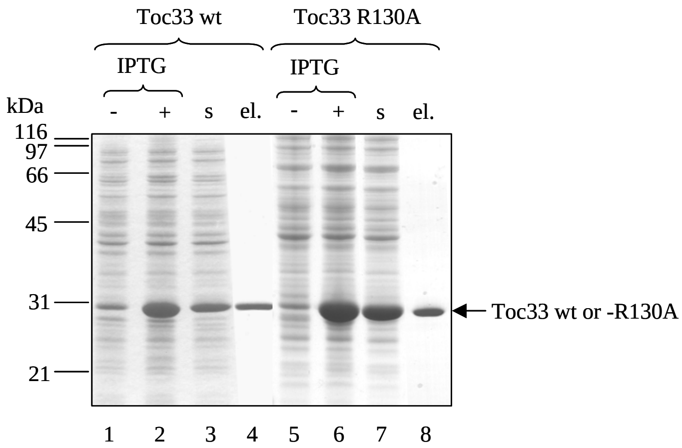


Fig. 7 Sequence alignment and expression of Toc33 wt and Toc33 R130A with hexahistidinyl-tag: **A)** Sequence alignment of pea Toc34, atToc33 and atToc159G. Amino acids identical in all three sequences are in red, whereas residues identical in only two sequences are in blue. The putative arginine finger is marked by an asterisk above the sequences (*). The arrow indicates the position of the hexahistidinyl-tag in Toc33 wt and Toc33 R130A. **B)** Expression and purification of hexahistidinyl-tagged Toc33 wt and Toc33 R130A. Both proteins were expressed in *E. coli* BL21(DE3) cells and purified under non-denaturing conditions using Ni-NTA affinity chromatography. The bound proteins were eluted with an imidazole gradient. Equivalents to 25 µl starting culture of the non-induced cultures (-IPTG, lanes 1 and 5), induced cultures (+IPTG, lanes 2 and 6), soluble fractions (s, lanes 3 and 7), as well as the purified proteins (1 µg each) (el., lanes 4 and 8) were subjected to SDS-PAGE, followed by Coomassie blue staining.

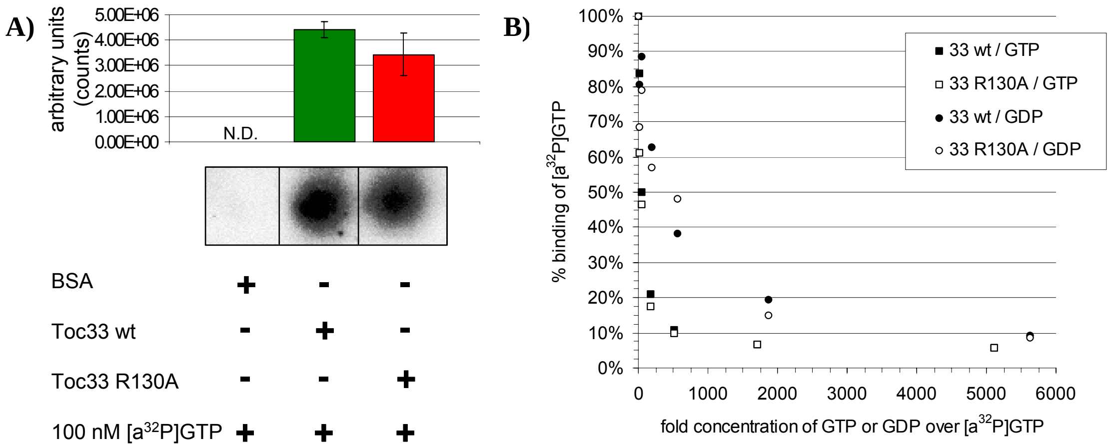
3.1.3. Toc33 wt and Toc33 R130A bind to guanine nucleotides with similar efficiency

To determine the role of Arg130 in the GTPase functions, GTP binding and hydrolysis by both Toc33 wt and Toc33 R130A were measured and the results compared. We started with the examination of guanine nucleotide binding to both Toc33 wt and Toc33 R130A, even though, according to the crystal structure, nucleotide binding is not likely to be impaired in Toc33 R130A, because the putative arginine finger is not predicted to be involved in guanine nucleotide binding in either of the monomers (Sun et al., 2002).

In a first experiment, we examined the ability of recombinant Toc33 wt and Toc33 R130A to bind radioactive [α^{32}P]-GTP. At the [α^{32}P]-GTP concentration used (100 nM), both Toc33 wt and Toc33 R130A bound [α^{32}P]-GTP with almost the same efficiency (Fig. 8, A), supporting the conclusion from the structural data that Arg130 is not involved in guanine nucleotide binding. No unspecific binding of [α^{32}P]-GTP was detected (N.D.), when bovine serum albumin (BSA) was used in a control reaction (Fig. 8, A).

Using [α^{32}P]-GTP in the GTP binding assay allows only to determine total guanine nucleotide binding but not to discriminate between GTP and GDP binding, respectively. To circumvent this problem, we employed a competition assay using [α^{32}P]-GTP and either unlabeled GTP or GDP to determine the affinities of Toc33 wt and Toc33 R130A for GTP and GDP, having the advantage that only a small amount of radioactively labeled GTP is needed. In this assay, the GTPases are incubated with a small amount of [α^{32}P]-GTP and increasing concentrations of unlabeled GTP or GDP to compete for the binding of the radioactively labeled guanine nucleotide and reach saturating binding of the unlabeled guanine nucleotide to Toc33 wt and Toc33 R130A. Then a [α^{32}P]-GTP binding assay is performed as described above. From this, the equilibrium dissociation constants for GTP and GDP binding to Toc33 wt and Toc33 R130A are calculated according to K_d = [Toc33][nucleotide]/ [Toc33·nucleotide].

The recombinant proteins were incubated with 50 nM [α^{32}P]-GTP in the presence of increasing concentrations of either unlabeled GTP or GDP to compete for the binding of [α^{32}P]-GTP to either Toc33 wt or Toc33 R130A. Aliquots of the reactions were applied to nitrocellulose and the [α^{32}P]-GTP remaining bound to the protein after repeated washing, was quantitated (Fig. 8, B).



	K_d (GTP)	K_d (GDP)
Toc33 wt	~2.6 μM	~3.8 μM
Toc33 R130A	~4.5 μM	~8.8 μM

Fig. 8 Guanine nucleotide binding to Toc33 wt and Toc33 R130A: A) One μM of recombinant Toc33 wt and Toc33 R130A, respectively, was incubated with 0.1 μM [α^{32}P]-GTP. The same concentration of bovine serum albumine (BSA) was used in a control reaction. Aliquots of each reaction mix were spotted onto pretreated nitrocellulose and the non-bound [α^{32}P]-GTP was removed by washing. Bound [α^{32}P]-GTP was quantitated using a Phosphorimager (upper panel). Bars represent mean values of three independent experiments. Binding to BSA could not be detected (N.D.). Error bars indicate the standard deviation. The central panel shows the autoradiography of a representative [α^{32}P]-GTP binding experiment out of the three performed experiments. **B)** Determination of the affinity of Toc33 wt and Toc33 R130A for GTP and GDP, respectively. Both proteins (1 μM) were incubated with 50 nM [α^{32}P]-GTP and increasing concentrations of either unlabeled GTP or GDP, following the protocol as described above. From this, the binding of [α^{32}P]-GTP to Toc33 wt and Toc33 R130A (expressed as percentage of binding without competition) in presence of increasing concentrations of cold GTP and GDP was plotted against the ratio of unlabeled nucleotide against [α^{32}P]-GTP.

Both GTP and GDP were found to be competitive inhibitors of [α^{32}P]-GTP binding to Toc33 wt and Toc33 R130A. The wild type as well as the mutant Toc33 bind GDP with an affinity slightly lower than that for GTP (Fig. 8, B). Thus, the calculated dissociation constants for GTP binding to Toc33 wt (K_d = ~2.6 μM) and Toc33 R130A (K_d = ~3.8 μM) are somewhat smaller than the dissociation constants for GDP binding to Toc33 wt (K_d = ~4.5 μM) and Toc33 R130A (K_d = ~8.8 μM). Toc33 wt has a slightly higher affinity for the two nucleotides than Toc33 R130A (Fig. 8, B).

Nevertheless, the dissociation constants of both Toc33 wt and R130A for the guanine nucleotides are in the same order of magnitude, confirming that Arg130 does not play a key role in guanine nucleotide binding.
A comparison of the calculated dissociation constants obtained for Toc33 wt and Toc33 R130A with those for other well-known GTPases shows that the dissociation constants for GTP and GDP obtained for wild type and mutant Toc33 are higher than those for Ras (Manne et al., 1984; Feuerstein et al., 1987), but in the same range as those for Ffh, the bacterial homolog of SRP54 (Jagath et al., 1998).
Furthermore, the data indicate that the affinity of Toc33 wt and Toc33 R130 is sufficiently high to ensure saturation at cellular concentrations of GTP (>100 µM) (Bourne et al., 1991), suggesting that GTP binding to Toc33 is saturated under cellular conditions, similar to other GTPases (Bourne et al., 1991).

3.1.4. Guanine nucleotide binding to Toc33 wt and Toc33 R130A is dependent on Mg^{2+}

For most GTPases, Mg^{2+} is essential for high affinity binding of guanine nucleotides as well as for GTP hydrolysis (Wittinghofer and Pai, 1991; Pan and Wessling-Resnick, 1998; Sprang and Coleman, 1998). Because the affinity of the GTPases for guanine nucleotides is high, specific GEFs (guanine nucleotide exchange factors) are needed to accelerate the exchange of the bound GDP (Cherfils and Chardin, 1999). It was suggested that GEFs simply promote GDP dissociation from its respective GTPase by disturbing the Mg^{2+} coordination between the GTPase and the guanine nucleotide (John et al., 1993; Pan and Wessling-Resnick, 1998).
The crystal structure of the pea Toc34 dimer contains one Mg^{2+} ion coordinated in every psToc34 monomer (Sun et al., 2002). But the involvement of this Mg^{2+} ion in guanine nucleotide binding is not known. Therefore, we investigated the interconnnection between the availability of Mg^{2+} and [α^{32}P]-GTP binding to wild type and mutant Toc33 in an adapted [α^{32}P]-GTP binding assay. In this assay we examined the effect of EDTA, which strongly chelates divalent cations like Mg^{2+}, on [α^{32}P]-GTP binding to Toc33 wt and Toc33 R130A (for a schematical overview of the [α^{32}P]-GTP binding assay see Fig. 9, A).

Two µM of recombinant Toc33 wt and Toc33 R130A, respectively, was incubated on ice with [α^{32}P]-GTP in a buffer containing 50 µM $MgCl_2$. The same concentration of BSA was used as a negative control. After the incubation, half of the reaction was supplemented with either the same volume of buffer containing an equal concentration of Mg^{2+} (50 µM final concentration) or 10 mM EDTA (5 mM final concentration), followed by an incubation on ice. Thereafter, part of the reaction batch containing 50 µM of Mg^{2+} was again mixed with a buffer containing the same concentration of Mg^{2+} to keep the Mg^{2+} concentration constant, while half of the reaction batch containing 5 mM EDTA was mixed with the same volume of buffer including 5 mM EDTA, and the other half of the reaction batch was supplemented with a buffer containing an excess of Mg^{2+} (10 mM final concentration). After another incubation on ice, an aliquot of each reaction was spotted onto nitrocellulose, followed by washing steps and quantification of the radioactive spots. No [α^{32}P]-GTP binding to BSA was detected, suggesting that no unspecific [α^{32}P]-GTP binding occurred (data not shown).

Both recombinant proteins bind [α^{32}P]-GTP with similar efficiency in the presence of low levels of Mg^{2+} (50 µM). Upon addition of a high concentration of EDTA, which complexes Mg^{2+} and removes it from Toc33, [α^{32}P]-GTP binding to Toc33 wt and Toc33 R130A is no longer observed (Fig. 9, B), suggesting that GTP binding to both proteins is dependent on the presence of bound Mg^{2+}. This essential role of Mg^{2+} for GTP binding has also been found in many other GTPases (Wittinghofer and Pai, 1991; Pan and Wessling-Resnick, 1998; Sprang and Coleman, 1998; Paduch et al., 2001).

Binding of the guanine nucleotide to Toc33 wt and Toc33 R130A can be restored by addition of an excess of Mg^{2+} after prior removal by EDTA, demonstrating that the observed effect is reversible (Fig. 9, B). This suggests that the nucleotide-free form of Toc33 wt and Toc33 R130 is stable enough to allow re-binding of guanine nucleotides.

We conclude from this experiment that the mutation of Arg130 to alanine has no detectable effect on Mg^{2+}-dependent GTP binding (Fig. 9, B). As the mutation also only had a minor effect on nucleotide binding to Toc33 (Fig. 8), we decided to use the recombinant mutant protein (Toc33 R130A) to examine the function of the proposed arginine finger in GTPase activity.

