A Toc159 Import Receptor Mutant, Defective in Hydrolysis of GTP, Supports Preprotein Import into Chloroplasts*[s]

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The heterotrimeric Toc core complex of the chloroplast protein import apparatus contains two GTPases, Toc159 and Toc34, together with the protein-conducting channel Toc75. Toc159 and Toc34 are exposed at the chloroplast surface and function in preprotein recognition. Together, they have been shown to facilitate the import of photosynthetic proteins into chloroplasts in *Arabidopsis*. Consequently, the *ppi2* mutant lacking atToc159 has a non-photosynthetic albino phenotype. Previous mutations in the conserved G1 and G3 GTPase motifs abolished the function of Toc159 *in vivo* by disrupting targeting of the receptor to chloroplasts. Here, we demonstrate that a mutant in a conserved G1 lysine (atToc159 K868R) defective in GTP binding and hydrolysis can target and assemble into Toc complexes. We show that atToc159 K868R can support protein import into isolated chloroplasts, albeit at lower preprotein binding and import efficiencies compared with the wild-type receptor. Considering the absence of measurable GTPase activity in the K868R mutant, we conclude that GTP hydrolysis at atToc159 is not strictly required for preprotein translocation. The data also indicate that preprotein import requires at least one additional GTPase other than Toc159.

Chloroplast biogenesis requires the import of hundreds of cytoplasmically synthesized proteins into the chloroplast. Most of them are synthesized as preproteins with a transit sequence specifying import into the organelle (1). Inside the chloroplast, the transit sequence is cleaved by the stromal-processing peptidase (SPP) (2). A dual membrane envelope encloses the chloroplast. Translocon complexes at the outer (Toc)[2] and inner membranes (Tic) facilitate recognition and translocation of preproteins. In recent years, many components of the Toc and Tic complexes have been identified and characterized (3–5). The Toc complex is heteromeric, consisting of a core of three different proteins (the numbers indicate the molecular mass in kDa), Toc159, Toc34, and Toc75, which *in vitro* may be sufficient for translocation (6).

Toc75 represents the protein-conducting channel of the Toc complex and is related to bacterial Omp85 known to be involved in the insertion of hydrophobic β-barrel proteins in bacteria and the mitochondrial outer membrane (7). Toc159 and Toc34 are homologous GTP-binding proteins. Their GTP-binding domains (G-domains) are exposed at the surface of the chloroplast (8). In addition to the G-domain, Toc159 has a large cytosolic N-terminal acidic (A-) domain of unknown function and a C-terminal membrane anchoring (M-) domain, which is mostly hydrophilic in sequence. Toc34 consists largely of its G-domain anchored in the outer membrane by a C-terminal hydrophobic sequence (9).

Biochemical work has demonstrated the importance of the concerted (inter-)actions of the Toc159 and Toc34 G-domains and their GTPase activities in preprotein import (10–14). According to the current models either of the two may serve as the primary import receptor. The precise mechanisms and sequence of reactions occurring at the GTPases, however, remain, unclear. Two models have been proposed to explain the function of the Toc GTPases, the "targeting model" and the "motor model." In the targeting model, Toc159 functions as the primary receptor delivering the preprotein to Toc34 and assisting its insertion into the Toc75 channel. Toc159 will then pick up a new preprotein and repeat the cycle (11–14). In this model, one GTP binding and hydrolysis cycle of Toc159 is required for the delivery of each preprotein. In the motor model, Toc34 functions as the primary receptor delivering preproteins to Toc159. Subsequently, Toc159, functioning as a motor protein, drives the preprotein across the Toc75 channel (6, 15, 16). In this model, one or more GTP hydrolysis cycles at Toc159 are required to achieve translocation of preprotein across the outer chloroplast membrane.

[2] The abbreviations used are: Toc/Tic, translocon complexes at the outer/inner chloroplast membrane; TAP, tandem affinity purification; CAB, chlorophyll a/b-binding protein; SSu, small subunit of Rubisco; WT, wild type.

