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AtToc90, a new GTP-binding component of the *Arabidopsis* chloroplast protein import machinery

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Abstract

AtToc159 is a GTP-binding chloroplast protein import receptor. *In vivo*, atToc159 is required for massive accumulation of photosynthetic proteins during chloroplast biogenesis. Yet, in mutants lacking atToc159 photosynthetic proteins still accumulate, but at strongly reduced levels whereas non-photosynthetic proteins are imported normally: This suggests a role for the homologues of atToc159 (atToc132, -120 and -90). Here, we show that atToc90 supports accumulation of photosynthetic proteins in plastids, but is not required for import of several constitutive proteins. Part of atToc90 associates with the chloroplast surface *in vivo* and with the Toc-complex core components (atToc75 and atToc33) *in vitro* suggesting a function in chloroplast protein import similar to that of atToc159. As both proteins specifically contribute to the accumulation of photosynthetic proteins in chloroplasts they may be components of the same import pathway.

Introduction

Light triggers a developmental program in plants that leads to photoautotrophic capacity (Kendrick and Kronenberg, 1994). A key step in this process is the biogenesis of chloroplasts from undifferentiated proplastids (Bauer *et al.*, 2001). The assembly of the photosynthetic apparatus requires the import of approximately 2000 different nuclear encoded proteins (Cline, 2000). Nuclear encoded proteins are synthesized in the cytosol as precursors with an N-terminal targeting peptide (transit peptide) and must be imported into the nascent chloroplast (Keegstra and Cline, 1999; Schleiff and Soll, 2000; Chen *et al.*, 2000b). The chloroplast is enclosed by an envelope consisting of two membranes. Both membranes contain translocons to facilitate the import of precursor proteins. These are termed the Toc- (translocon at the outer chloroplast membrane) and Tic-complexes (translocon at the inner chloroplast membrane). In pea, the Toc-complex consists of three major components forming a stable complex (Schnell *et al.*, 1994; Ma *et al.*, 1996). Toc159 (the number indicates the molecular mass in kD) and Toc34 are surface exposed, GTP-binding integral membrane proteins (Hirsch *et al.*, 1994; Kessler *et al.*, 1994; Seedorf *et al.*, 1995; Chen *et al.*, 2000a). The two proteins share a highly conserved GTP-binding domain (Kessler *et al.*, 1994). The available evidence indicates that the two proteins act in concert to recognize the chloroplast targeting peptide (Kouranov and Schnell, 1997; Sveshnikova *et al.*, 2000; Chen *et al.*, 2000a). Toc159 is thought to act as the primary receptor of transit sequences (Hirsch *et al.*, 1994; Perry and Keegstra, 1994;

Kouranov and Schnell, 1997). An alternative model in which Toc34 functions as the primary receptor and Toc159 as a GTP-dependent translocation motor has also been proposed (Schleiff *et al.*, 2003). Toc75, the third component, forms at least part of a hydrophilic channel through which precursors are translocated across the outer membrane (Schnell *et al.*, 1994; Hinnah *et al.*, 1997; Hinnah *et al.*, 2002).

Sequencing of the *Arabidopsis* genome revealed an unexpected complexity of Toc-components (The *Arabidopsis* Genome Initiative, 2000; Jackson-Constan and Keegstra, 2001; Hiltbrunner *et al.*, 2001a). All of the known pea Tic- and Toc-proteins have *Arabidopsis* homologues (Jackson-Constan and Keegstra, 2001). Strikingly the *Arabidopsis* homologues of the Toc34 and Toc159 GTP-binding proteins are encoded by a small gene family (Jackson-Constan and Keegstra, 2001; Hiltbrunner *et al.*, 2001a). AtToc33 and atToc34, the two homologues of pea Toc34, consist of a soluble GTP-binding domain (G-domain), containing GTP-binding motifs, followed by a C-terminal hydrophobic transmembrane helix anchoring the proteins in the outer membrane, while the G-domain faces the cytosol (Jarvis *et al.*, 1998). AtToc159, atToc132 and atToc120, highly homologous to pea Toc159, have a tripartite structure (Bauer *et al.*, 2000). In this group of proteins, the G-domain is flanked by an N-terminal A-domain (acidic domain) of unknown function. The A- and G-domains are both exposed to the cytosol (Chen *et al.*, 2000a). A C-terminal M-domain (membrane anchor domain) lacking predicted hydrophobic transmembrane helices anchors the proteins in the outer membrane (Hirsch *et al.*, 1994; Kessler *et al.*, 1994). In addition, precursor proteins were shown to directly interact with the M-domain (Kouranov and Schnell, 1997).

Disruption of atToc33 in the *ppi1* mutant (plastid protein import mutant 1) results in a chlorophyll-deficient seedling phenotype due to retarded chloroplast biogenesis, but mature plants are indistinguishable from the wild type (Jarvis *et al.*, 1998). Ectopic expression of atToc34 rescues *ppi1* indicating that atToc34 and atToc33 are functionally equivalent *in vivo* (Jarvis *et al.*, 1998). There are four homologues of pea Toc159 in *Arabidopsis,* atToc159, -132, -120 and -90 (Hiltbrunner *et al.*, 2001a; Jackson-Constan and Keegstra, 2001). The initial characterization of the last, atToc90, is presented in this study. Disruption of atToc159 in the *ppi2* mutant causes a lethal block in chloroplast biogenesis resulting in albino seedlings lacking thylakoids and starch granules from their undifferentiated plastids (Bauer *et al.*, 2000). Furthermore, *ppi2* plastids fail to normally accumulate highly abundant photosynthetic proteins (Bauer *et al.*, 2000). Nevertheless, *ppi2* plants have basal import activity as residual amounts of photosynthetic proteins as well as wild type levels of non-photosynthetic proteins are imported into the undifferentiated plastids (Bauer *et al.*, 2000). Thus, atToc159 is not required for the import of constitutive proteins and even its function in the import of photosynthetic proteins is partially substituted for, presumably by the activity of its homologues atToc132, -120 and -90 (Bauer *et al.*, 2001).