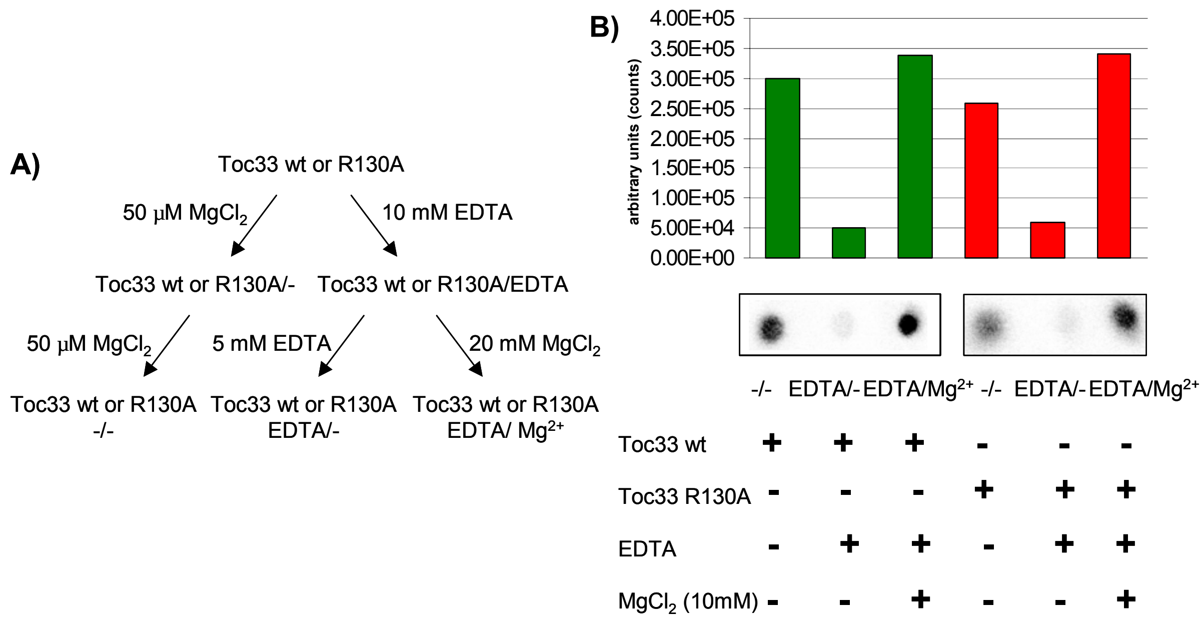


Fig. 9 Mg^{2+} is essential for [α^{32}P]-GTP binding to Toc33 wt and Toc33 R130A: A) Schematical overview of the assay described under B). **B)** Two µM Toc33 wt and Toc33 R130A, respectively, was incubated on ice with 0.1 µM [α^{32}P]-GTP in buffer containing 50 µM Mg^{2+} in a final volume of 40 µl. A 15 µl aliquot of the reaction batch was subsequently mixed with a buffer containing 50 µM Mg^{2+}, while the other half of the reaction batch was supplemented with the same volume of a buffer containing EDTA (5 mM final concentration), followed by incubation on ice. Thereafter, 10 µl of the reaction containing 50 µM Mg^{2+} was mixed with the same volume of buffer containing Mg^{2+} to keep the Mg^{2+} concentration constant. A 10 µl aliquot of the reaction batch including EDTA was mixed with the same amount of buffer containing 5 mM EDTA and the other half was mixed with buffer containing an excess of Mg^{2+} (10 mM final concentration). After another incubation on ice, 2 µl of each reaction mix were spotted onto nitrocellulose, followed by extensive washing and quantification of the bound [α^{32}P]-GTP. The bars represent [α^{32}P]-GTP binding to Toc33 wt (in green) and Toc33 R130A (in red), respectively. The panels below the graph show the autoradiogram of the [α^{32}P]-GTP binding experiment. The spots represent the [α^{32}P]-GTP bound to Toc33 wt and Toc33 R130A, respectively.

3.1.5. The GTPase activities of Toc33 wt and Toc33 R130A are similar

If Arg130 of Toc33 functions as an arginine finger, mutation of Arg130 is expected to lead to a decrease in the GTPase activity of the mutant Toc33 R130A, whereas Toc33 wt should have a GTPase activity similar to other GTPases in presence of their respective GAPs (Gideon et al., 1992; Eccleston et al., 1993). For this purpose we determined the rate of GTP hydrolysis by Toc33 wt and Toc33 R130A.

Recombinant Toc33 wt and Toc33 R130A, respectively, were incubated with 50 nM [α^{32}P]-GTP at room-temperature, allowing GTP hydrolysis in the course of four hours. Subsequently, the reactions were stopped and the guanine nucleotides were separated by polyethyleneimine (PEI)-cellulose thin layer chromatography (Fig. 10, A). The spots corresponding to [α^{32}P]-GTP and [α^{32}P]-GDP were visualized using a Phosphorimager (Fig. 10, A). BSA was used as a negative control (Fig. 10, A, lanes 9-12). Both wild type and mutant Toc33 have low, but similar GTPase activities, as judged by the autoradiogram (Fig. 10, A, compare lanes 1-4 with lanes 5-8). No GTP hydrolysis was detected in the negative control with BSA (Fig. 10, A, lanes 9-12), excluding spontaneous GTP hydrolysis. This experiment indicates that the two proteins hydrolyze 50 nM [α^{32}P]-GTP at similar rates, suggesting that Arg130 does not function as an arginine finger.

To substantiate this conclusion, we determined the catalytic constants of GTP hydrolysis by Toc33 wt and Toc33 R130A (Fig. 10, B and C). Recombinant Toc33 wt and Toc33 R130A were incubated with increasing concentrations (0-1000 µM) of unlabeled GTP in the presence of 50 nM [α^{32}P]-GTP as a marker. Subsequently, a [α^{32}P]-GTP hydrolysis assay was performed as described previously.

The rate of the GTP hydrolysis (in nmol/min) was calculated for the concentration of wild type and mutant Toc33 used in the reaction and plotted against the concentration of GTP in the reaction (Fig. 10, B and C). The maximal hydrolysis rate (v_{max}) and the concentration of enzyme employed in every reaction were then used to calculate the catalytic constants (k_{cat}) for both Toc33 wt and Toc33 R130A according to Michaelis Menten kinetics ($k_{cat} = v_{max}/[E]$) (Fig. 10, B and C).

Toc33 wt ($k_{cat} = 2.2*10^{-4}\ s^{-1}$) and Toc33R130A ($k_{cat} = 3.2*10^{-4}\ s^{-1}$) have very similar catalytic constants, corroborating our assumption that Arg130 of Toc33 does not function as an arginine finger. Moreover, the hydrolysis rate calculated for Toc33 wt is very low, comparable to the intrinsic activity of Ras ($1.3*10^{-4}$-$5*10^{-4}\ s^{-1}$) (Bourne et al., 1990), suggesting that Toc33 was not in an activated state. In the case of a (auto-)activated GTPase, we would expect a rate of GTP hydrolysis comparable to that of Ras stimulated by a GAP, which is increased up to 5 orders of magnitude (Gideon et al., 1992).

We conclude that Toc33 is not an activated GTPase and that Arg130 does not function as arginine finger, as suggested by Sun et al. (2002).

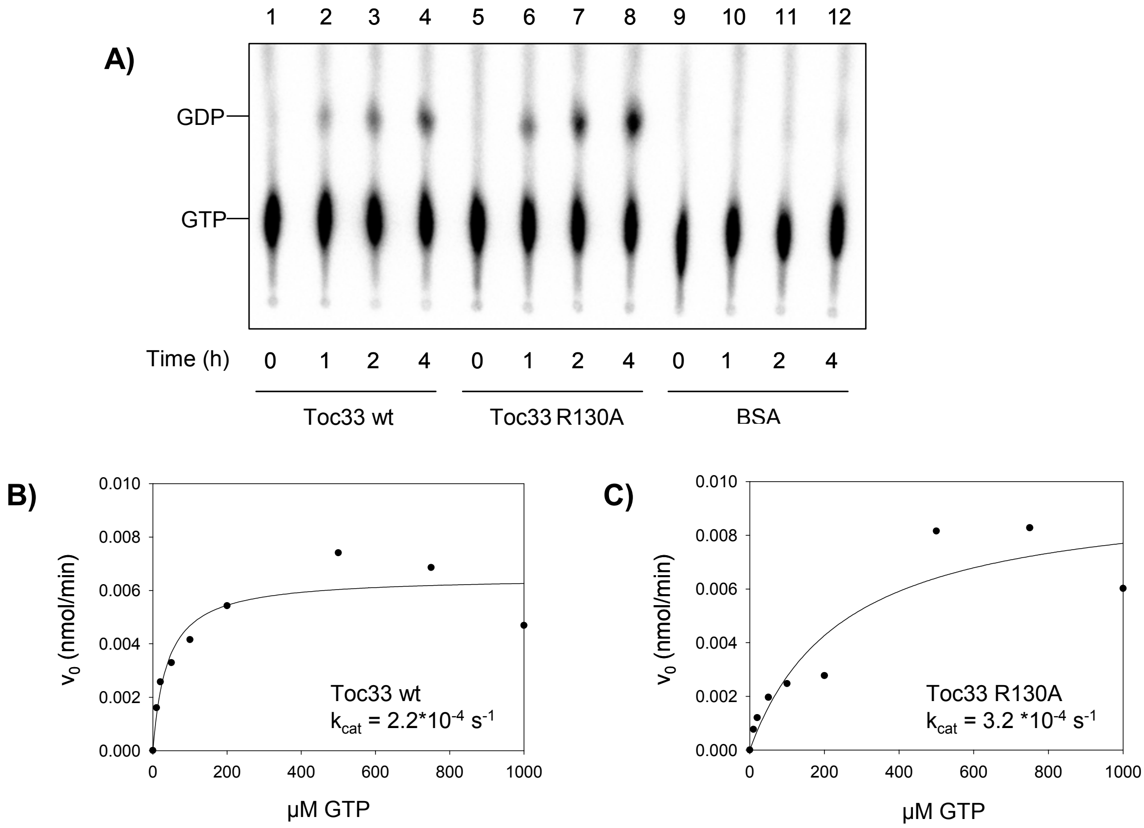


Fig. 10 Kinetics of [α^{32}P]-GTP hydrolysis by Toc33 wt and Toc33 R130A: A) Time-course of [α^{32}P]-GTP hydrolysis. Recombinant Toc33 wt and Toc33 R130A (0.5 µM), respectively, was incubated at 25°C with 50 nM [α^{32}P]-GTP. After 0, 1, 2 and 4 h, an aliquot of the reaction mix was stopped and 2 µl of the samples were spotted onto PEI-cellulose TLC plates to separate [α^{32}P]-GTP and [α^{32}P]-GDP. The spots representing [α^{32}P]-GTP or [α^{32}P]-GDP were visualized using a Phosphorimager. **B)** Determination of k_{cat} for GTP hydrolysis of Toc33 wt. Recombinant Toc33 wt (10 µM) was incubated with 50 nM [α^{32}P]-GTP and increasing concentrations of non-radioactive GTP (1-1000 µM). After 0, 30, 60 and 120 minutes samples were taken and spotted onto PEI-cellulose plates to separate [α^{32}P]-GTP and [α^{32}P]-GDP. Quantification of the spots corresponding to GTP and GDP was done using a Phosphorimager. The total GTP hydrolysis rate (in nmol/min) was plotted against the GTP concentration and k_{cat} was calculated according to $k_{cat} = v_{max}/[E]$. **C)** Determination of the k_{cat} for GTP hydrolysis by Toc33 R130A was accomplished as described under B).

3.1.6. Toc33 R130A is impaired in homodimerization

The arginine residue corresponding to Arg130 in Toc33 was suggested to be essential for GTPase activity as well as dimerization (Sun et al., 2002). As Toc33 R130A hydrolyzes GTP to GDP with kinetics similar to wild type Toc33, it was still possible that homo- and/or heterodimerization of Toc33 R130A is affected by the point mutation. Therefore, the homodimerization of Toc33 wt and Toc33 R130A was examined using blue native PAGE (Fig. 11) as well as a pull-down assay (Fig. 12).

3.1.6.1. Examination of Toc33 homodimerization using blue native PAGE

Blue native PAGE allows the separation of protein-complexes under native conditions (Schagger and von Jagow, 1991; Schagger et al., 1994), and is therefore a suitable method to study the dimerization of Toc33. We subjected increasing amounts of recombinant Toc33 wt and Toc33 R130A to blue native PAGE, followed by Coomassie blue staining. BSA was chosen as a standard, because the molecular weight of its monomer is 66 kDa, which is similar to the calculated molecular mass of the Toc33 dimer (around 60 kDa).