The crystal structures of the Toc34 G-domains of *Arabidopsis* (atToc33) and pea (psToc34) have shed light on the structure of the G-domains of Toc-GTPases (17–19). The overall fold and the G1 and G3 motifs are conserved with the GTPase superfamily. But when compared with the prototypical Ras protein, Toc GTPase G-domains contain additional stretches of amino acids including a motif that appears to be involved in the hetero- and homodimerization. The activities of a number of mutations of the G1-motif have been characterized *in vitro* using the recombinant, purified G-domains. The atToc159 A864R/K868N/S869R triple mutation (known as mGTP mutant) reduced GTP binding to below 5% of the wild type and GTP hydrolysis was no longer detectable (14). Similar results were obtained for the atToc159 K868R mutation (around 20% GTP binding, no detectable GTP hydrolysis) (12). In contrast, the atToc159 A864R mutant bound GTP as the wild type but showed a lower hydrolytic rate than the wild type (12, 20).

In *Arabidopsis*, both the T-DNA insertion mutants of atToc159 (plastid protein import mutant 2; *ppi2*; albino) (21) and atToc33 (*ppi1*; pale green) (22) have photosynthetic phenotypes suggesting their involvement in the import of photosynthetic proteins into the nascent chloroplast. But many non-photosynthetic proteins are imported normally into both of these mutants. This import activity has been attributed to the homologs of atToc159 (atToc132, -120, and -90) and atToc33 (atToc34) (23–25). Moreover, atToc159/-33/-75 and atToc132/-120/-34/-75 may be present in separate heteromeric Toc complexes and represent pathways specific to photosynthetic and non-photosynthetic, housekeeping preproteins, respectively (9, 25).

The *ppi2* mutant has been exploited to dissect the domain requirements of atToc159 using complementation assays (14, 26). The complementation experiments demonstrated that the G-domain is essential. The characterized GTPase mutations, the atToc159 mGTP triple point mutation (A864R, K868N, S869R) (G1 motif) (14), S869N (G1), and D909L (G3) (26) prevented atToc159 from inserting into the outer chloroplast membrane and from assembling into the Toc complex. These experiments clearly demonstrate an essential role of the Toc159 G-domain in the assembly of Toc159 into Toc complex. But because the mutants stayed in the cytosol, the functions of the G-domain in protein translocation could not be analyzed further in these mutants. A recent study demonstrated that atToc159 A864R increases preprotein import into isolated chloroplasts despite its reduced hydrolysis activity (20).

Here, we identify the atToc159 K868R single point mutation as a non-lethal Toc159 GTPase mutant still able to assemble into the Toc complex. We demonstrate an inhibitory effect of the K868R mutation on preprotein binding and import. Because of its strong defect in GTP hydrolysis, we conclude that hydrolysis is not directly implicated in the preprotein binding and translocation mechanism. Import experiments in the atToc159 K868R background using the non-hydrolyzable GTP analog GMP-PNP indicate preprotein import requires additional GTPases other than Toc159.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—Plants were grown on soil ("Rasenerde Top Dressing," Ricoter AG) or *in vitro* on 0.8% Phyto Agar (Duchefa, Haarlem, The Netherlands) containing $0.5\times$ Murashige and Skoog (MS) medium (Duchefa) under short day conditions (8-h light, 16-h dark, 120 μmol m^{-2} s^{-1}, 21 °C). For import experiments, the *in vitro* growth medium was complemented with 0.8% w/v sucrose.