A poorly characterized aspect of chloroplast protein import comprises the events occurring in the cytosol before membrane translocation. It appears unlikely that newly synthesized chloroplast precursor proteins randomly diffuse to the chloroplast surface. Instead, it has been proposed that cytosolic factors may target precursors to the chloroplast surface (Schnell and Blobel, 1993). Indeed, a cytosolic member of the 14-3-3 family of proteins has been identified that binds to phosphorylated transit sequences (Waegemann and Soll, 1996) and thus forms a guidance complex to deliver precursor proteins to the Toc-complex (May and Soll, 2000). Recently, we have discovered that atToc159 also exists in an abundant soluble form, partitioning between the outer chloroplast membrane and the cytosol (Hiltbrunner *et al.*, 2001b). Cytosolic atToc159 is directly targeted to the Toc-complex in a homotypic interaction with atToc33 mediated by the G-domains of the two proteins (Hiltbrunner *et al.*, 2001b; Bauer *et al.*, 2002; Smith *et al.*, 2002; Lee *et al.*, 2003; Weibel *et al.*, 2003). These results are supported by the crystal structure of the pea Toc34 homodimer identifying sequence motifs conserved in both atToc33 and atToc159 which are required for dimerization and chloroplast targeting of soluble atToc159 (Sun *et al.*, 2002; Weibel *et al.*, 2003). These findings suggest that atToc159 and its homologues may function as soluble factors in chloroplast protein import (Hiltbrunner *et al.*, 2001b; Bauer *et al.*, 2002; Smith *et al.*, 2002). The Toc GTPase-system is strikingly analogous to the SRP system (which also employs a targeting mechanism based on two homotypic GTP-binding proteins) (Keenan *et al.*, 2001) as well as to the Sec-system: SecA is present as soluble protein in the cytosol but also as a membrane-attached protein in the outer bacterial membrane and may drive protein translocation across the periplasmic membrane (Economou and Wickner, 1994) in an ATP-dependent, sewing machine-type fashion. Similarly, Toc159 may provide a GTP-dependent push to aid precursor translocation across the outer chloroplast envelope (Schleiff *et al.*, 2003; Wallas *et al.*, 2003).

Here, we present the initial characterization of atToc90, a component of the protein import machinery functioning in the accumulation of photosynthetic proteins in the chloroplast.

Materials and methods

DNA constructs used in in vitro *synthesis of proteins*

AtToc90 was amplified by PCR from EST-clone H4C12T7 (Acc. No. W43716) using primers including a *BspHI* (CAT GTC ATG AAA GGC TTC AAA GAG TG) and *Eco*RI site (CCG GAA TTC TTA GGA AAC GAG AAA ATT CAC AA), respectively. The PCR product was then ligated into the *N*coI/*E*coRI site of pET21d resulting in pET21d-atToc90. pET21d-atToc159 has been described (Bauer *et al.*, 2000). pPCR-Script-atToc159$_{1\text{-}731}$, which contains the A-domain of atToc159 under the control of the T7 promoter, has been described (Bauer *et al.*, 2002). The constructs described above were used for *in vitro* synthesis of [^{35}S]methionine labeled proteins in a reticulocyte-based coupled transcription/translation system (Promega, Madison WI, USA).

Chloroplast isolation, insertion assay and immunoprecipitation

Arabidopsis thaliana plants var. Columbia 2 were grown under long-day conditions (16 h light, 100 μmol s^{-1} m^{-2}, 21 °C) on agar plates containing 0.5 × Murashige and Skoog medium supplemented with 1% w/v sucrose and used for isolation of intact chloroplast as previously described (Fitzpatrick and Keegstra, 2001), but with minor modifications. 1% w/v cellulase (Serva, Heidelberg, Germany) was used instead of 4% and 0.25% w/v macerozyme (Yakult Honsha Co., Tokyo, Japan) instead of 1%. For purification of intact chloroplasts a 40%/85% w/v Percoll (Amersham Biosciences, Buckinghamshire, England) step gradient was used rather than a linear gradient.

Insertion assays contained chloroplasts corresponding to 60 μg chlorophyll in import buffer (330 mM sorbitol, 50 mM Hepes/KOH pH 7.5, 40 mM KOAc, 2 mM $Mg(OAc)_2$, 25 μM DTT) supplemented with 3 mM ATP, 1 mM GTP, and 10 mM methionine in a final volume of 400 μl. The insertion reaction was started by adding 16 μl [^{35}S]atToc90, incubated for 15 min at 25 °C and stopped by adding 4 ml HS (330 mM sorbitol, 50 mM Hepes/KOH pH 7.5).

Chloroplasts were then washed once with HS, resuspended in 200 μl 0.6 M sucrose/TE (50 mM Tricine/KOH pH 7.5, 2 mM EDTA) and frozen at −20° C. After thawing the sample was diluted 5-fold with TE, sonicated and centrifuged for 15 min at 100 000 × *g* at 4 °C. The pellet was resuspended in 200 μl TE supplemented with 250 mM NaCl, 10% v/v glycerol and 2% v/v Triton X-100 and incubated for 10 min on ice to solubilize chloroplast membranes. After 15 min centrifugation at 100 000 × *g* at 4 °C the supernatant was removed and used for immunoprecipitation. Hundred microlitres solubilized membranes were incubated on a rolling shaker for 60 min at 4 °C with 3 μg affinity-purified atToc75 antibody (Hiltbrunner *et al.*, 2001b); as a control an equal amount of unspecific rabbit IgG was used. Twenty microlitres packed Protein A Sepharose beads (Amersham Biosciences) were added and the samples were incubated for another 60 min. Protein A Sepharose beads were then washed with TE supplemented with 250 mM NaCl, 10% v/v glycerol and 2% v/v Triton X-100 and incubated in 2% w/v SDS at 65 °C for elution. Samples were then concentrated as described elsewhere (Wessel and Flügge, 1984) and analyzed by SDS-PAGE and autoradiography according to standard protocols. Immunodetection of atToc75 was done as described (Hiltbrunner *et al.*, 2001b).

Soluble phase binding assay

Expression and purification of atToc33$_{1\text{-}265}$ and soluble phase binding assays were done as described (Hiltbrunner *et al.*, 2001b).

GFP-constructs and transient transformation of A. thaliana *protoplasts*

Plants used for protoplast isolation were grown as described above. Transient transformation of *A. thaliana* protoplasts was done as described (Jin *et al.*, 2001) using the following modification. Instead of 4% and 1%, respectively, we used only 1% w/v cellulase (Serva) and 0.25% w/v macerozyme (Yakult Honsha Co.).

The construct for transient expression of GFP-atToc159GM as well as pSH11 and pCL60 have been described (Bauer *et al.*, 2002). The construct for transient expression of GFP-atToc90 was obtained as follows: A DNA fragment encoding atToc90 was amplified from EST-clone H4C12T7 using 5′ and 3′ primers including *NotI* sites (5′ primer: 5′-ATA AGA ATG CGG CCG CGA TGA AAG GCT TCA AAG ACT G-3′; 3′ primer: 5′-ATA AGA ATG CGG CCG CTT AGG AAA CGA GAA AAT TCA CA-3′). Ligating the PCR fragment into the *NotI* site of pSH11 resulted in pSH11-atToc90. pRbcS-GFP was obtained by ligating a DNA fragment coding for the transit peptide of pea RbcS-3A (Acc. No. X04333) (5′ primer: 5′-CAT GCC ATG GCT TCT ATG ATA TCC TCT TCA-3′; 3′ primer: 5′-CAT GCC ATG GGC CAC ACC TGC ATG CAC TTT ACT CTT CCA-3′) into the *NcoI* site of pCL60.