Blue native PAGE of recombinant Toc33 wt produced two bands, one migrating below the BSA standard (66 kDa) (Fig. 11, A, lanes 2-5, monomer), the other migrating above (Fig. 11, lanes 2-5, dimer), suggesting that the lower band (~30 kDa) corresponds to the Toc33 monomer and the upper band (~70 kDa) to the Toc33 dimer (Fig. 11, A). Higher molecular weight bands were not detected, suggesting the existence of two major species of Toc33 in the preparation (Fig. 11, A, lanes 2-5). Blue native PAGE of Toc33 R130A produced two bands as well, a strong monomer band around 30 kDa and, in comparison to Toc33 wt, a very faint putative dimer band (~70 kDa) (Fig. 11, A, lanes 6-9, dimer), suggesting an impaired dimerization of Toc33 R130A.

To confirm that the upper ~70 kDa band indeed contained Toc33 wt or Toc33 R130A, respectively, both upper and lower bands were excised from the gel. The proteins were eluted from the gel and analyzed by SDS-PAGE followed by Western blotting (Fig. 11, B). SDS-PAGE of the lower bands of both Toc33 wt (Fig. 11, B, lane 1, m) and Toc33 R130A (Fig. 11, B, lane 3, m) resulted in ~31 kDa proteins as visualized by Amido black staining after transfer of the proteins to nitrocellulose (Fig. 11, B, lower panel). The ~70 kDa bands of both Toc33 wt (Fig. 11, B, lane 2, d) and Toc33 R130A (Fig. 11, B, lane 4, d) resulted in much weaker bands of about 31 kDa when stained with Amido black.

Subsequently, the blot was probed with antibodies recognizing Toc33 (αatToc33) to detect Toc33 wt and Toc33 R130A (Fig. 11, B, upper panel). The lower bands of both Toc33 wt (Fig. 11, B, lane 1, m) and Toc33 R130A (Fig. 11, B, lane 3, m) were recognized by αatToc33, giving a strong signal, suggesting that the lower bands indeed contained Toc33 wt and Toc33 R130A, respectively, most likely in a monomeric form. Also the upper blue native PAGE bands of Toc33 wt (Fig. 11, B, lane 2, d) and Toc33 R130A (Fig. 11, B, lane 4, d) were recognized by αatToc33 after Western blotting, thus probably representing dimers. However, the intensities of the two bands were different. The upper band of Toc33 wt (Fig. 11, B, lane 2, d) gave a relatively strong signal, whereas the signal from the upper band of Toc33 R130A (Fig. 11, B, lane 4, d) was weak, again suggesting that mutant Toc33 is most likely impaired in homodimerization. This finding supports the hypothesis that Arg130 is involved in Toc33 homodimerization (Sun et al., 2002).

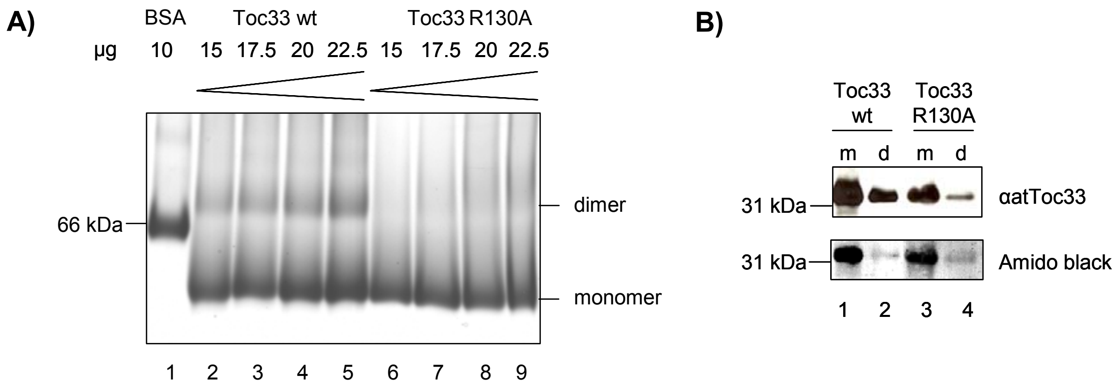


Fig. 11 Blue native PAGE analysis of recombinant Toc33 wt and Toc33 R130A: A) Increasing amounts of Toc33 wt and Toc33 R130A (15-22.5 µg) were analyzed by BN-PAGE followed by additional Coomassie blue staining. BSA was used as standard (molecular weight of the monomer ~66 kDa). **B)** Bands corresponding to the putative monomers (m) and dimers (d) of Toc33 wt (lanes 1 and 2) and Toc33 R130A (lanes 3 and 4) were excised from the native gel, followed by elution of the proteins and subsequent analysis by SDS-PAGE and Western blotting. The blot was stained with Amido black (lower panel, Amido black) and probed with antibodies recognizing Toc33 (upper panel, αatToc33).

3.1.6.2. Examination of the homodimerization of Toc33 wt and Toc33 R130A using a Ni-NTA agarose pull-down assay

To obtain further evidence for the involvement of Arg130 in Toc33 homodimerization, we examined the interaction of Toc33 wt and Toc33 R130A in a soluble-phase pull-down assay in which recombinant, hexahistidinyl-tagged Toc33 wt or Toc33 R130A, respectively, in increasing concentrations (0-40 µM), were incubated with radioactively labeled Toc33 ([^{35}S] wt) or Toc33 R130A ([^{35}S] R130A) lacking a hexahistidinyl-tag. After the incubation of the purified protein with its radioactive binding partner, Ni-NTA agarose was added to every sample to reisolate the hexahistidinyl-tagged protein including any radioactive binding partner. Subsequently, the Ni-NTA agarose was washed and the bound protein eluted with imidazole. The eluates were subjected to SDS-PAGE followed by autoradiography (Fig. 12, A, upper panel, autoradiography). Additionally, a part of the Coomassie blue stained SDS-PAGE gel is shown to demonstrate that comparable protein amounts were used for analysis (Fig. 12, A, lower panel, Coomassie blue).

To calculate the percentage of the bound [^{35}S]-labeled protein, an aliquot was taken from each reaction before the incubation with Ni-NTA agarose, analyzed by SDS-PAGE and quantitated. From this, the percentage of binding of the [^{35}S]-labeled protein to the hexahistidinyl-tagged protein could be determined (Fig. 12, B).

Although the nature of the pull-down assay made it impossible to determine the exact binding constants, it still allowed us to observe quantitative differences between the homodimerization of Toc33 wt and the homodimerization of Toc33 R130A (Fig. 12, A and B). Hexahistidinyl-tagged Toc33 wt (wt (H_6)) pulled down 20% at most of the radioactive wild type protein ([^{35}S] wt) (Fig. 12, A, lanes 1-6), whereas Toc33 R130A (R130A (H_6)) pulled down only trace amounts (up to approximately 1%) of the radioactive mutant protein ([^{35}S] R130A) (Fig. 12, A, lanes 7-9). These results confirm the finding of the blue native PAGE analysis (Fig. 11) that Arg130 is important for proper homodimerization of Toc33.

To examine if Arg130 has to be present in both monomers in the Toc33 dimer to allow stable dimerization, we incubated hexahistidinyl-tagged Toc33 wt (wt (H_6)) with [^{35}S]-labeled Toc33 R130A ([^{35}S] R130A) and vice versa hexahistidinyl-tagged Toc33 R130A (R130A (H_6)) with [^{35}S]-labeled Toc33 wt ([^{35}S] wt) and performed the same soluble-phase pull-down as described above. Surprisingly, purified hexahistidinyl-tagged Toc33 wt (wt (H_6)) also pulled down very low amounts of radioactive Toc33 R130 ([^{35}S] R130A) (Fig. 12, A, lanes 10-12) and the same holds for the pull-down between purified hexahistidinyl-tagged Toc33 R130A (R130A (H_6)) and radioactive wild type Toc33 ([^{35}S] wt) (Fig. 12, A, lanes 13-15), suggesting that Arg130 must be present in both Toc33 monomers to allow stable homodimerization.

Thus, even though Toc33 R130 does not seem to be dramatically affected in GTP binding (Fig. 8) and GTP hydrolysis (Fig. 10), it appears to be strongly impaired in homodimerization (Fig. 11 and 12). Thus, GTP hydrolysis may be uncoupled from dimerization, contradicting an earlier hypothesis according to which dimerization and GTPase activity were thought to be interrelated (Sun et al., 2002).

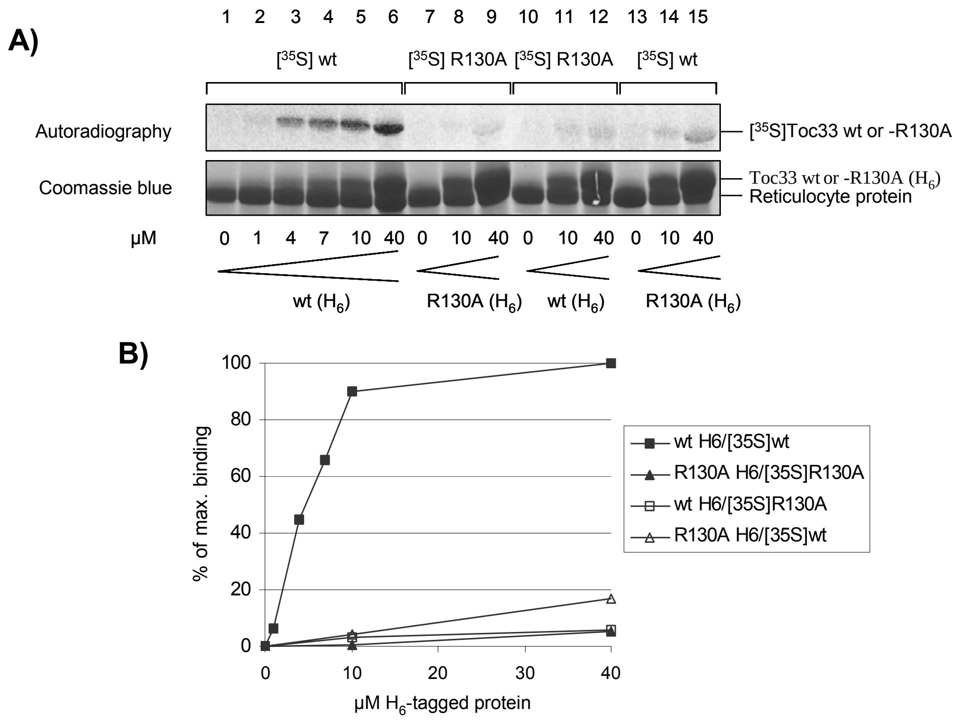


Fig. 12 Homodimerization of Toc33 wt and Toc33 R130A: A) [^{35}S]-labeled Toc33 wt ([^{35}S] wt) and Toc33 R130A ([^{35}S] R130A), lacking the hexahistidinyl-tag, were incubated with increasing concentrations (0-40 µM) of purified, hexahistidinyl-tagged Toc33 wt (wt (H_6)) or Toc33 R130A (R130A (H_6) in the four possible permutations. Ni-NTA agarose chromatography was used to reisolate recombinant hexahistidinyl-tagged proteins and any bound [^{35}S]-labeled proteins. Subsequently, the resin was washed and the proteins were eluted using imidazole. Eluates were analyzed by SDS-PAGE and Coomassie blue staining (lower panel), followed by autoradiography (upper panel). **B)** Quantification of the homodimerization assay using a Phosphorimager. The amount of [^{35}S]-labeled Toc33 wt ([^{35}S] wt) bound to 40 µM hexahistidinyl-tagged purified Toc33 wt (wt (H_6)) was defined as 100% binding. Binding without any added hexahistidinyl-tagged protein was adjusted to zero.

3.1.7. Toc33 R130A is impaired in heterodimerization with Toc159 and Toc159G

There is no evidence for a function of atToc33 homodimerization be it *in vitro* or *in vivo*. However, atToc33 and atToc159 share a highly conserved G-domain, including the dimerization motifs (Sun et al., 2002), suggesting that the two proteins known to interact may do so by dimerization of their G-domain. Furthermore, it has been suggested that atToc33 could function as a membrane-bound receptor for the targeting of the soluble form of atToc159 to the Toc-complex (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002). The observation that atToc33, lacking its C-terminal membrane domain, inhibits the insertion of the soluble form of atToc159 into isolated *Arabidopsis* chloroplasts, supports this hypothesis (Hiltbrunner et al., 2001b). Moreover, the direct binding of recombinant atToc33 and [^{35}S]-labeled atToc159 was demonstrated *in vitro* (Hiltbrunner et al., 2001b; Bauer et al., 2002). In addition, the interaction of the G-domains of purified atToc33 and [^{35}S]-labeled atToc159 was shown in a solid-phase *in vitro* pull-down assay (Smith et al., 2002).