atToc159 Constructs: Binary Vectors for Stable Plant Transformation—For cloning of pCHF7-NTAPi-Toc159GM, the coding sequence for the tandem affinity purification tag (NTAPi) was amplified from plasmid pTN289-NTAPi (27) with the primer pair NTAPi_Nco_forw (5′-CAT GCC ATG GTG GTC GAC AA-3′) and NTAPi_RevBsphXba (5′-TGC TCT AGA AGT CATG AGC CCT CCA CTA GAC AGT-3′) and cloned NcoI/XbaI into BspHI/SpeI-digested pCHF7-H6Toc86. pCHF7-H6Toc86 contains BspHI/XbaI the coding sequence for Toc159aa728–1503 with an N-terminal hexahistidine tag. The binary vector pCHF7 (Dr. Christian Fankhauser, Center for Integrative Genomics, University of Lausanne, CH) contains two CaMV35S eukaryotic promotors, one of them having a duplicated enhancer region, an rbcs terminator, and the phosphinotricin acetyltransferase (*pat*) gene for transgene selection. For the cloning of pCHF7-NTAPi-Toc159GM K868R, the coding sequence for the G-domain of atToc159 was amplified from pET21d-Toc159aa727–1093 (28) with sense primer 159GB5′NcoI (5′-CAT GCC ATG GCA AGT CAG GAT GGT ACG AAA-3′) and reverse primer pETR (5′-TTA TGC TAG TTA TTG CTC AG-3′) and cloned NcoI/NotI into vector pETM40 (EMBL, Heidelberg, Germany). The resulting plasmid pETM40-Toc159G was the template to introduce the K868R point mutation along with an additional AseI restriction site using complementary primers qc_K868R_s (5′-CCG GGG TGG GAA GAA GTG CTA CTA TTA ATT CCA TTT TGG G-3′) and qc_K868R_r (5′-CCC AAA ATG GAA TTA ATA GTA GCA CTT CTT CCC ACC CCG G-3′) via QuikChange® Site-directed Mutagenesis (Stratagene). The construct pETM40-Toc159G K868R was digested with NcoI and StuI, and the fragment was ligated into BspHI/StuI-digested pCHF7-NATPi-Toc159GM. pCHF7-NTAPi-Toc159 and pCHF7-NTAPi-Toc159 K868R were obtained by cloning the NcoI/StuI fragment derived from pET21-Toc159 (21) or pET21-Toc159 K868R to BspHI/StuI-digested pCHF7-NTAPi-Toc159GM. pCHF7-Toc159 and pCHF7-Toc159 K868R were obtained were obtained by cloning the NcoI/StuI fragment derived from pET21-Toc159 or pET21-Toc159 K868R to BspHI/StuI-digested pCHF7-H6Toc86.

AtToc159 Vectors Used for in Vitro Translation—For pET21d-Toc159GM, the coding sequence of Toc159aa728–1503 was amplified from pET21d-Toc159 (21) using primers 5′-CAT GTC ATG ACT AGT CAG GAT GGT ACG AA-3′ and 5′-CCC AAG CTT GAA TTC TTA GTA CAT GCT GTA CTT GTC G-3′ and cloned BspHI/HindIII into the NcoI/HindIII sites of pET21d. For pET21d-Toc159GM mGTP, a cDNA fragment encoding atToc159aa728–852 was amplified from pET21d-Toc159 using primers 5′-CAT GTC ATG ACT AGT CAG GAT GGT ACG AA-3′ and 5′-CAT GGA GCT

CTT CGT TGC CCT CAG CCT CAG-3′. A second cDNA fragment coding for Toc159aa851–1503 (A864R/K868N/S869R) was amplified from the same template using primers 5′-CAT GGA GCT CAT CTT CTC CCT AAA CAT ACT GGT CCT TGG AAA ACG CGG GGT GGG AAA CAG AGC TAC TAT AAA TTC ATT TTT G-3′ and 5′-CCC AAG CTT GAA TTC TTA GTA CAT GCT GTA CTT GTC G-3′. The cDNA fragments were digested either with BspHI and SacI or with SacI and HindIII and ligated in one step into the NcoI/HindIII site of pET21d resulting in pET21d-Toc159GM mGTP. pET21d-Toc159GM K868R was cloned in the same way as pET21d-Toc159GM mGTP but with primer 159K868R (5′-CAT GGA GCT CAT CTT CTC CCT AAA CAT ACT GGT CCT TGG AAA AGC CGG GGT GGG AAg AAG TGC TAC TAT AAA-3′) as sense primer in the second PCR reaction.

Plant Transformation—Stable transformation of heterozygous *Arabidopsis thaliana* (ecotype Wassilewskija) *ppi2* plants was carried out using the floral dip method (29). Transformants were selected by screening the T1 generation for BASTA (phosphinotricin). To determine the genotypes and the presence of transgenes, DNA isolated from seedlings was analyzed by PCR using primers specific for *ppi2* allele (forward 5′-GAA TAG GGT TTT AAT CGG AAG-3′ and reverse 5′-GAT GCA ATC GAT ATC AGC CAA TTT TAG AC-3′), the wild-type atTOC159 gene (forward 5′-GAA TAG GGT TTT AAT CGG AAG-3′ and reverse 5′-TGC CAC ATC AAC ATG CAC TG-3′) or the transgene (forward 5′-TCT CCA CTG ACG TAA GGG AT-3′ and reverse 5′-GCC TGT CCT ATA GAC TGT TG-3′). The presence of the K868R mutation was confirmed by digestion of the transgene specific PCR product with AseI and sequencing.