DNA extraction, diagnostic PCR, Southern and Northern analysis

DNA extraction and diagnostic PCR was done as described (Bauer *et al.*, 2002). Plants containing a T-DNA insertion in *TOC90* were identified by PCR using the primer set 5′-ATA AGA ATG CGG CCG CGA TGA AAG GCT TCA AAG ACT G-3′/5′-AGA TAG GCT GAC ACG CCA AG-3′. The primers 5′-ATA AGA ATG CGG CCG CGA TGA AAG GCT TCA AAG ACT G-3′ and 5′-TTC AAT AAA CGG GCC TAA AG-3′ were used for identification of plants containing at least one wild type copy of *TOC90*.

Genomic DNA for Southern analysis was extracted from plants grown on plates using the DNeasy Plant Maxi Kit (Qiagen, Basle, Switzerland). *BglII* digest of genomic DNA and Southern transfer were done according to standard protocols. A PCR fragment amplified from pCAMBIA3300 (primers: 5′-TCG TCA ACC ACT ACA TCG AG-3′ and 5′-TGC CAG AAA CCC ACG TCA TG-3′) was used as a template for generation of a *pat*-specific probe. Labeling with [α^{32}P]dATP was done using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla CA, USA).

RNA used for Northern blotting was extracted from plants grown on plates using the RNeasy Plant Mini Kit (Qiagen). Formaldehyde agarose gel electrophoresis was done as described in the RNeasy Mini Handbook (Qiagen). Alkaline treatment, neutralization and subsequent Northern transfer were done according to standard protocols. Labeling of radioactive probes for detection of atToc90 and atToc159 mRNA was done as described above. To enhance the sensitivity, three separate probes were synthesized for each atToc90 and -159 and pooled prior to use. The templates were obtained by PCR amplification from pSH11-atToc90 and pSH11-atToc159GM, respectively, using primer pairs specific for atToc90 (5′-CAA GTT CCA CTT GAA AGC TTG-3′ and 5′-AAC TTT AAC TCC ACT GAC AG-3′; 5′-GCA TGG ATG TGG TTC AAC ATT-3′ and 5′-CTG CCG ATA CGC ATC GAT ATC-3′; 5′-GAG AAG ATC TGG TGT ACT CG-3′ and 5′-AGT CAT TTC AGT CTT ACT CCG CCG-3′) and atToc159 (5′-GCA GCG CAG GTT CTT TAG CG-3′ and 5′-GAA GCA GTA ATC GTC CTT AG-3′; 5′-CTT GCT AAG GAC GAT TAG TG-3′ and 5′-TTA GGA TGT GCA CGG GAC TG-3′; 5′-TAA GCT CCC TGG AGA CCA AG-3′ and 5′-CCT CGA GGT TCG CAC CAT AG-3′).

Protein extraction and immunoblot analysis

Extraction of total protein from *Arabidopsis* plants has been described (Rensink *et al.*, 1998). Immunoblot analysis was done according to standard protocols. Antibodies specific to atToc75 have been described (Hiltbrunner *et al.*, 2001b). Antibodies against the chlorophyll a/b binding protein (CAB) were provided by Prof. K. Apel (Swiss Federal Institute of Technology, Zurich, Switzerland).

Accession numbers

Accession numbers of sequences mentioned in this article are W43716 (EST-clone H4C12T7), At5g20300 (*TOC90*), U89959 (atToc33), AF069298 (atToc159) and X04333 (pea RbcS-3A). The cDNA sequence of atToc90 as determined by sequencing of EST-clone H4C12T7 has been deposited in the database (AY465351).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for non-commercial research. To obtain materials, please contact F. Kessler, fax: +41 32 718 22 71, felix.kessler@unine.ch.

Results

AtToc90, a new member of a family of GTP-binding proteins in the chloroplast protein import machinery

Sequencing of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000) has revealed a family of GTP-binding proteins related to pea Toc34 and Toc159. Apart from atToc159, -132 and -120 (Bauer *et al.*, 2000) the *Arabidopsis* databases contain a fourth and previously uncharacterized homologue of Toc159 which, based on the sequence similarity, we designate a Toc protein (Hiltbrunner *et al.*, 2001a). A full length EST-clone (H4C12T7) with a 2379 bp open reading frame (ORF) encoding the protein is available (Kieber *et al.*, 1993), indicating that the gene is expressed. The open reading frame accounts for a protein of 793 amino acids with a molecular mass of 89 338 Da (Figure 1). However, in the *Arabidopsis* databases a methionine residue 14 amino acids downstream of the first methionine in the ORF is predicted as the start codon (Figure 1a and c, indicated by an arrow) resulting in a predicted protein of 779 amino acids with a molecular mass of 87 726 Da. Comparison of the genomic sequence and the cDNA sequence derived from EST-clone H4C12T7 revealed the presence of two introns, a 98 bp intron in the 5′ untranslated region and a 407 bp intron 73 bp downstream of the predicted start codon. The coding sequence of atToc90 predicted in the databases is probably incorrect as it assumes a splice site 97 bp downstream of the predicted initiation codon. Thus, the protein predicted in the databases is by eight amino acids larger than the one encoded by EST-clone H4C12T7. With the uncertainties remaining regarding the correct start codon and splicing, we suggest to designate the protein atToc90, although its most likely mass is 87 726 Da. The protein (atToc90) deduced from the cDNA has extensive similarity to atToc159 (BLASTP value: 10^{-123}) (Figure 1c) as well as to atToc132 and atToc120 (data not shown). AtToc90 has G- (46.0% identity with the G-domain of atToc159; Figure 1c, underlined with a double line) and M-domains (30.9% identity with the M-domain of atToc159) but lacks the N-terminal A-domain characteristic of atToc159, -132 and -120 (Figure 1b). The sequence comparison of atToc90 with atToc159 and atToc33 revealed regions conserved above average. These include the GTP-binding motifs (Figure 1c, G1 and G3) and a newly identified dimerization motif (Sun *et al.*, 2002) (Figure 1c, D1) in the G-domain. But amino acid tracts of unknown function in the M-domain (corresponding to amino acids 435–483 and 509–561 of atToc90) (Figure 1c, overlined with dotted lines) are also conserved.