3.1.7.1. Binding of [^{35}S]Toc159 to hexahistidinyl-tagged Toc33 wt and Toc33 R130A

To examine the influence of Arg130 on heterodimerization of Toc159 and Toc33, we incubated both purified, hexahistidinyl-tagged Toc33 wt and Toc33 R130A in increasing concentrations (0-40 µM) with [^{35}S]-labeled Toc159 ([^{35}S]Toc159), lacking a hexahistidinyl-tag. Ni-NTA agarose chromatography was used to reisolate the hexahistidinyl-tagged protein with any bound radioactive Toc159. Subsequently, the resin was washed, the bound proteins were eluted and the eluates analyzed by SDS-PAGE, followed by Coomassie blue staining (Fig. 13, A, lower panel) and autoradiography (Fig. 13, A, upper panel). The stained SDS-gel shows that equal protein amounts of the eluates were used for SDS-PAGE analysis (Fig. 13, A, lower panel) and therefore allowed us to qualitatively compare the binding of [^{35}S]Toc159 to hexahistidinyl-tagged Toc33 wt and Toc33 R130A, respectively.

In the pull-down assay, hexahistidinyl-tagged Toc33 bound soluble radioactively labeled Toc159 (~5% of the total radioactive protein) (Fig. 13, A, lanes 1-4) more efficiently than Toc33 R130A (less than 2% of the total radioactive protein) (Fig. 13, A, lanes 5-8), suggesting that Arg130 is essential for homodimerization of Toc33 (Fig. 11 and 12) as well as for heterodimerization of Toc33 and Toc159 (Fig. 13).

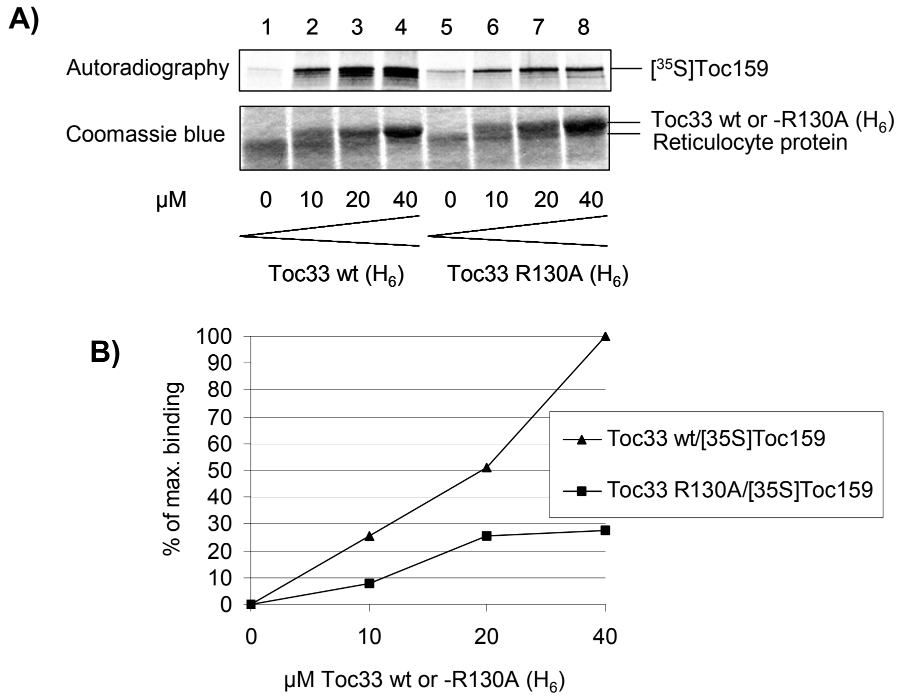


Fig. 13 Binding of [^{35}S]Toc159 to hexahistidinyl-tagged wild type and mutant Toc33: A) [^{35}S]Toc159 was incubated with increasing concentrations (0-40 µM) of hexahistidinyl-tagged Toc33 wt and Toc33 R130A. Any bound radioactive Toc159 was isolated together with the hexahistidinyl-tagged protein by Ni-NTA agarose chromatography. All bound protein was eluted with imidazole and analyzed by SDS-PAGE, followed by Coomassie blue staining and autoradiography. **B)** Quantification using a Phosphorimager. The amount of [^{35}S]Toc159 bound to 40 µM Toc33 wt was defined as 100% binding. [^{35}S]Toc159 binding to the Ni-NTA resin in absence of any hexahistidinyl-tagged protein was adjusted to zero.

3.1.7.2. [^{35}S]Toc159G binding to Toc33 wt and Toc33 R130A

It has been shown in *in vitro* pull-down assays that the G-domains of atToc33 and atToc159 interact directly (Smith et al., 2002). We therefore analyzed the binding of [^{35}S]Toc159G, corresponding to the G-domain of Toc159 encompassing amino acids 727 to 1093, to hexahistidinyl-tagged Toc33 wt and Toc33 R130A. [^{35}S]-labeled Toc159G ([^{35}S]Toc159G) was incubated with increasing concentrations (0-10 µM) of hexahistidinyl-tagged Toc33 wt and Toc33 R130A, followed by a soluble-phase pull-down assay that was accomplished as described for the binding of [^{35}S]Toc159 to hexahistidinyl-tagged Toc33 wt and Toc33 R130A (see 3.1.7.1.).

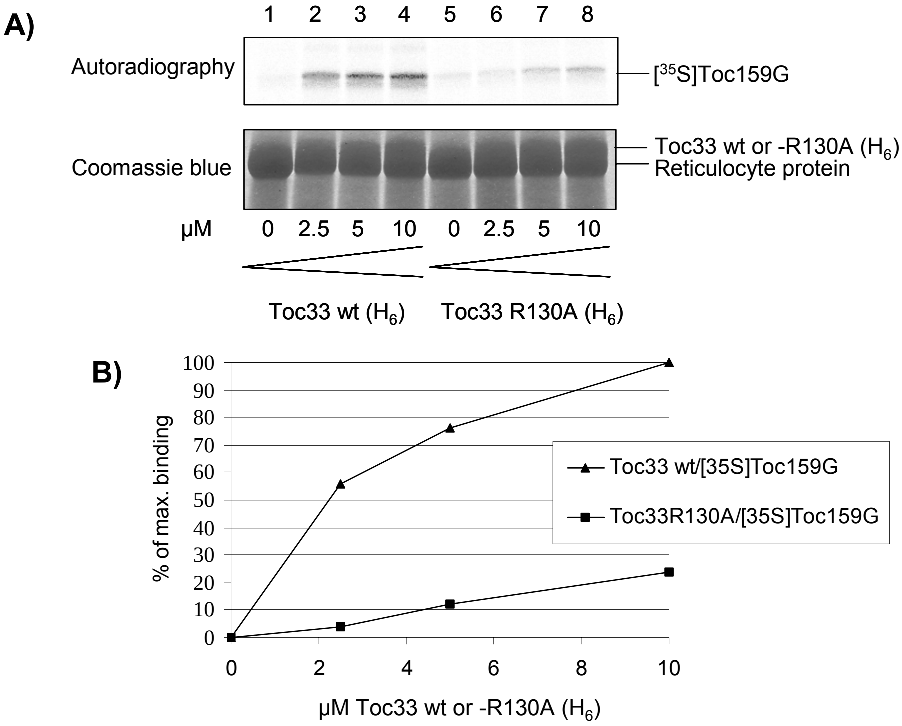


Fig. 14 Binding of [^{35}S]Toc159G to Toc33 wt and Toc33 R130A: A) [^{35}S]Toc159G was incubated with increasing concentrations (0-10 µM) of hexahistidinyl-tagged Toc33 wt and Toc33 R130A. Isolation of the hexahistidinyl-tagged protein and any bound [^{35}S]Toc159G was achieved by Ni-NTA agarose chromatography. The resin was washed and the bound protein eluted with imidazole. The eluted protein fractions were subjected to SDS-PAGE, followed by Coomassie blue staining and autoradiography. **B)** Quantification of [^{35}S]Toc159G bound to hexahistidinyl-tagged Toc33 wt and Toc33 R130A. Binding of [^{35}S]Toc159G to 10 µM purified Toc33 wt was defined as 100% binding. Any binding to the Ni-NTA resin in absence of the hexahistidinyl-tagged protein was adjusted to zero.

Reminiscent of the results of the pull-down assay using full length [^{35}S]Toc159, recombinant hexahistidinyl-tagged Toc33 bound more efficiently to soluble [^{35}S]Toc159G (Fig. 14, A, lanes 1-4) (around 5% of the total [^{35}S]-labeled Toc159G) than hexahistidinyl-tagged Toc33 R130A (Fig. 14, A, lanes 5-8) (up to 1% of the total radioactive Toc159G). These results further substantiate that Arg130 of Toc33 is critical for heterodimerization between Toc33 and the G-domain of Toc159.

Moreover, the fact that both homodimerization of Toc33 (Fig. 11 and 12) and heterodimerization of Toc33 with either Toc159 (Fig. 13) or Toc159G (Fig. 14) are dependent on Arg130, suggests that homodimerization as well as heterodimerization may rely on similar molecular mechanisms.

3.2. Guanine nucleotide dependency of Toc33 homodimerization and heterodimerization of Toc33 and Toc159

As seen in the crystal structure, pea Toc34 may dimerize in its GDP bound form (Sun et al., 2002). Moreover, it has been reported that in a soluble-phase binding assay the heterodimerization between the G-domains of atToc33 and atToc159 occurs preferentially in the GDP bound state (Smith et al., 2002). In contrast to this, another group has demonstrated that the interaction between atToc33 and atToc159GM, encompassing the G- and M-domains of atToc159, is stabilized by a non-hydrolyzable analog of GTP. Moreover, it was reported by the same group, that GDP even favours the dissociation of the two GTPases (Becker et al., 2004b), giving rise to two contradictory models for the course of the interaction between Toc34 and Toc159 at the chloroplast membrane. In the first model, GTP hydrolysis is a prerequisite for the interaction between the two GTPases, leading to the insertion of soluble Toc159 into the outer chloroplast membrane (Smith et al., 2002; Kessler and Schnell, 2004). In the second model, GTP hydrolysis by both GTPases causes their dissociation (Becker et al., 2004b).

It has been shown that the G-domain is essential for the targeting of atToc159 to *Arabidopsis* chloroplasts (Bauer et al., 2002). Moreover, the targeting is dependent on the GTPase activity of both atToc33 and atToc159 (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003). The guanine nucleotide dependent interaction between Toc34 and Toc159 at the outer chloroplast membrane appears to be a crucial step for preprotein import into chloroplasts and we therefore investigated the requirement of guanine nucleotides for the homodimerization of atToc33 as well as of the heterodimerization of atToc33 and atToc159 *in vitro*. We used a soluble pull-down assay, in which one of the binding partners was a recombinant GST-tagged Toc-GTPase and the other a hexahistidinyl-tagged recombinant Toc-GTPase, in the absence or presence of different guanine nucleotides. The reisolation of the differently tagged binding partners was accomplished using GST-sepharose chromatography.

3.2.1. Expression and purification of the differently tagged recombinant proteins

For the soluble-phase binding assay we needed recombinant hexahistidinyl-tagged Toc33 as well as N-terminally GST-tagged Toc33 (Toc33-GST) and Toc159G (Toc159G-GST). Expression and non-denaturing purification of hexahistidinyl-tagged Toc33 (Toc33 wt) was accomplished as described in 3.1.2.

GST-tagged Toc33 (Toc33-GST) and Toc159G (Toc159G-GST), respectively, were expressed in *E. coli* BL21(DE3) cells and purified under non-denaturing conditions using GST-sepharose chromatography, followed by SDS-PAGE analysis and Coomassie blue staining (Fig. 15). The expression of both Toc33-GST and Toc159G-GST was highly induced by IPTG (Fig. 15, compare lanes 1 and 2 as well as lanes 5 and 6). Bacterial extracts were separated into soluble and insoluble fractions and the GST-tagged proteins were purified from the soluble fractions by GST-sepharose chromatography.