Detection of Protein Expression by Western Blotting—Proteins were isolated from *Arabidopsis* leaves according to (30). 0.5% v/v protease inhibitor mixture for plant cell extracts (Sigma P9599) was added to the extraction buffer to avoid proteolytic degradation. After concentration of proteins by chloroform/methanol precipitation (31) 50 μg were separated by SDS-PAGE and blotted onto nitrocellulose membranes.

Chlorophyll Measurements—Chlorophyll levels were determined by the method of Lichtenthaler (32).

Antibodies—Purified atToc33 was used to produce rabbit antibodies (Eurogentec S. A., Belgium). Antibodies were affinity purified using the antigen coupled to Affi-Gel 10 (BioRad) according to the supplier's recommendation. Other primary antibodies or sera used in this study include anti-atToc75 (13), anti-atToc159 serum or purified against the recombinant A-domain (anti-atToc159A) (21), anti-chlorophyll a/b-binding protein (CAB) (Dr. K. Apel, Institute of Plant Science, ETH Zurich, Switzerland), anti-atToc132 (Dr. D. J. Schnell, University of Massachusetts, Amherst, MA), monoclonal anti-actin (Sigma A0480), anti-rubisco and anti-phosphoribulokinase (PRK) (Dr. Pia Stieger, University of Neuchâtel, Switzerland), anti-BIP (Dr. Guillaume Gouzerh, University of Neuchâtel, Switzerland) anti-PGL40,[3] rabbit IgGs (ICN Immunobiologicals), and anti-GFP (Living Colors® A.V. monoclonal Antibody JL-8, Clontech, Cat. 632381).

Subcellular Fractionation and Membrane Extraction—Seedlings were ground in mortar in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1% v/v protease inhibitor mixture for plant cell extracts (Sigma P9599). The protein extract was centrifuged for 10 min at $1,500 \times g$. The supernatant was passed through a 200-μm nylon mesh and subjected to centrifugation (Beckmann, SW55-Ti, $100,000 \times g$, 1 h, 4 °C). The $100,000 \times g$ pellet fraction was treated with extraction buffer or extraction buffer supplemented with 2 M NaCl or 100 mM Na_2CO_3, pH 11.5 followed by centrifugation (Beckmann, SW55-Ti, $100,000 \times g$, 1 h, 4 °C).

Transient Expression and in Vivo Targeting of RbcS-nt:GFP in ppi2 Protoplasts—Protoplasts were isolated from leaf tissue of *ppi2* plants using the method described previously (33). 10 μg of the plasmid encoding the N-terminal 90 amino acids of the small subunit of Rubisco fused to GFP (RbcS-nt:GFP) together with 20 μg of empty vector, T7-attoc159 (26), pCHF7-NTAPi-Toc159GM, pCHF7-NTAPi-Toc159GM K868R, pCHF7-NTAPi-Toc159, and pCHF7-NTAPi-Toc159 K868R were cotransformed by PEG (polyethylene glycol)-mediated transformation (33). At 12 h after transformation, total protein extracts from transformed protoplasts were analyzed by Western blotting using anti-GFP and atToc159 antibodies.

Chloroplast Import Experiments—Intact chloroplasts of 4-week-old *in vitro* grown *Arabidopsis* plants were isolated according to Ref. 34 with the following modifications. Cellulase Onozuka R-10 and macerozyme R-10 (Serva 16419, 28302) were used at 1.5% and 0.375% w/v, respectively. The breakage buffer was supplemented with 0.1% v/v protease inhibitor mixture for plant cell extracts (Sigma P9599). Chloroplasts were purified on a Percoll step gradient (40% v/v and 85% v/v in 300 mM sorbitol, 20 mM Tricine-KOH, pH 8.5, 5 mM $MgCl_2$, 2.5 mM EDTA) rather than a linear gradient. For each import reaction chloroplasts corresponding to 15 μg of chlorophyll and 5 μl of *in vitro* translated (TNT® T7 Quick-coupled Transcription/Translation System, Promega) preprotein of the small subunit of Rubisco (pSSu) were used. Where indicated chloroplasts were preincubated with 10 mM GMP-PNP for 5 min at room temperature in the dark and for 15 min at 4 °C in the light prior to starting the import reactions with the preprotein substrate. For preprotein binding reactions/early import intermediate formation, the preprotein substrate was dialysed against 330 mM sorbitol, 50 mM Hepes-KOH, pH 8 to remove nucleotides. Chloroplasts were held on ice in the dark for 20 min prior to the assays to deplete of internal ATP. Energy-depleted chloroplasts corresponding to 25 μg of chlorophyll were incubated in the presence or absence of 0.1 mM ATP and 0.1 mM GTP with the dialysed preprotein at 25 °C for 10 min in the dark. Chloroplasts were reisolated by centrifugation through a 35% v/v Percoll cushion. All samples of chloroplast binding and import reactions were resolved by SDS-PAGE and analyzed by phosphorimaging (BioRad, Personal Molecular Imager Fx). For quantification the Quantity One® software (BioRad) was used.