Light-induced upregulation of atToc90 transcript levels

The existence of EST-clones (H4C12T7, RZL46g05F, RAFL07-09-E20, RAFL07-18-F22) (Kieber *et al.*, 1993; Asamizu *et al.*, 2000; Seki *et al.*, 2002) indicates that *TOC90* is an expressed gene. To confirm that *TOC90* is functional and to determine whether the atToc90 mRNA level is similarly upregulated by light as the transcript levels of atToc159, -132 and -120 (Bauer *et al.*, 2000) total RNA was isolated from dark and light grown plants, respectively. In a Northern blotting experiment the RNA was separated by formaldehyde agarose gel electrophoresis and transferred to nitrocellulose. The blot was sequentially hybridized to probes specific for either atToc159 or atToc90 whereas 25S rRNA was used to control loading amounts (Figure 2). Binding of the gene-specific probes was quantified using a phosphorimager. Functionality of *TOC90* was confirmed by the presence of atToc90 mRNA (Figure 2, atToc90). Moreover, in light grown plants the transcript level of at Toc90 was upregulated 1.8-fold, comparable to the 2-fold light-induced upregulation of atToc159 mRNA. Thus, all Toc159 homologues in *Arabidopsis* are subject to light-induced upregulation at the transcript level (Figure 2) (Bauer *et al.*, 2000).

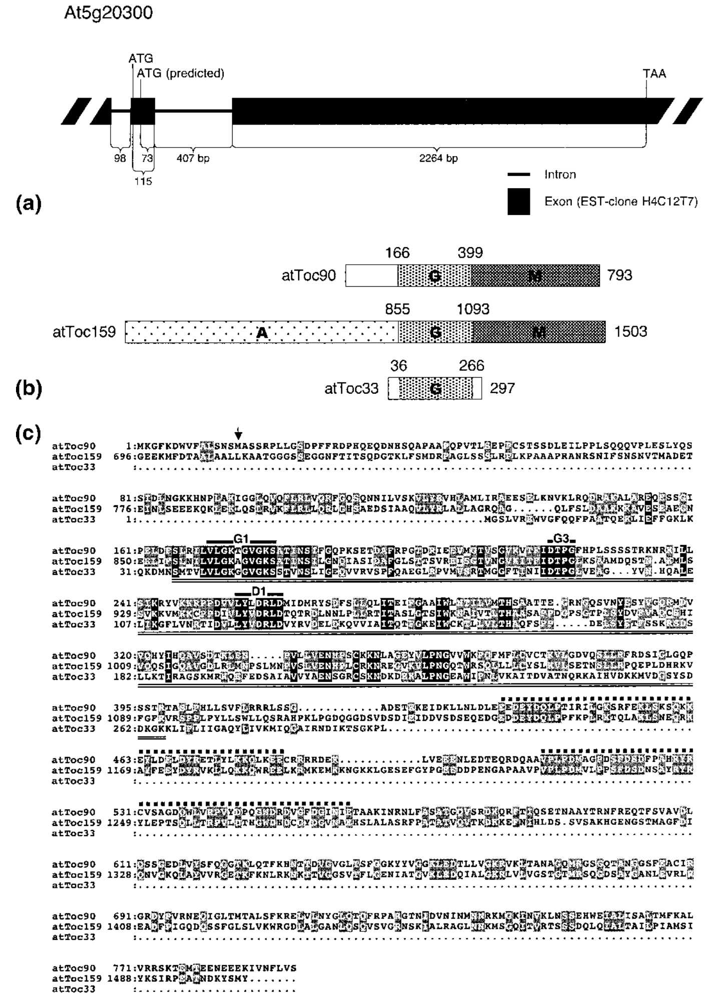

Figure 1. Intron/exon structure of *TOC90* and alignment of atToc90 to atToc159 and -33. (a) Intron/exon structure of *TOC90*. The first ATG codon in the open reading frame as well as the second ATG codon predicted to be the start codon *in vivo* are indicated. Black boxes and lines represent exons and introns, respectively. (b) Schematic sequence alignment of atToc90, -159 and -33. The A-, G- and M-domains are given in different shades of grey. Other amino acid tracts are given in white. (c) Detailed sequence alignment. The ClustalX (1.8) software (Thompson *et al.*, 1997) was used to align atToc90, -159 and -33. The G-domain is underlined with a double line. The G1, G3 and D1 motifs are overlined with solid lines. Conserved amino acid tracts in the M-domain are overlined with dotted lines. The methionine predicted to be the start codon of atToc90 is indicated by an arrow. For clarity, only residues 696-1503 of atToc159 are shown.

Association of atToc90 with chloroplasts and the Arabidopsis *Toc-complex*

Although we produced specific antibodies that recognize atToc90 synthesized in a reticulocyte lysate, they were not sensitive enough to detect the protein in plant protein extracts (data not shown). We therefore employed strategies not relying on antibodies to localize atToc90 at the subcellular level and to demonstrate its association with the Toc-complex.

To determine the subcellular distribution *in vivo,* we transiently expressed atToc90 fused N-terminally to GFP in *Arabidopsis* protoplasts. In *Arabidopsis* protoplasts, GFP-atToc90 (Figure 3a) was present at the chloroplast resulting in fuzzy peripheral fluorescence at the chloroplast surface. A portion of GFP-atToc90 also appeared to be present in the cytosol, similar to GFP alone (Figure 3d). GFP-atToc90 was not localized within the chloroplast, using GFP fused to the C-terminus of the transit peptide of the

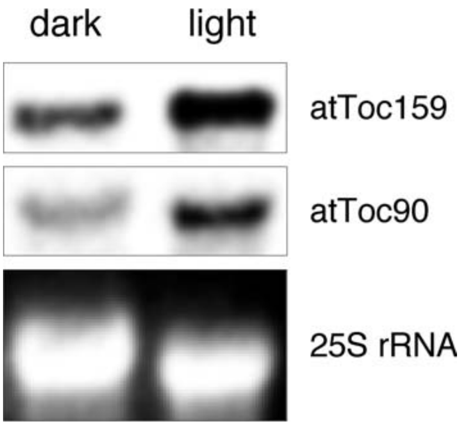

Figure 2. The atToc90 transcript level is enhanced by light. Total RNA was extracted from *Arabidopsis* plants grown for 10 days in the dark (dark) or under long-day conditions (light) and used for formaldehyde agarose gel electrophoresis and Northern blot analysis. AtToc159 (atToc159) and atToc90 (atToc90) mRNAs, respectively, were detected using [^{32}P]labeled gene-specific probes. A phosphorimager was used to detect radioactive signals. Equal loading amounts were confirmed by identical 25S rRNA levels (25S rRNA).

small subunit of RubisCO as a stromal reference (pRbcS-GFP, Figure 3c). Although we lack supporting immunological evidence, we conclude that atToc90 is targeted to the chloroplast envelope, but do not exclude that, similar to atToc159 (Figure 3b) (Bauer *et al.*, 2002), a cytosolic pool of the protein may also exist.