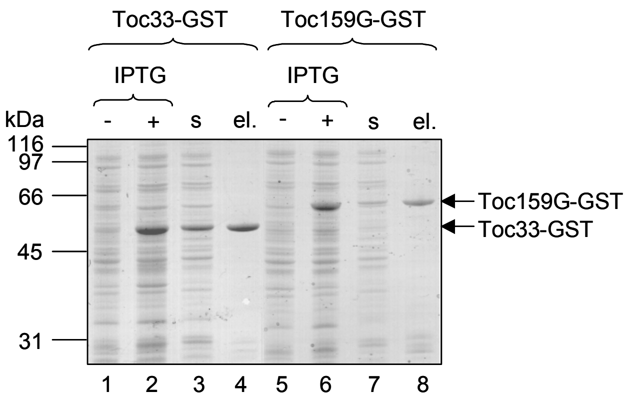


Fig. 15 Expression and purification of Toc33-GST and Toc159G-GST: The two GST-tagged proteins were expressed in *E. coli* BL21(DE3) cells. The non-denaturing purification of the recombinant proteins was accomplished using GST-sepharose chromatography. Bound proteins were eluted using 10 mM reduced glutathione in 50 mM Tris/HCl pH 8.0. Aliquots equivalent to 25 µl of non-induced cultures (-IPTG, lanes 1 and 5), induced cultures (+IPTG, lanes 2 and 6), soluble fractions (s, lanes 3 and 7), as well as the purified, recombinant proteins (1 µg each) (el, lanes 4 and 8) were used for SDS-PAGE analysis followed by Coomassie blue staining.

While half of the expressed Toc33-GST was soluble (Fig. 15, lane 3, s), only about 10% of Toc159G-GST was found in the soluble fraction (Fig. 15, lane 7, s). The purification yielded about 4 mg recombinant Toc33-GST and approximately 3 mg recombinant Toc159G-GST (Fig. 15, lanes 4 and 8, el.). Beside the purified proteins, only few contaminating protein bands with a molecular weight of about 30 kDa are visible on the Coomassie blue stained gel. They most likely represent GST (~26 kDa) and its degradation products (Fig. 15, lanes 4 and 8).

3.2.2. Examination of the functionality of the recombinant proteins

Because Toc33 and Toc159G were expressed with a GST-tag, i.e. a protein tag of almost the same molecular mass as the tagged proteins, we wanted to ensure that the tag did not affect the functionality of the recombinant proteins. Therefore we performed a [α^{32}P]-GTP binding assay with Toc33-GST and Toc159G-GST. As we had already determined the functionality of the hexahistidinyl-tagged Toc33 (Toc33 wt) (see section 3.1.3.), we included hexahistidinyl-tagged Toc33 as positive control in the [α^{32}P]-GTP binding assay.

One µM of each recombinant protein was incubated with 0.1 µM [α^{32}P]-GTP. The same amount of BSA was used in a negative control reaction. Subsequently, 2 µl of every reaction mix was spotted onto pretreated nitrocellulose and the unbound [α^{32}P]-GTP was removed by repeated washing. After drying, the spots corresponding to bound [α^{32}P]-GTP were quantified (Fig. 16).

The autoradiogram (Fig. 16, lower panel) and its quantitative analysis (Fig. 16, graph) indicate binding of [α^{32}P]-GTP to the three recombinant proteins with comparable efficiency, suggesting that the GST-tag of Toc33 and Toc159G does not interfere with GTP binding. No binding to BSA was detected (Fig. 16).

Thus, the purified and tagged recombinant proteins were functional and could be used for further experiments.

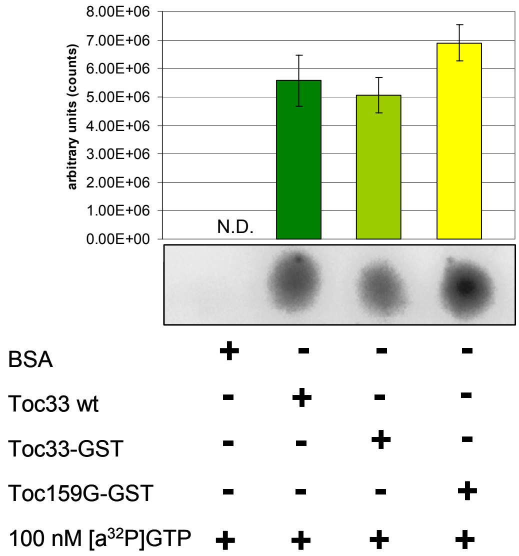


Fig. 16 Binding of [α^{32}P]-GTP to hexahistidinyl-tagged Toc33, Toc33-GST and Toc159G-GST: One µM of the respective recombinant protein was incubated with 0.1 µM [α^{32}P]-GTP. The same concentration of BSA was used as a negative control. After the incubation, 2 µl of every reaction mix was spotted onto pretreated nitrocellulose, followed by extensive washing to remove unbound [α^{32}P]-GTP. The bound [α^{32}P]-GTP was finally quantitated using a Phosphorimager (upper panel). The lower panel shows the autoradiogram resulting of a representative binding experiment. No [α^{32}P]-GTP binding to BSA was detected in the negative control (N.D.). Bars represent mean values from three performed experiments. Error bars indicate the standard deviation.

3.2.3. Homodimerization of Toc33 as well as heterodimerization of Toc33 and Toc159G is supported by GDP

The fact that GDP was found bound to pea Toc34 in the crystal structure, suggests that pea Toc34 has a sufficient affinity to stably bind guanine nucleotides under the conditions used in the purification and crystallization process (Sun et al., 2002). The finding that K_d for GTP binding (K_d = ~2.6 µM) to atToc33 (Toc33 wt) (see 3.1.3.) is smaller than required to ensure tight GTP binding at cytoplasmic concentrations of GTP (>100 µM) (Bourne et al., 1991) supports this suggestion, even though Ras has a higher affinity for guanine nucleotides (Manne et al., 1984; Feuerstein et al., 1987). Thus, it is essential to deplete the proteins of nucleotides prior to loading them with a given guanine nucleotide for the pull-down assay.

We have already demonstrated that the guanine nucleotide binding to hexahistidinyl-tagged Toc33 depends on the presence of Mg^{2+} and is abolished by EDTA (Fig. 9).

Moreover, it was shown that the ability of hexahistidinyl-tagged Toc33 to bind guanine nucleotides was restored upon re-addition of an excess of Mg^{2+} over EDTA (Fig. 9). The same was also demonstrated for Toc33-GST and Toc159G-GST (data not shown). Therefore, the recombinant proteins were dialyzed for two hours against a buffer containing 5 mM EDTA prior to the binding assays. The resulting “empty” Toc-GTPases were subsequently used for the pull-down assays in presence of Mg^{2+} alone, or Mg^{2+} including GTP, GDP or GMP-PNP (5'-Guanylyl-imidodiphosphate), a non-hydrolyzable analog of GTP.

3.2.3.1. Homodimerization of Toc33 is favoured in presence of GDP

To examine Toc33 homodimerization, we used both recombinant Toc33-GST and hexahistidinyl-tagged Toc33. After dialysis of an aliquot of either protein against a buffer containing 5 mM EDTA, 2 µM Toc33-GST was incubated on ice with hexahistidinyl-tagged Toc33 in two concentrations (0.5 and 1.5 µM) in the presence of 10 mM $MgCl_2$ and either GTP, GDP or GMP-PNP (5 mM final concentration) or no guanine nucleotide. GST-sepharose was added to reisolate GST-tagged Toc33 including any hexahistidinyl-tagged Toc33 bound to it. Subsequently, the sepharose was washed and the bound protein eluted with reduced glutathione. The eluted protein was subjected to SDS-PAGE, followed by Western blot analysis. The Western blot was stained with Amido black (Fig. 17, A, upper panel, Amido black) and any hexahistidinyl-tagged Toc33 bound to Toc33-GST was detected using antibodies directed against the hexahistidinyl-tag (Fig. 17, A, lower panel).
Two different negative controls were performed. The first showed that hexahistidinyl-tagged Toc33 did not interact with GST-Sepharose in absence of Toc33-GST (Fig. 17, B, lane 1, NC1), thus excluding unspecific binding of hexahistidinyl-tagged Toc33 to the GST-sepharose. The second demonstrated that the antibody specific for the hexahistidinyl-tag did not crossreact with Toc33-GST (Fig. 17, B, lane 2, NC2).
The comparable amounts of Toc33-GST reisolated during the binding assay with different nucleotides (Fig. 17, A, upper panel, lanes 1-8) allowed us to qualitatively compare binding of the hexahistidinyl-tagged Toc33 under the different guanine nucleotide conditions tested.

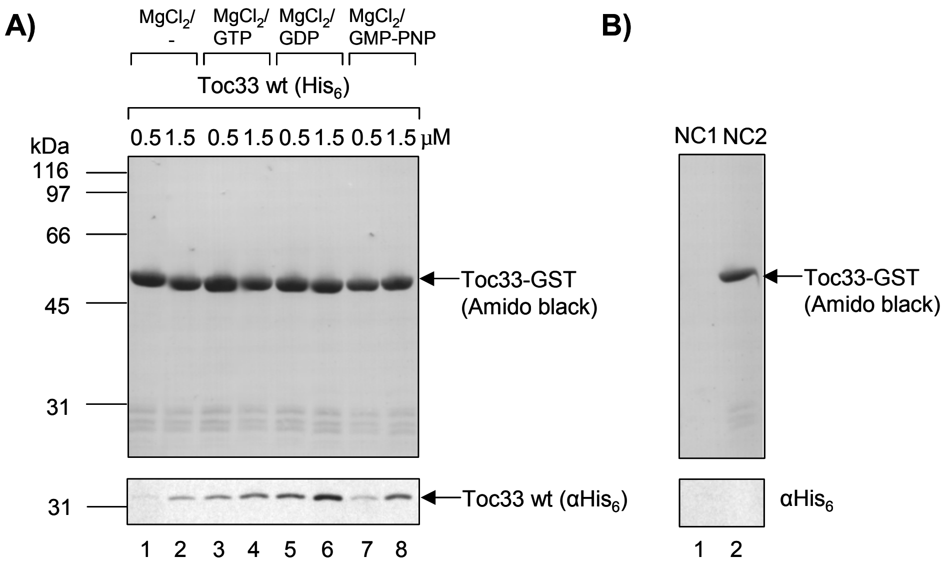


Fig. 17 Homodimerization of Toc33-GST and hexahistidinyl-tagged Toc33 is favoured by GDP: **A)** After dialysis of recombinant hexahistidinyl-tagged Toc33 and Toc33-GST against a buffer containing 5 mM EDTA, 2 µM Toc33-GST was incubated with two different concentrations of hexahistidinyl-tagged Toc33 (0.5 and 1.5 µM, respectively) and supplemented with 10 mM $MgCl_2$ and either no guanine nucleotide (lanes 1 and 2), GTP (lanes 3 and 4), GDP (lanes 5 and 6) or GMP-PNP (lanes 7 and 8) (final concentration 5 mM). GST-sepharose chromatography was used to reisolate Toc33-GST and any hexahistidinyl-tagged Toc33 bound to it. The protein was eluted from the sepharose with 10 mM reduced gluathione in 50 mM Tris/HCl pH 8.0 and the eluates were subjected to SDS-PAGE followed by Western blot analysis. The Western blot was stained with Amido black (upper panel) and hexahistidinyl-tagged Toc33 bound to Toc33-GST was detected with antibodies specific for the tag (αHis_6) (lower panel). **B)** Negative control (NC) reactions. The negative control reactions were done as described above. In the first reaction, the binding of 1.5 µM hexahistidinyl-tagged Toc33 to GST-Sepharose in presence of 5 mM GTP was tested (NC1), whereas in a second reaction 2 µM Toc33-GST supplemented with 5 mM GTP was used in the pull-down assay (NC2).

The binding efficiency of Toc33-GST and hexahistidinyl-tagged Toc33 was lowest in the absence of any guanine nucleotide (Fig. 17, A, lower panel, lanes 1 and 2), while in presence of GTP or GMP-PNP they were elevated and appeared to be similar (Fig. 17, A, lower panel, lanes 3 and 4 or lanes 7 and 8, respectively). The similar results in presence of GTP and GMP-PNP indicate that no or very slow GTP hydrolysis takes place on ice, which was confirmed by the finding that GTP hydrolysis did not occur under these conditions. However, the highest binding efficiency for Toc33-GST and hexahistidinyl-tagged Toc33 was observed in the presence of GDP (Fig. 17, A, lower panel, lanes 5 and 6), suggesting that Toc33 homodimerization of Toc33 is favoured by GDP, which is in agreement with the crystal structure data for pea Toc34 (Sun et al., 2002).

3.2.3.2. Heterodimerization of Toc33 and Toc159G is favoured by GDP

The finding that atToc33 and atToc159 interact directly (Hiltbrunner et al., 2001b; Bauer et al., 2002) via their G-domains (Smith et al., 2002) is critical for the targeting model of protein import into chloroplasts (Kessler and Schnell, 2004). Therefore we also examined the heterodimerization between hexahistidinyl-tagged Toc33 and Toc159G-GST in a soluble-phase binding assay. We chose Toc159G-GST, including the G-domain with an N-terminal GST-tag, because it was not possible to overexpress and purify Toc86, a functional form of Toc159 including the G- and the M-domains, under non-denaturing conditions, even though we tried a number of different tags (data not shown).