Toc159 Targeting Assays—Toc159 chloroplast targeting reactions were done according to Ref. 14 with the following modifications: *A. thaliana* (ecotype Wassilewskija) plants were grown for 4–5 weeks under short day conditions. After the
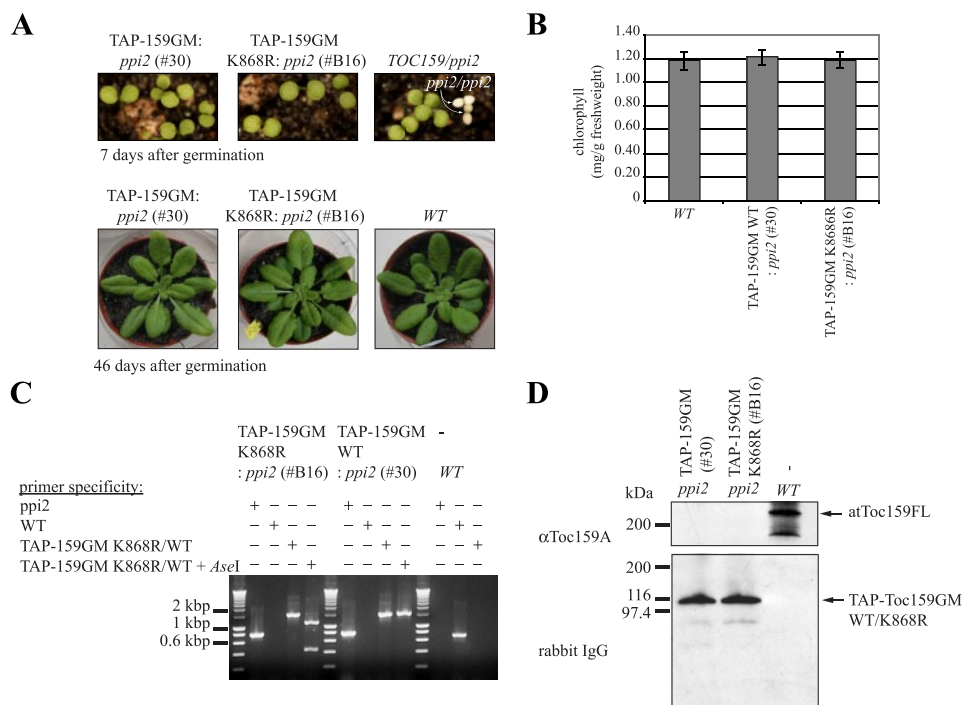
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[3] F. Kessler, unpublished data.

FIGURE 1. **Complementation of the *ppi2* mutant by GTPase deficient Toc159GM K868R.** *A*, phenotypes of homozygous *ppi2* plants transformed with TAP-Toc159GM and TAP-Toc159GM K868R and untransformed *A. thaliana* plants. In the *upper panel*, untransformed plants are seedlings descended from a heterozygous *ppi2* plant; in the *lower panel* an untransformed homozygous wild-type plant ecotype Wassilewskija is shown. *B*, chlorophyll levels in leaves of untransformed wild-type plants and homozygous *ppi2* plants complemented by TAP-Toc159GM or TAP-Toc159GM K868R at 19 days after germination. *C*, confirmation of genotypes and the presence of the K868R single point mutation. PCR analysis of genomic DNA from plants shown above with primer sets specific for the *ppi2* allele, the non-disrupted TOC159 gene (*WT*) and the transgene. Digestion of the transgene-specific PCR product by AseI is indicative of the presence of the K868R mutation that was introduced along with an AseI cleavage site. *D*, expression of the TAP-tagged proteins in the absence of endogenous Toc159. 50 µg of total protein extracts each were used for Western blotting with rabbit IgG for the detection of TAP-tagged protein and anti-Toc159 A-domain for the detection of endogenous full-length atToc159. The numbers in *brackets* represent the numbers of the transgenic plant lines (see supplemental Table S1).