To provide additional evidence for a chloroplast envelope location and to determine whether atToc90 may function in chloroplast protein import, we analyzed its ability to associate with isolated chloroplasts and the Toc-complex *in vitro*. For this purpose we synthesized radioactively labeled atToc90 ([^{35}S]atToc90) by coupled *in vitro* transcription/translation in a reticulocyte lysate (Figure 3e, lane 1). The reticulocyte lysate containing [^{35}S]atToc90 was incubated with isolated *Arabidopsis* chloroplasts. Subsequently, we isolated total chloroplast membranes and solubilized the membrane proteins with a buffer containing Triton X-100. After clearing the solubilized fraction by centrifugation at 100 000 × g, affinity-purified antibodies specific to atToc75 (Figure 3e, αatToc75) were used to isolate solubilized Toc-complexes. Immunocomplexes were collected using Protein A covalently linked to Sepharose, washed with buffer and released by heating in 2% SDS. In a control experiment, the same procedure was repeated using unspecific purified IgG (Figure 3e, PI). Upon incubation with chloroplasts, [^{35}S]atToc90 was indeed present in the solubilized and cleared membrane protein fraction (Figure 3e, lane 2, longer exposure) indicating that atToc90 had associated with chloroplasts. Furthermore, [^{35}S]atToc90 was present in the αatToc75 immunoprecipitate (Figure 3e, lane 5) but absent from the unspecific antibody control eluate (Figure 3e, lane 8), suggesting that atToc90 functions as a component of the chloroplast protein import machinery.

Direct interaction between soluble atToc90 and the G-domain of atToc33

The recently determined crystal structure of pea Toc34 (the pea ortholog of atToc33) has revealed that the protein forms dimers involving a dimerization motif, D1, in its G-domain (Sun *et al.*, 2002). D1 is highly conserved throughout the *Arabidopsis* family of Toc-GTPases (Figure 1c). Thus, conceivably all of these GTPases may be able to form homodimers as well as heterodimers in all permutations (Hiltbrunner *et al.*, 2001a). We have recently demonstrated that soluble synthetic atToc159 binds directly to the purified recombinant G-domain of atToc33 (Hiltbrunner *et al.*, 2001b) and that the interaction contributes to targeting of atToc159 to the Toc-complex. *Vice versa,* soluble synthetic atToc33 also binds directly to atToc159 (Smith *et al.*, 2002). To test whether atToc90 may also function as binding partner for atToc33, we incubated [^{35}S]atToc90, synthesized in a reticulocyte lysate, with purified recombinant atToc33$_{1\text{-}265}$-H$_6$ in an *in vitro* pull down assay using nickel-nitrilotriacetic acid-agarose (Ni-NTA-agarose) to reisolate atToc33$_{1\text{-}265}$-H$_6$ and the proteins associated with it (Figure 4). Soluble [^{35}S]atToc159 was used as a positive control known to bind to atToc33$_{1\text{-}265}$-H$_6$ (Hiltbrunner *et al.*, 2001b) whereas the isolated A-domain of atToc159 (atToc159$_{1\text{-}731}$) was employed as a negative control as it was previously demonstrated to be dispensable for chloroplast association of atToc159 (Bauer *et al.*, 2002; Smith *et al.*, 2002). Binding of [^{35}S]atToc90 and [^{35}S]atToc159 increased with rising concentrations of atToc33$_{1\text{-}265}$-H$_6$ (Figure 4a, lanes 2–5) whereas no binding to

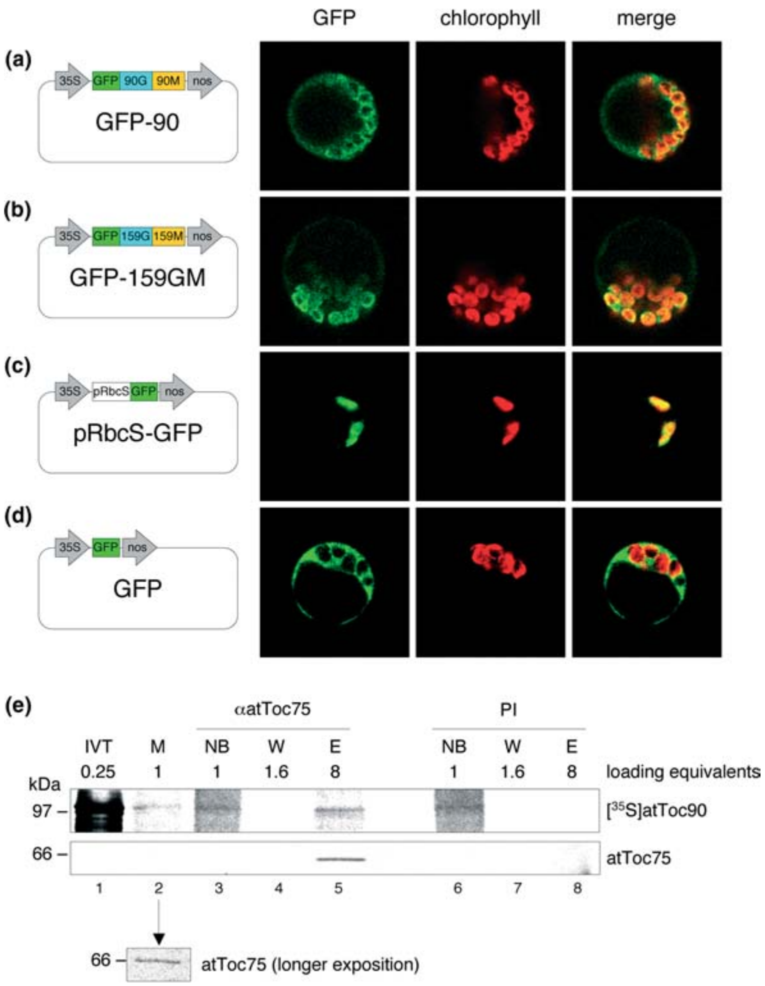

Figure 3. Subcellular localization of atToc90. (a)–(d) Transient expression of GFP-tagged atToc90 in protoplasts. GFP fusion constructs were transformed into isolated *Arabidopsis* protoplasts. Expression of GFP fusion proteins was monitored by confocal laser scanning microscopy. The schematic drawings of the vectors on the left side depict the constructs used for transient expression in protoplasts. The G- and M-domains are colored in blue and yellow, respectively. GFP is shown in green, promoter and terminator in grey. GFP, GFP fluorescence; chlorophyll, chlorophyll autofluorescence; merge, merged GFP and chlorophyll fluorescence. (a) GFP-90. N-terminal GFP fusion to atToc90. (b) GFP-159GM. N-terminal GFP fusion to the combined G- and M-domains of atToc159. (c) pRbcS-GFP. C-terminal GFP fusion to the transit peptide of pea RbcS. (d) GFP control. (e) Association of atToc90 with the *Arabidopsis* Toc-complex. $[^{35}S]$labeled atToc90 was synthesized by coupled *in vitro* transcription/translation (IVT) and incubated with isolated *Arabidopsis* chloroplasts. Total chloroplast membranes were then isolated and solubilized in buffer containing Triton X-100 (M). Affinity-purified antibodies specific to atToc75 were used to isolate solubilized Toc-complexes (αatToc75). Unspecific purified IgG was used as a control (PI). Immunocomplexes were isolated using Protein A covalently linked to Sepharose, washed (W) and eluted (E). Samples of the fractions (including the non-bound protein (NB)) were used for SDS-PAGE and Western blot transfer. Proteins were detected either by autoradiography ($[^{35}S]$atToc90) or by affinity-purified antibodies specific to atToc75 followed by enhanced chemiluminescence (atToc75). Loading equivalents are indicated above the lanes. For the solubilized membrane fraction a longer exposure is shown.