Prior to the binding assay aliquots of both purified hexahistidinyl-tagged Toc33 and Toc159G-GST were dialyzed against a buffer containing an excess of EDTA (5 mM), to remove any bound guanine nucleotides. Subsequently, 2 µM Toc159G-GST was incubated with increasing amounts of hexahistidinyl-tagged Toc33 and the same pull-down assay was performed as described above for the examination of the homodimerization between Toc33-GST and hexahistidinyl-tagged Toc33 (see section 3.2.3.1.). Following the assay, eluted Toc33-GST and any bound hexahistidinyl-tagged Toc33 were separated using SDS-PAGE, followed by Western blotting. The blot was stained with Amido black (Fig. 18, A, upper panel) and the hexahistidinyl-tagged Toc33 subsequently detected using specific antibodies for the hexahistidinyl-tag (Fig. 18, A, lower panel).

Two different negative control reactions were performed (NC1 and NC2). In the first control reaction the ability of 1.5 µM hexahistidinyl-tagged Toc33 to bind to GST-sepharose in presence of 5 mM GTP was examined. As can be seen on the Western blot stained with Amido black (Fig. 18, B, upper panel, lane 1, NC1) and on the blot tested with antibodies against the hexahistidinyl-tag (Fig. 18, B, lower panel, lane 1, NC1), hexahistidinyl-tagged protein did not bind to GST-Sepharose in the absence of any GST-tagged protein. Thus, no unspecifig binding of hexahistidinyl-tagged Toc33 to the GST-sepharose occurs in the absence of GST-tagged Toc159G.

In the second negative control reaction, 2 µM Toc159G-GST was incubated in presence of 5 mM GTP and reisolated using GST-sepharose chromatography. The Western blot stained with Amido black (Fig. 18, B, upper panel, lane2, NC2) reveals that a similar amount of Toc159G-GST was bound to the GST-sepharose as in the binding experiments including hexahistidinyl-tagged Toc33 (compare Fig. 18, B, upper panel, lanes 1 to 8 with Fig. 18, B, upper panel, lane 2) and that no crossreaction can be detected on the Western blot using antibodies specific for the hexahistidinyl-tag (αHis$_6$) (Fig. 18, B, lower panel, lane 2, NC2).

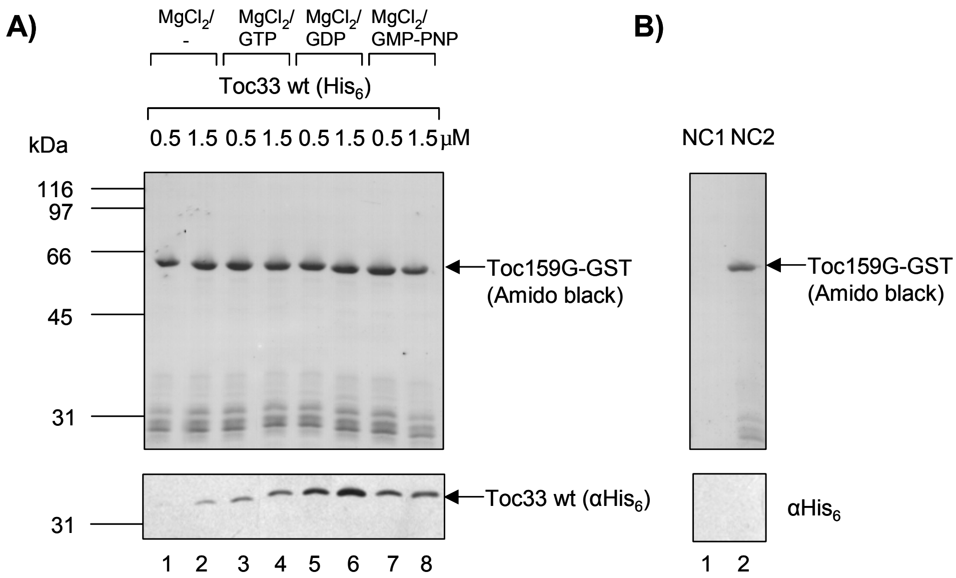


Fig. 18 Heterodimerization between hexahistidinyl-tagged Toc33 and Toc159G-GST is stabilized in the presence of GDP: A) After dialysis against a buffer containing 5 mM EDTA, 2 µM Toc159G-GST was incubated with either 0.5 or 1.5 µM hexahistidinyl-tagged Toc33 and supplemented with 10 mM $MgCl_2$ and either GTP, GDP or GMP-PNP (final concentration 5 mM) or no additional guanine nucleotide. The reisolation of GST-tagged Toc159G together with any bound hexahistidinyl-tagged Toc33 was accomplished using GST-sepharose chromatography. After elution with 10 mM reduced gluathione in 50 mM Tris/HCl pH 8.0, the eluted protein was analyzed by SDS-PAGE, followed by Western blotting. Subsequently, the blot was colored with Amido black to visualize Toc159G-GST (upper panel). The hexahistidinyl-tagged protein was detected using antibodies specific for the hexahistidinyl-tag (αHis$_6$) (lower panel). **B)** Negative control (NC) reactions. First, 1.5 µM recombinant hexahistidinyl-tagged Toc33 supplemented with 5 mM GTP was used for the pull-down assay as described above (NC1). Second, the same was done with 2 µM Toc159G-GST in the presence of 5 mM GTP (NC2).

Similar to the homodimerization for Toc33, the binding efficiency for the heterodimerization between hexahistidinyl-tagged Toc33 and Toc159G-GST was lowest in absence of any guanine nucleotide (Fig. 18, A, lower panel, lanes 1 and 2), whereas the efficiency of the heterodimerization supplemented with either GTP or GMP-PNP was similar (Fig. 18, A, lower panel, compare lanes 3 and 4 with lanes 7 and 8) and higher than in absence of any guanine nucleotide. The highest hetero-dimerization efficiency was detected in the presence of GDP (Fig. 18, A, lower panel, lanes 5 and 6), suggesting that heterodimerization between Toc33 and Toc159G, like Toc33 homodimerization, is favoured in presence of GDP. This supports an earlier experiment, showing that the interaction between [^{35}S]-labeled atToc33G and immobilized, recombinant hexahistidinyl-tagged atToc159G is stabilized by GDP (Smith et al., 2002).

4. Discussion

4.1. Characterization of Arg130, the putative arginine finger of atToc33

4.1.1. The GTP binding proteins of the Toc-complex

Toc34 and Toc159 are components of the trimeric Toc-complex (Kessler et al., 1994) that are involved in preprotein recognition and binding (Ma et al., 1996; Kouranov and Schnell, 1997). They both contain a GTP binding domain and are able to bind GTP (Kessler et al., 1994). Because non-hydrolyzable, or slowly hydrolyzable, analogues of GTP inhibit protein import into chloroplasts, it was suggested that GTP is involved in protein import into chloroplasts (Olsen and Keegstra, 1992; Kessler et al., 1994). Because no other factors requiring GTP are known to be involved in protein import into chloroplasts, interest has focused on Toc34 and Toc159, the two known GTP binding proteins at the outer chloroplast envelope.

Solving the crystal structure of pea Toc34 in 2002, was a first step towards the understanding of the molecular function of psToc34. Pea Toc34 was found to exist exclusively as a dimer in the crystal, whereas in solution monomers and dimers of psToc34 coexist (Sun et al., 2002). In the psToc34 dimer, every monomer coordinates one GDP molecule and one Mg^{2+} ion. The fact that every psToc34 monomer had a GDP molecule bound in absence of added guanine nucleotides during the purification and crystallization process, suggests that psToc34 has a sufficient affinity for nucleotides to allow for stable binding. Like in many other GTPases, a Mg^{2+} ion is found in every monomer of the pea Toc34 dimer (Sun et al., 2002), suggesting that this Mg^{2+} ion contributes to the affinity for guanine nucleotides, as is the case for a wide variety of GTPases (John et al., 1993). This is supported by crystal structures of Ras GTPases revealing that Mg^{2+} contributes directly to the coordination of the guanine nucleotide (Pai et al., 1990; Tong et al., 1991; Amor et al., 1994).

Because the overall fold of the pea Toc34 dimer resembles a small Ras-like GTPase together with its corresponding GAP, it was suggested that each monomer acts as a GAP for the other monomer in the pea Toc34 dimer (Sun et al., 2002), similar to Ffh and FtsY, the bacterial homologues of SRP54 and SRα which have been shown to reciprocally activate each other's GTPase activity (Powers and Walter, 1995; Rapiejko and Gilmore, 1997; Shan et al., 2004). This has been substantiated by a mutant psToc34 impaired in dimerization, which showed a reduced GTPase activity in comparison with wild type psToc34 (Sun et al., 2002).

Many GAPs contain an essential arginine residue, called the arginine finger, which protrudes into the active site of their corresponding GTPase (Ahmadian et al., 1997; Scheffzek et al., 1997, 1998; Li and Zhang, 2004). This arginine finger neutralizes negative charges arising during the GTP hydrolysis (Ahmadian et al., 1997; Scheffzek et al., 1998; Li and Zhang, 2004). Due to the position of Arg133 at the interface of the pea Toc34 dimer, it has been suggested that this arginine residue may work as an arginine finger (Sun et al., 2002).

4.1.2. Examination of the arginine finger hypothesis for Arg130 of atToc33

4.1.2.1. Heterologous expression and examination of the functionality of atToc33 wt and atToc33 R130A

To examine the arginine finger hypothesis for Arg133 of pea Toc34 proposed by Sun and colleagues (Sun et al., 2002), we used atToc33, the *Arabidopsis* ortholog of pea Toc34. A construct of atToc33, lacking the C-terminal transmembrane tail, which was replaced by a hexahistidinyl-tag, was used to mutate the putative arginine finger (Arg130) of atToc33 to alanine. Both wild type and mutant atToc33 were expressed in *E. coli* BL21(DE3) cells at high levels and purified from the soluble fraction under non-denaturing conditions (Fig. 7, B).

To ensure that the mutation of Arg130 to alanine does not disrupt the folding and hence the function of mutated atToc33, we first examined the ability of atToc33 wt and atToc33 R130A to bind GTP in a rapid [α^{32}P]-GTP binding assay, which revealed that both proteins bind GTP with similar efficiency (Fig. 8, A). Second, we determined the affinity of mutant atToc33 for GTP as well as for GDP and compared it to that of wild type atToc33. Both mutant and wild type atToc33 bound GTP and GDP with similar affinity, with slight preference for GTP (Fig. 8, B), suggesting that atToc33 R130A is still functional and can be used for the examination of the arginine finger hypothesis for the function of Arg130 of atToc33. Moreover, the calculated

dissociation constants for binding of GTP and GDP to wild type and mutant atToc33 indicate that the respective affinities are high enough to ensure saturation of GTP binding at cellular concentrations of GTP (>100 µM) (Bourne et al., 1991).
Comparing the affinities of atToc33 wt and atToc33 R130A for GTP and GDP with the respective affinities of other GTPases, it can be seen that wild type and mutant atToc33 bind GTP and GDP with a lower affinity than Ras (k_d = 0.01-0.00001 µM) (Manne et al., 1984; Feuerstein et al., 1987), but with a similar affinity like Ffh, the bacterial homolog of SRP54 (k_d = 1-2 µM) (Jagath et al., 1998) and SRα (k_d = ~10 µM) (Miller et al., 1995; Bacher et al., 1999). Because Ras-like GTPases have a very high affinity for guanine nucleotides, GEFs are needed to catalyze the exchange of GDP for GTP (Cherfils and Chardin, 1999). In contrast to this, no GEFs are known for SRP54 and SRα and their bacterial homologues, most likely due to the fact that their low affinity for guanine nucleotides and the rapid nucleotide dissociation allows the spontaneous exchange of GDP for GTP (Moser et al., 1997; Jagath et al., 1998; Peluso et al., 2001). In support of this, it has been shown that the intrinsic nucleotide dissociation rate of Ffh and FtsY is similar to those of other GTPases in presence of their corresponding GEFs (Moser et al., 1997; Jagath et al., 1998). The dissociation constants for GTP and GDP binding to atToc33 (Fig. 8, B), which are in the same range as the respective dissociation constants of Ffh and SRα, suggest that no GEFs may be needed for the exchange of GDP for GTP of atToc33. But this needs to be examined more closely.