Ni-NTA-agarose alone was detectable (Figure 4a, lane 1) suggesting a specific interaction. By comparison, $[^{35}S]$atToc159$_{1\text{-}731}$ did not bind to atToc33$_{1\text{-}265}$-H$_6$ confirming that binding of soluble $[^{35}S]$atToc90 and -159 to atToc33$_{1\text{-}265}$-H$_6$ is specific (Figure 4b).

T-DNA insertional disruption of TOC90

To investigate the function of atToc90 *in vivo* we searched the SAIL database for *Arabidopsis* mutant lines containing a T-DNA insertion in *TOC90* and identified line 1236.c11. Among the descen-

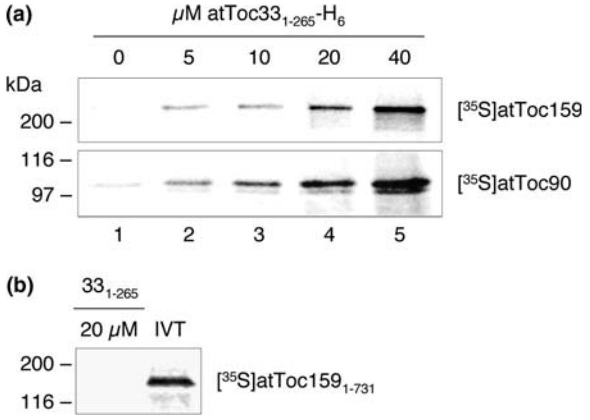

Figure 4. Direct binding of soluble [^{35}S]atToc90 and -159 to atToc33$_{1\text{-}265}$. (a) Soluble phase binding assay. Radioactively labeled soluble atToc90 and atToc159 were synthesized by coupled *in vitro* transcription/translation. Increasing concentrations of purified, soluble His$_6$-tagged atToc33$_{1\text{-}265}$ (concentrations indicated in μM) were incubated with labeled atToc90 ([^{35}S]atToc90) and atToc159 ([^{35}S]atToc159), respectively, and reisolated by Ni-NTA chromatography. The samples were analyzed by SDS-PAGE and autoradiography. (b) The A-domain of atToc159 does not bind to atToc33. Radioactively labeled A-domain of atToc159 ([^{35}S]atToc159$_{1\text{-}731}$) was synthesized by coupled *in vitro* transcription/translation (IVT) and used in a soluble phase binding assay in presence of 20 μM atToc33$_{1\text{-}265}$-H$_6$. The sample was analyzed as described in (a).

dants of T-DNA insertion line 1236.c11 several individual plants (e.g. 1236.c11-4 and -33) contained a homozygous T-DNA insertion at *TOC90* as determined by PCR (Figure 5a). More detailed analysis of the T-DNA insertion at *TOC90* showed that T-DNA left borders were present both at the 3′ and at the 5′ ends of the insertion, suggesting that the T-DNA insertion at this locus consists of two (or more) T-DNA molecules (Figure 5b). In a Southern blotting experiment the genomic DNA of individual plants (1236.c11-4 and -33) was digested with *Bgl*|II and hybridized to a phosphinothricin acetyl transferase probe (*pat*). Sequence analysis predicts the formation of 1.4 and 3.2 kb Bgl|II fragments in case of two T-DNA molecules inserted right border to right border at *TOC90*. In fact, line 1236.c11-4 (Figure 5c, 1236.c11-4) yielded two hybridizing fragments corresponding in mass to those predicted (Figure 5b). Additional Bgl|II fragments hybridizing in line 1236.c11-33 (Figure 5c, 1236.c11-33) suggest that the parental line 1236.c11 also contains T-DNA insertions at loci other than *TOC90,* which in line 1236.c11-4 apparently were lost through segregation. DNA sequencing revealed that the T-DNAs are inserted in the 407 bp intron of *TOC90* four nucleotides downstream of the exon/intron border. Northern blotting using a probe specific for atToc90 did not reveal detectable levels of atToc90 mRNA in line 1236.c11-4 (Figure 5d, atToc90) indicating that the homozygous T-DNA insertion in the intron results in a gene knock out. In contrast, the level of atToc159 transcript is not affected (Figure 5d, atToc159). None of the plants of line 1236.c11 had a visible phenotype (Figure 5e). Yet, we propose to name the mutant *ppi4* (plastid protein import mutant 4), in accordance with the nomenclature used for knock out mutants of import components, such as *TOC33* (*ppi1*) (Jarvis *et al*., 1998) *TOC159* (*ppi2*) (Bauer *et al*., 2000) and *TOC34* (*ppi3*) (P. Jarvis, personal communication).

ppi2 ppi4 *double knock out mutants*

The previously described plastid protein import mutants *ppi1* (Jarvis *et al*., 1998) and *ppi2* (Bauer *et al*., 2000), as well as *tic40* (Chou *et al*., 2003) have pale, lack of chlorophyll phenotypes. These phenotypes reflect effects of the mutations on chloroplast biogenesis. The absence of such a phenotype in plants lacking atToc90 (Figure 5e, *ppi4*) suggests that atToc90 has a non-essential function. However, single knock out mutants often lack a visible phenotype (Bouché and Bouchez, 2001) and, not unlikely, atToc159, -132 and -120 may fully substitute for atToc90 in the *ppi4* mutant. Despite a T-DNA insertion in the gene encoding the major plastid protein import receptor, atToc159, *ppi2* mutant plants still import detectable residual amounts of photosynthetic proteins (Bauer *et al*., 2000). Furthermore, import of non-photosynthetic proteins (e.g. atToc75 or atTic110) into plastids is not affected in the *ppi2* mutant (Bauer *et al*., 2000). Thus, alternative import receptors, such as atToc132, -120 and -90, may substitute to support import activity in *ppi2* plants.