In the pea Toc34 dimer, each monomer coordinates a Mg^{2+} ion in addition to one GDP molecule (Sun et al., 2002), suggesting that Mg^{2+} plays a role in GTP binding and/or hydrolysis, as is the case for many GTPases (Wittinghofer and Pai, 1991; Pan and Wessling-Resnick, 1998; Sprang and Coleman, 1998). In support of this, we found in a [α^{32}P]-GTP binding assay, employing EDTA to remove any bound guanine nucleotides, that the [α^{32}P]-GTP binding to atToc33 wt as well as atToc33 R130A is dependent on Mg^{2+} (Fig. 9, B). In the presence of EDTA only background [α^{32}P]-GTP binding to wild type and mutant atToc33 was detected (Fig. 9, B). Upon re-addition of an excess of Mg^{2+} over EDTA, [α^{32}P]-GTP binding to atToc33 wt and atToc33 R130A was restored (Fig. 9, B), suggesting that the nucleotide-free form of wild type and mutant atToc33 is stable enough to allow Mg^{2+}-dependent rebinding of guanine nucleotides.

Despite the fact that the members of the SRP family show an affinity for GTP and GDP very similar to wild type and mutant atToc33, it has been shown that binding of GTP and GDP to Ffh is not dependent on Mg^{2+} (Jagath et al., 1998). Nonetheless, Mg^{2+} ions were found to be bound to the complex between Ffh and FtsY, the bacterial homologues of SRP54 and SRα, in the presence of a non-hydrolyzable analogue of GTP in the crystal (Egea et al., 2004; Focia et al., 2004b). Furthermore, in the crystals of Ffh, Mg^{2+} was coordinated additionally to GDP (Focia et al., 2004a).
However, the finding that GTP binding to atToc33 is dependent on Mg^{2+} suggests that GEFs must exist specific for atToc33 which catalyze GDP dissociation from atToc33 by disturbing its coordination with Mg^{2+} as hypothesized for other GTPases (Pan and Wessling-Resnick, 1998). Currently, we have no information on the nature of these GEFs.

4.1.2.2. The GTPase activities of atToc33 wt and atToc33 R130A are comparable

The similar affinities of atToc33 wt and atToc33 R130A for GTP and GDP (Fig. 8), as well as the Mg^{2+}-dependent guanine nucleotide binding of wild type and mutant atToc33 (Fig. 9), suggest that recombinant atToc33 wt and atToc33 R130A are functional. This allowed us to examine the arginine finger hypothesis of Sun and colleagues (Sun et al., 2002).
GAPs accelerate the low intrinsic GTPase activity of their corresponding GTPases (Wittinghofer et al., 1997; Scheffzek et al., 1998). Many GAPs have a catalytic arginine residue, called arginine finger, which protrudes into the active site of the corresponding GTPase and directly participates in GTP hydrolysis by stabilizing emerging negative charges during the transition state (Ahmadian et al., 1997; Scheffzek et al., 1998; Li and Zhang, 2004). This arginine finger is necessary for the full functionality of the GAP, since arginine finger mutants of RasGAPs have a drastically reduced ability to increase the slow intrinsic GTPase activity of Ras (Ahmadian et al., 1997; Sermon et al., 1998), even though the affinity of the mutant GAPs for Ras did not change significantly (Sermon et al., 1998). Thus, it was suggested that the arginine finger is essential for efficient catalysis of GTP hydrolysis of the corresponding GTPase (Sermon et al., 1998). Therefore, if Arg130 acts as an arginine finger, one would expect that the GTPase activity of wild type atToc33 would

be much higher than the GTPase activity of mutant atToc33. However, both proteins hydrolyzed GTP at a very low rate (Fig. 10). The fact that Toc33 wt and Toc33 R130A have similar GTPase activities, indicates that Arg130 is most likely not an arginine finger.

Furthermore, the catalytic constants for GTP hydrolysis of atToc33 wt and atToc33 R130A are in the range of those for Ras (k_{cat} = 1.3*10^{-4}-5*10^{-4} s^{-1}) (Bourne et al., 1990) and Rho (1.5*10^{-4} s^{-1}) (Graham et al., 1999) without their corresponding GAPs, suggesting that atToc33 is not an activated GTPase. The conclusion that one monomer does not act as a GAP for the other monomer in the atToc33 dimer was corroborated by another group (Jelic et al., 2002, 2003), which found a similar GTPase activity for psToc34 (k_{cat} = 3.3*10^{-4} s^{-1}) (Jelic et al., 2002).

Only the GTPase activities reported recently for atToc33 (k_{cat} = 5.2*10^{-3} s^{-1}) and atToc34 (k_{cat} = 4.5*10^{-3} s^{-1}) (Jelic et al., 2003) are slightly higher in comparison with the GTPase activity found for atToc33 wt in this work (Fig. 10, B). Still, the GTPase activity is much lower than the accelerated GTPase activity of other GTPases such as Ras (k_{cat} = 10-23 s^{-1}) (Gideon et al., 1992; Eccleston et al., 1993; Sermon et al., 1998) or Rho (k_{cat} = 5.4 s^{-1}) (Graham et al., 1999) in the presence of their corresponding GAPs. However, GAP-like activities have been reported for precursor proteins. Rates of GTP hydrolysis by pea Toc34 and its *Arabidopsis* homologues were accelerated up to 30 times by preproteins, e.g. preSSU and preFNR (Jelic et al., 2002, 2003). The slight increase of the intrinsic GTP hydrolysis activity of pea Toc34 and its *Arabidopsis* homologues by preproteins may be too small for preproteins being a GAP for these proteins *in vivo*. Therefore, GAPs may exist which are specific for atToc33 and accelerate the GTPase activity much more than the preproteins found until now. But as recombinant proteins were used to determine the GAP activity of precursor proteins with atToc33, it is still possible that the properties of the recombinant proteins do not reflect the *in vivo* situation. Furthermore, components may be missing in the *in vitro* system, which, in addition to the precursor proteins, contribute to the activation of atToc33 hydrolysis. Thus, the search for GAPs for atToc33 should be continued, may be by using the yeast two-hybrid system.

4.1.3. Arg130 is essential for homodimerization of atToc33

It has been hypothesized that the putative arginine finger of pea Toc34 is involved in activation of GTP hydrolysis by the pea Toc34 dimer (Sun et al., 2002), but our results with the homologous *Arabidopsis* protein discredit this hypothesis.
Additionally, the crystal structure suggests that the putative arginine finger of pea Toc34 forms hydrogen bonds with amino acids of the other monomer and also with the GDP bound to the other monomer in the dimer (Sun et al., 2002). Thus, it has been argued that this arginine residue has an important role in dimerization (Sun et al., 2002). Another arginine, Arg128, of pea Toc34, may also be involved in dimerization of pea Toc34. This role was supported by the finding that the mutant psToc34, in which Arg128 was mutated to alanine, only existed in monomeric form in solution (Sun et al., 2002).
Therefore, to elucidate the function of the putative arginine finger Arg130 of atToc33, we examined the effect of the mutation of the putative arginine finger to alanine on homodimerization of atToc33. Using blue native PAGE, it was shown that atToc33 R130A is impaired in homodimerization (Fig. 11). This data has been corroborated by a soluble-phase pull-down assay (Fig. 12), suggesting that Arg130 is essential for atToc33 homodimerization.

4.1.4. Arg130 has to be present in both atToc33 monomers to allow for stable homodimerization

We examined whether Arg130 has to be present in both atToc33 monomers to make a stable interaction possible, expecting that the lack of Arg130 in one of the partners reduces homodimerization only slightly. Surprisingly, the presence of Arg130 in only one of the two atToc33 monomers is not sufficient for stable homodimerization (Fig. 12). Thus, Arg130 is absolutely essential for atToc33 homodimerization, suggesting that the interactions of Arg130 of one monomer with residues of the other monomer as well as with the GDP bound to the other monomer may be one of the driving forces for atToc33 homodimerization.
Thus, blue native PAGE (Fig. 11) and pull-down assay (Fig. 12) show that Arg130 is essential for stable atToc33 homodimerization. The pull-down experiment additionally

shows that Arg130 has to be present in both atToc33 monomers to allow for homodimerization (Fig. 12).

4.1.5. Arg130 is essential for heterodimerization of atToc33 and atToc159

Toc34 and Toc159 share a conserved G-domain. The most conserved regions beside the G-motifs, are in the region responsible for dimerization (Kessler and Schnell, 2002; Sun et al., 2002). Both Toc34 and Toc159 contain a dimerization motif specific for Toc-GTPases, suggesting that they interact via their G-domains (Kessler and Schnell, 2002). This interaction is crucial for the targeting model of protein import into chloroplasts, in which Toc34 functions as the membrane bound receptor for soluble, cytosolic Toc159 (Kessler and Schnell, 2004).
Therefore we examined the heterodimerization of atToc159 and either atToc33 wt or atToc33 R130A in a pull-down assay. We incubated recombinant, hexahistidinyl-tagged atToc33 wt or atToc33 R130A with radioactively labeled atToc159 and performed a pull-down assay using Ni-NTA agarose chromatography. AtToc33 R130A was found to have a strongly reduced ability to form heterodimers with atToc159 (Fig. 13), suggesting that stable heterodimerization between atToc33 and atToc159 is, like atToc33 homodimerization (Fig. 11 and 12), dependent on the presence of Arg130.

AtToc33 and atToc159 directly interact *in vitro* via their G-domains (Smith et al., 2002). Moreover, the G-domain of atToc159 is essential for the targeting of cytosolic atToc159 to the outer chloroplast membrane *in vivo* (Bauer et al., 2002). Thus, we examined also the role of Arg130 for the interaction between atToc33 and the G-domain of atToc159 in a pull-down assay. Using only the G-domain of atToc159 for the assay has also a technical advantage. It has been reported that Toc159 is very susceptible to proteolytic degradation (Bölter et al., 1998a; Chen et al., 2000). The degradation of [^{35}S]-labeled atToc159 can be seen on the autoradiogram of the pull-down experiment with full-length atToc159 (Fig. 13, A). Thus, employing only the G-domain of atToc159, instead of full-length atToc159, in the assay allows us to circumvent this degradation.

The pull-down experiment indicated that the heterodimerization of atToc33 R130A and atToc159G is impaired (Fig. 14). This suggests that, in addition to atToc33 homodimerization (Fig. 12) and heterodimerization of atToc33 and full-length atToc159 (Fig. 13), heterodimerization of atToc33 and atToc159G is also dependent on Arg130 (Fig. 14).

The finding that both atToc33 homodimerization as well as heterodimerization of atToc33 and atToc159 are dependent on Arg130, suggests that complex formation may rely on the same molecular mechanisms. The fact that the dimerization motif within the G-domains is very conserved in the two Toc-GTPases (Sun et al., 2002) supports this hypothesis.

4.1.6. Summary of the characterization of Arg130 of atToc33

We have presented evidence that Arg130 is not involved in GTP binding (Fig. 8) and GTP hydrolysis by atToc33 (Fig. 10). Furthermore, we have shown that Arg130 is essential for atToc33 homodimerization (Fig. 11 and 12) as well as for heterodimerization of atToc33 and atToc159 (Fig. 13), or the G-domain of atToc159 (Fig. 14), respectively.

4.2. Requirement for guanine nucleotides for atToc33 homodimerization and heterodimerization of atToc33 and atToc159

4.2.1. AtToc33 homodimerization is favored in the presence of GDP

Despite the fact that no function of atToc33 homodimerization is known either *in vitro* or *in vivo*, it has been examined here because of the finding that recombinant pea Toc34 exists as a dimer in the crystal and as both monomer and dimer in solution (Sun et al., 2002). Because it has been shown that each monomer coordinates a GDP molecule in the structure of the pea Toc34 dimer, it was of interest which guanine nucleotide favors the homodimerization of pea Toc34 and its *Arabidopsis* homologues in solution. As mentioned, the crystal structure suggests that psToc34 homodimerization is supported in the presence of GDP (Sun et al., 2002). In contrast to this, another group reported that the homodimerization of atToc33 is favored in the unliganded, nucleotide-free form (Jelic et al., 2003).

Thus, to re-examine the question of the requirement for guanine nucleotides for atToc33 homodimerization, we used a pull-down assay with GST- and hexahistidinyl-tagged recombinant atToc33 in the presence of $MgCl_2$ and either GTP, GDP, GMP-PNP, or in absence of guanine nucleotides.

4.2.1.1. Expression and examination of the functionality of recombinant hexahistidinyl-tagged atToc33 and atToc33-GST

Hexahistidinyl-tagged atToc33 and atToc33-GST were expressed in *E. coli* BL21(DE3) cells and purified from the soluble bacterial fraction under non-denaturing conditions to near homogeneity (Fig. 15). Because we did not remove the GST-tag, which has almost the same molecular weight as the soluble construct of atToc33, we had to ensure that the tagged protein is still functional after the purification process. To examine this, we performed a [α^{32}P]-GTP binding assay and included hexahistidinyl-tagged atToc33 as a positive control. This GTP binding experiment allowed a qualitative assessment of the GTP binding ability of the tested proteins and showed that both hexahistidinyl- and GST-tagged atToc33 bind GTP (Fig. 16), suggesting that the purified recombinant proteins are functional.