To test this hypothesis we crossed the *ppi4* mutant line 1236.c11-4, containing no other T-DNA insertions but that in *TOC90,* into the *ppi2* background resulting in *ppi2 ppi4* double knock out lines disrupted in both *TOC159 and TOC90*. Immunoblot analysis revealed residual

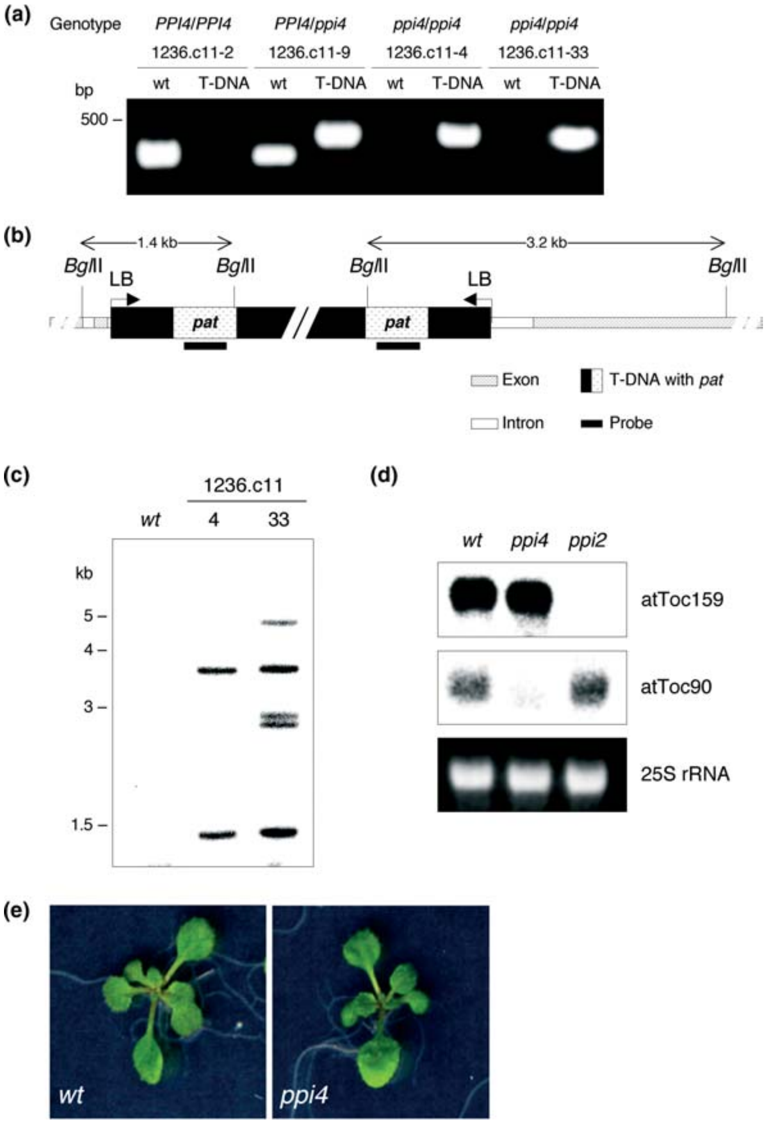

Figure 5. Characterization of the *ppi4* mutant containing a T-DNA insertion in *TOC90*. (a) PCR analysis. DNA was extracted from individual plants (1236.c11-2, -4, -9 and -33) derived from T-DNA insertion line 1236.c11 and analyzed by PCR to determine the respective genotype (indicated at the top). PCR reactions were designed to detect the presence of a wild type copy of *TOC90* (*wt*) and the T-DNA insertion in *TOC90* (T-DNA), respectively. The PCR products were analyzed by agarose gel electrophoresis. *PPI4*, wild type allele, *ppi4*, mutant allele. (b) T-DNA insertion in *TOC90*. Two T-DNA molecules (black) are inserted at the *TOC90* locus (grey, exons; white, introns) with their left borders (LB) towards the 5′- and 3′-end of *TOC90*, respectively. The Bgl||II fragments detected by a *pat*-specific probe in the Southern blot experiment described in (c) are indicated as well. *pat,* phosphinothricin acetyltransferase (the gene was used as a selection marker during T-DNA mutagenesis). (c) Southern blot analysis. Genomic DNA was extracted from wild type (*wt*) and *TOC90* knock out plants (lines 1236.c11-4 and 1236.c11-33), digested with *Bg*|II and used for Southern blot analysis. A [^{32}P]labeled probe specific to the phosphinothricin acetyltransferase gene (*pat*) was used to detect the *Bg*|II fragments. (d) Northern blot analysis. Total RNA was extracted from wild type (*wt*), *TOC90* (line 1236.c11-4) (*ppi4*) and *TOC159* (line CS11072) (*ppi2*) knock out plants and used for Northern blot analysis. AtToc159 (atToc159) and atToc90 (atToc90) RNA was detected using [^{32}P]labeled gene-specific probes. The 25S rRNA (25S rRNA) indicates equal loading amounts. (e) *TOC90* knock out plants have no visible phenotype. Wild type (*wt*) and *TOC90* knock out plants (line 1236.c11-4) (*ppi4*) were grown for 16 days under long-day conditions.

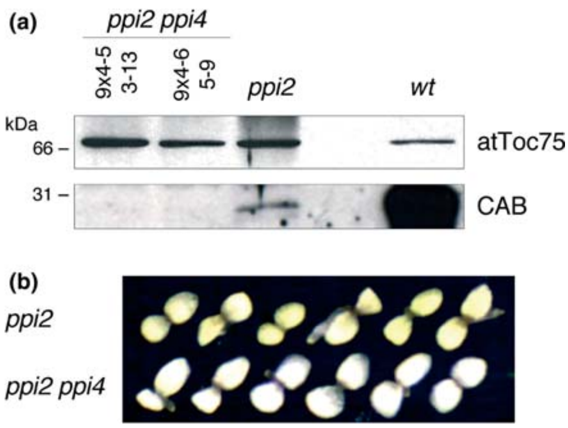

Figure 6. Initial characterization of *ppi2 ppi4* double knock out plants. (a) Immunoblot analysis of total protein extracts from *ppi2 ppi4* double knock outs. Two independent *ppi2 ppi4* double knock out lines (9 × 4-5 3-13 and 9 × 4-6 5-9) as well as *ppi2* single mutant plants were grown for 6 days on 0.5 × Murashige and Skoog medium without sucrose and used for protein extraction. Total protein corresponding to 3 μg (atToc75) and 13 μg (CAB), respectively, were analyzed by immunoblotting using antibodies specific to either atToc75 or CAB. (b) Phenotype of *ppi2 ppi4* double knock out plants. Photograph of individuals of one representative *ppi2 ppi4* double knock out line (9 × 4-5 3-11) and *ppi2* seedlings (line CS11072) grown for 5 days on 0.5 × Murashige and Skoog medium without sucrose.

amounts of chlorophyll a/b binding protein (CAB) in 6-days-old *ppi2* single knock out plants (Figure 6a, *ppi2,* CAB). In contrast, *ppi2 ppi4* double mutant lines did not accumulate detectable amounts of CAB (Figure 6a, *ppi2 ppi4,* CAB) and appeared even paler than *ppi2* single knock out plants (Figure 6b). However, atToc75 (Figure 6a, atToc75) and atTic110 (data not shown) were present at equal levels in both single and double knock out plants. Thus, atToc90 is probably involved in the import of CAB and other photosynthetic proteins, but not in that of constitutive proteins, in the absence of the major plastid protein import receptor, atToc159.