4.2.1.2. Examination of the requirement for guanine nucleotides for atToc33 homodimerization

In the absence of any guanine nucleotides during the purification and crystallization process, GDP was found bound to pea Toc34 in the crystals (Sun et al., 2002). Because we could not exclude the possibility that guanine nucleotides remain bound to recombinant hexahistidinyl-tagged atToc33 and atToc33-GST during purification, we had to remove any bound guanine nucleotides prior to the pull-down assay. To do so, we took advantage of the finding that guanine nucleotide binding to atToc33 is dependent on the presence of Mg^{2+} (Fig. 9) and used EDTA to remove any bound guanine nucleotides from the purified recombinant proteins. The guanine nucleotide "free" atToc33-GST and hexahistidinyl-tagged atToc33 were subsequently used for the pull-down assay in presence of Mg^{2+} and either GTP, GDP, GMP-PNP or no guanine nucleotide, revealing that atToc33 homodimerization is favored in the presence of GDP (Fig. 17), as it was assumed according to the structure of pea Toc34 (Sun et al., 2002).

Contrary to a recent report (Jelic et al., 2003), we found the lowest atToc33 homodimerization efficiency in the guanine nucleotide "free" form. We suspect that the absence of both Mg^{2+} and guanine nucleotide may lead to aggregation of atToc33. In the course of our experiments designed to find a suitable system to study the guanine nucleotide dependency of atToc33 homodimerization, we also observed this aggregation of atToc33 in the nucleotide and Mg^{2+} "free" form as higher molecular mass bands on blue native gels (data not shown).

4.2.2. Heterodimerization of atToc33 and atToc159G is favored by GDP

It was shown earlier that protein import into chloroplasts is dependent on GTP (Olsen and Keegstra, 1992; Kessler et al., 1994), suggesting that the two GTP binding Toc-proteins at the outer chloroplast membrane, i.e. Toc34 and Toc159, play a central role in these import processes. The understanding of the GTPase cycle of both GTPases could tell us much about how preprotein recognition, binding and translocation is achieved at the outer chloroplast envelope.

Presently, two models have been proposed for the mechanism of protein import into chloroplasts. The principle difference between the two models refers to the nature of the GTPase cycle (Kessler and Schnell, 2004). In the targeting model, the GTPase cycle coordinates the interaction of Toc159, Toc34 and Toc75 thus ensuring unidirectionality of the import (Weibel et al., 2003; Kessler and Schnell, 2004). In the motor model, GTP-hydrolysis at Toc34 serves to hand the preprotein from Toc34 to Toc159, and cycles of GTP hydrolysis and exchange of GDP for GTP at Toc159 push the preprotein across the outer chloroplast membrane (Becker et al., 2004b; Kessler and Schnell, 2004).

In both models, the interaction between Toc34 and Toc159 plays a central role (Kessler and Schnell, 2004). However, the targeting and the motor model, respectively, do not agree on the question of which guanine nucleotide is bound to Toc34 and Toc159 when they dimerize at the outer chloroplast envelope. According to the targeting model, they form a stable heterodimer in the presence of GDP (Smith et al., 2002; Kessler and Schnell, 2004), whereas according to the motor model the heterodimerization is stable in the presence of GTP (Becker et al., 2004b; Kessler and Schnell, 2004).

Thus, to answer the question in which form Toc34 and Toc159 preferably interact, we performed a soluble-phase pull-down assay with hexahistidinyl-tagged atToc33 and GST-tagged atToc159G. The functionality of atToc159G-GST was examined using a [α^{32}P]-GTP binding assay, revealing that atToc159G-GST binds GTP (Fig. 16), and is therefore likely to be functional.

Heterodimerization of empty proteins was supported by GDP (Fig. 18), as previously proposed by Smith and colleagues (Smith et al., 2002) and in the targeting model (Kessler and Schnell, 2004). This experiment suggests that GTP hydrolysis by both atToc33 and atToc159 is a prerequisite for their stable interaction.

Furthermore, the finding that both atToc33 homo- and heterodimerization of atToc33 and atToc159G are favored by GDP, supports the hypothesis that the interactions may rely on the same molecular mechanisms.

4.3. Similarities between the Toc-GTPase cycle and the SRP GTPase cycle

AtToc159 exists in an integral membrane as well as in a soluble cytosolic form, respectively (Hiltbrunner et al., 2001b; Lee et al., 2003), suggesting that soluble Toc159 is involved in protein import into chloroplasts. Furthermore, atToc33 functions as the membrane receptor for soluble atToc159 (Hiltbrunner et al., 2001b; Kessler and Schnell, 2002; Smith et al., 2002). This scenario has striking similarity with the SRP system involving two homologous GTPases, SRP and SRα, and one more distantly related GTPase, SRβ.

4.3.1. GTPases involved in protein import into the ER

SRP is a ribonucleoprotein consisting of six polypeptides and one RNA molecule. One of its subunits, SRP54, interacts with the α subunit of the SRP receptor (SRα). Both SRP54 and SRα have very similar GTP binding domains as can be judged from the structures of their bacterial homologues (Freymann et al., 1997; Montoya et al., 1997). They form a special subfamily of GTPases, characterized by a relatively low affinity for guanine nucleotides (Miller et al., 1995; Bacher et al., 1996).
In contrast to this, the second subunit of the SRP receptor, SRβ, has a higher affinity for guanine nucleotides (Bacher et al., 1999) and is more closely related to Ras-like GTPases than to the SRP subfamily, including SRP54 and SRα (Miller et al., 1995).
In this work it has been shown that atToc33 has an affinity for guanine nucleotides similar to that of the members of the SRP subfamily (Fig. 8, B). Furthermore, atToc33 functions as membrane receptor for soluble atToc159, resembling the situation at the ER membrane where SRα is the receptor for soluble SRP at the ER membrane. This suggests that the Toc-GTPases may functionally be more closely related to the subfamily of SRP GTPases than to the Ras-like GTPases. Additionally, it supports the hypothesis that the targeting of atToc159 to atToc33 at the chloroplast membrane may occur in a way similar to the targeting of SRP54 to SRα at the ER membrane (Kessler and Schnell, 2002).

4.3.2. The homotypic interaction of homologous GTPases

The homotypic interaction between Toc34 and Toc159 plays a crucial role in protein import into chloroplast. Similar to the homotypic interaction between Toc34 and Toc159, SRP54 and SRα also form a homotypic heterodimer, supporting the suggestion that the Toc-GTPases may functionally be more closely related to the SRP family of GTPases than to the Ras-like GTPases (see above). Thus, the heterodimeric interaction between SRP54 and SRα may serve as a model for the interaction between Toc34 and Toc159.
The structure of the heterodimeric GTPase core of Ffh and FtsY, the bacterial homologues of SRP54 and SRα, provided more insight into this homotypic interaction (Egea et al., 2004; Focia et al., 2004b). Both GTPases contain a G-domain similar to

the G-domain of Ras-like GTPases and an additional α-helical N-domain forming the NG-domains, specific for the SRP subfamily (Freymann et al., 1997; Montoya et al., 1997). Ffh and FtsY interact in a symmetric heterodimeric complex via their NG-domains (Egea et al., 2004; Focia et al., 2004b; Mandon and Gilmore, 2004). It has been suggested that complex formation is driven mainly by the G-domain (Egea et al., 2004). In the heterodimer between Ffh and FtsY, the G- and N-domain of one protein interact with its counterpart in the other protein, forming a quasi two-fold symmetrical heterodimer. The active site is located at the interface between Ffh and FtsY surrounding the two bound guanine nucleotides, suggesting that complex formation and GTPase activation are coupled (Egea et al., 2004; Focia et al., 2004b; Mandon and Gilmore, 2004). This has been supported by experiments showing that Ffh and FtsY reciprocally activate the GTPase activity of each other (Powers and Walter, 1995; Shan et al., 2004).

It is shown here that the G-domain of atToc159 interacts with atToc33 (Fig. 14), supporting the finding that atToc33 and atToc159 interact homotypically via their G-domains (Smith et al., 2002). Furthermore, Arg130, located in the G-domain of atToc33, is essential for this interaction (Fig. 14). Guanine nucleotide binding to atToc33 is dependent on the presence of Mg^{2+} (Fig. 9), in contrast to Ffh, the bacterial homologue of SRP54. Furthermore, atToc33 in its dimeric form is not a self-activated GTPase (Fig. 10) as is the case for Ffh and FtsY (Powers and Walter, 1995; Shan et al., 2004). Because of the conserved G-domains of atToc33 and atToc159, we hypothesize that atToc159 also does not function as a GAP for atToc33 in the heterodimer, but data supporting this hypothesis are lacking. Thus, while the Toc-GTPase and the SRP systems show strong overall similarity, GTP binding and GTP hydrolysis at the Toc-GTPases are probably regulated in a manner different from that of the SRP-GTPases.

4.3.3. The targeting model for protein import into chloroplasts

In the targeting model for chloroplast protein import (Kessler and Schnell, 2004), Toc159-GTP serves as soluble primary receptor for preproteins in the cytosol and Toc34 is the receptor for soluble Toc159 at the outer chloroplast membrane. Toc34-GTP and Toc159-GTP interact with low affinity via their G-domains. The assembly of the functional Toc-complex with Toc34, Toc159, Toc75, including the preprotein, increases the GTP hydrolysis by both Toc34 and Toc159, leading to the stable dimerization of Toc34-GDP and Toc159-GDP. Upon this steady interaction, the preprotein is handed over to the translocon, Toc75. Thereafter, the GDP bound to Toc34 and Toc159 is exchanged for GTP and Toc159 is released into the cytosol for a new round of preprotein import. In support of the targeting model, it has been shown here that the interaction between atToc33, the *Arabidopsis* ortholog of psToc34, and atToc159 is favored by GDP (Fig. 18).

We think that the homotypic interaction between Toc34 and Toc159 is critical for protein import into chloroplast. In support of this, the interaction between Toc34 and Toc159 plays a central role in the targeting model as well as in the motor model (Kessler and Schnell, 2004), even though the two models disagree on the question which guanine nucleotide is bound to Toc34 and Toc159 in the stable dimer. Thus, independent of the question which of the two models describes protein import into chloroplasts more accurately, we believe that the interaction between Toc34 and Toc159 is an essential step in this import process and suggest that the two GTPases form a stable dimer in their GDP bound form (Fig. 18). This implies that GTP hydrolysis at both Toc-GTPases is a prerequisite for their interaction.

4.3.4. The implications of the SRP GTPase cycle for the targeting hypothesis

SRP and SR are thought to be components of a proofreading mechanism to ensure efficient and unidirectional targeting to the ER. In a nutshell, this simplified model proposes that SRP recruits ribosomes with exposed signal sequences in the cytosol, whereas SR recruits the translocon. As a result of these processes, the affinity of SRP and SR for each other is increased, thereby facilitating the assembly of the ribosome-membrane junction (Keenan et al., 2001).

Based on striking similarities with the SRP GTPase cycle, as well as experimental findings, we suggest the following model for the Toc-GTPase cycle at the outer chloroplast membrane. Toc159 serves as a primary receptor for precursor proteins destined for the chloroplast. In turn, Toc34 recruits the protein-conducting channel, Toc75. These two processes initialize Toc159 and Toc34 to undergo their subsequent stable interaction, most likely by enhancing the GTP hydrolysis at both

Toc-GTPases. Subsequently, the stable interaction of both Toc159 and Toc34 in their GDP bound form leads to the assembly of the functional Toc-complex, resulting in transport of the precursor protein across the outer chloroplast membrane.

Thus, similar to the SRP system, we suggest that the main function of the Toc-GTPase cycle during protein import into the chloroplast is to coordinate the action of Toc159, Toc34 and Toc75, leading to the assembly of the functional Toc-complex and ensuring the unidirectionality of the import process. But even though we have made notable progress in revealing the Toc-GTPase cycle lately, most of the precise details of this cycle still remain elusive.

The two recent models for protein import into chloroplasts brought up a variety of hypotheses. According to the targeting model Toc159 shuttles as a cytosolic preprotein receptor between soluble and membrane bound form. However, it is not known how Toc159 inserts into and deinserts from chloroplast membranes. Thus, one important question that has to be addressed is if and how Toc159 can achieve membrane insertion and deinsertion. Furthermore, it has to be confirmed that soluble Toc159 interacts with preproteins in the cytosol.

Regarding the motor model the role of precursor phosphorylation prior to import has to be clarified. In addition, the proposed motor function of Toc159 has to be re-examined carefully.

In general, the contradictory results which lead to the two different models have to be reconciled. Because most of the data incorporated in the two models resulted from *in vitro* systems, combined *in vivo* and *in vitro* experiments may lead to more elaborate models in the future. It is likely that future models will include the valid features of both models.

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Publications

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