Discussion

We report on the characterization of a sixth, and last, member of the Toc-family of GTP-binding proteins, a group of proteins involved in the early stages of chloroplast protein import (Hiltbrunner *et al.*, 2001a; Jackson-Constan and Keegstra, 2001). Based on its sequence similarity and molecular mass, we have designated the protein encoded by the ORF in *Arabidopsis* EST-clone H4C12T7 atToc90. Sequence alignment of atToc90 to atToc159 and atToc33 revealed high conservation of GTP-binding and dimerization motifs in the G-domain (Figure 1c, overlined with solid lines). Comparison of the M-domains of atToc90 and atToc159 also revealed several highly conserved stretches of amino acids (Figure 1c, overlined with dotted lines). These conserved stretches of amino acids may reflect common functional properties such as the ability to associate with the outer membrane or transit sequence binding and suggest that atToc90 may function in ways similar to atToc159. However, atToc90 lacks the N-terminal A-domain typical of atToc159, -132 and -120 (Figure 1b) supporting the notion that this domain has an auxiliary, yet unidentified role (Bauer *et al.*, 2002; Lee *et al.*, 2003).

To determine the subcellular location of atToc90, the protein was expressed as a GFP fusion in isolated *Arabidopsis* protoplasts (Figure 3). GFP was fused to the N-terminus of atToc90 (GFP-atToc90) to avoid obstruction of the C-terminal M-domain which is predicted to function in membrane association. GFP-atToc90 was present at the chloroplast periphery suggesting a chloroplast outer membrane location (Figure 3a). Some GFP-atToc90 fluorescence was observed in the cytosol. This fluorescence may be due either to unassembled protein or a pool of the protein functional in the cytosol. A similar observation has previously been reported for atToc159 (Bauer *et al.*, 2002) and was confirmed using both biochemical and immunohistochemical methods (Hiltbrunner *et al.*, 2001b; Lee *et al.*, 2003). Those data are consistent with partitioning of the protein between the cytosolic compartment and the outer chloroplast membrane. Possibly, GFP-atToc90 is also present in a soluble, cytosolic pool but this notion needs to be confirmed independently once high affinity antibodies become available.

To confirm the chloroplast location and to determine whether atToc90 is likely to function in chloroplast protein import, we analyzed its ability to associate with isolated chloroplasts and the Toc-complex. Synthetic atToc90 bound to chloroplast membranes and a portion of the protein specifically co-immunoprecipitated with atToc75 (Figure 3). These findings suggest that atToc90 functions as a component of the chloroplast protein import machinery in *Arabidopsis*. It now has been demonstrated that all four Toc159 homologues in *Arabidopsis* associate with other components of the Toc-complex (Figures 3 and 4) (Bauer *et al.*, 2000). However, the distinct function of the Toc159 homologues are not clearly understood, nor is it known whether they all assemble into a large holocomplex or rather into distinct smaller trimeric complexes.

We recently demonstrated that soluble, cytosolic atToc159 is targeted to the Toc-complex via a direct interaction with atToc33 (Hiltbrunner *et al.*, 2001b; Bauer *et al.*, 2002). Here, we demonstrate that atToc90, synthesized in a reticulocyte lysate, binds specifically to the recombinant G-domain of atToc33 in a soluble phase binding assay as does synthetic atToc159 (Figure 4). The results indicate that atToc90 directly interacts with atToc33 and suggests that this interaction may contribute to the targeting of atToc90 to the Toc-complex. The interaction between atToc159 and atToc33 has been characterized in detail indicating that the two proteins heterodimerize *via* their G-domains (Smith *et al.*, 2002; Weibel *et al.*, 2003). Whether this also applies to the G-domain of atToc90, while it appears likely, needs to be addressed in the future.

Some of the most intriguing questions surrounding the *Arabidopsis* chloroplast protein import receptors are those regarding their distinct functions. It has been proposed that atToc159, -132, -120 and -90 represent distinct substrate specific receptors (Cline, 2000). However, so far no evidence has been presented to substantiate this model. On the other hand, it has been proposed that the receptors have overlapping function and that atToc159, the knock out of which is lethal, is required for bulk import of very abundant photosynthetic proteins (Bauer *et al.*, 2000). Here we show that atToc90 is upregulated by light (Figure 2) suggesting that atToc90 may be involved in chloroplast development. We have demonstrated earlier that despite the absence of atToc159 in the *ppi2* mutant a number of constitutively expressed proteins and residual amounts of the normally abundant photosynthetic proteins are still imported (Bauer *et al.*, 2000). Thus, a function of atToc90, possibly together with atToc132 and -120, may be to support import activity in the background of atToc159. To determine the function of atToc90 *in vivo,* we isolated a T-DNA insertional knock out of the corresponding gene (Figure 5). The knock out of the gene had no visible effect on the mutant plants (Figure 5e) suggesting that atToc159, -132 and -120 substitute for atToc90 in the *ppi4* mutant. Yet, atToc90 may contribute to import of (photosynthetic) proteins as *ppi2 ppi4* double knock out mutants, disrupted in both *TOC159* and *TOC90* did not accumulate detectable amounts of CAB whereas *ppi2* plants, which are wild type regarding *TOC90*, did (Figure 6a, CAB). Furthermore, *ppi2 ppi4* double knock outs appeared even paler than *ppi2* plants suggesting even more severely impaired chloroplast biogenesis (Figure 6b). In contrast, neither *ppi2* single nor *ppi2 ppi4* double knock out plants were affected in accumulation of atToc75 (Figure 6a, atToc75), atTic110 (data not shown) and possibly other non-photosynthetic plastid proteins. These findings strongly suggest that the highly homologous atToc132/-120 receptor pair may be required for import of constitutively expressed plastid proteins and have an essential function in plant development. As both atToc90 and -159 contribute to the accumulation of photosynthetic proteins and behave as components of the Toc-complex, they may both function in the same import pathway.

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