(Toc159$_{aa728-1503}$) or the same construct carrying the K868R mutation, respectively. The A-domain is known to be non-essential. For detection and purification we replaced its sequence by an N-terminal TAP tag consisting of two IgG binding domains of protein A and calmodulin-binding protein (CBP) (27). This resulted in TAP-Toc159GM and TAP-Toc159-GM K868R, respectively. With both constructs *Agrobacterium*-mediated transformation of heterozygous *ppi2* plants yielded four transformants heterozygous for the *ppi2* insertion. In case of transformation with the TAP-Toc159GM construct an additional transformant homozygous for the *ppi2* insertion was isolated. Starting from these transgenic lines non-segregating lines homozygous for *ppi2* and the TAP-Toc159GM (line 30) or TAP-Toc159-GM K868R (line B16) insertions were isolated (Fig. 1*A* and supplemental Table S1). The genotypes were confirmed using wild-type and *ppi2* specific primer pairs as well as a primer pair specific for the T-DNA construct (Fig. 1*C*). An AseI restriction site had been introduced along with the K868R mutation allowing identification of the PCR product with the mutation by AseI digestion (Fig. 1*C*). Western blotting using A-domain specific antibodies (Fig. 1*D*, *anti-Toc159A*) revealed the absence of endogenous atToc159 in the homozygous *ppi2* lines. Instead, nonspecific rabbit IgG (Fig. 1*D*, *lower panel*) specifically decorated the TAP-tagged transgenic Toc159GM proteins at around 110 kDa. Wild-type and mutated TAP-Toc159GM appeared to be expressed at equivalent levels (Figs. 1*D* and 2). Both constructs resulted in a green phenotype in the *ppi2* background suggesting complementation. Under normal growth conditions as well as in de-etiolation experiments (data not shown) the transgenic plants exhibited phenotypes indistinguishable from wild-type plants. Furthermore no difference in chlorophyll accumulation compared with wild-type plants was observed (Fig. 1*B*). The homozygous *ppi2* mutant has reduced levels of some photosynthetic proteins (21). Therefore antibodies against the small subunit (SSu) of Rubisco as well as CAB were used as molecular markers for complementation of *ppi2* (Fig. 2). As expected, the two proteins were detected only at low levels in the *ppi2* mutant but were accumulated to wild-type levels in the *ppi2* lines expressing TAP-Toc159GM and TAP-Toc159-GM K868R. Furthermore, in the same Western blotting experiment we observed higher levels of the atToc159 homolog atToc132 in the *ppi2* mutant, whereas TAP-

targeting reactions, chloroplasts were reisolated via a 35% v/v Percoll cushion and thermolysin was used at 50 µg/ml.

TAP Purification Procedure— 4-week-old *in vitro* grown seedlings were homogenized in extraction buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.1% v/v protease inhibitor mixture (Sigma P9599) followed by incubation on an overhead shaker for 20 min at 4 °C. Insoluble material was separated by centrifugation. 900 µg of protein of each supernatant fraction was incubated with 50 µl of pre-equilibrated IgG Sepharose fast flow (GE Healthcare) for 2 h at 4 °C. After washing five times with 1 ml of extraction buffer, proteins bound to IgG Sepharose were eluted with 0.1 M glycine-HCl pH 3.0, concentrated by chloroform/methanol precipitation and subjected to SDS-PAGE analysis.

RESULTS

Complementation of ppi2— Earlier work demonstrated reduced GTP binding as well as the absence of measurable GTP hydrolysis activity in the recombinant atToc159 G-domain containing the K868R mutation (12). To analyze the effects of the K868R mutation *in vivo*, we engineered a T-DNA construct encoding the wild-type G- and M-domains of atToc159
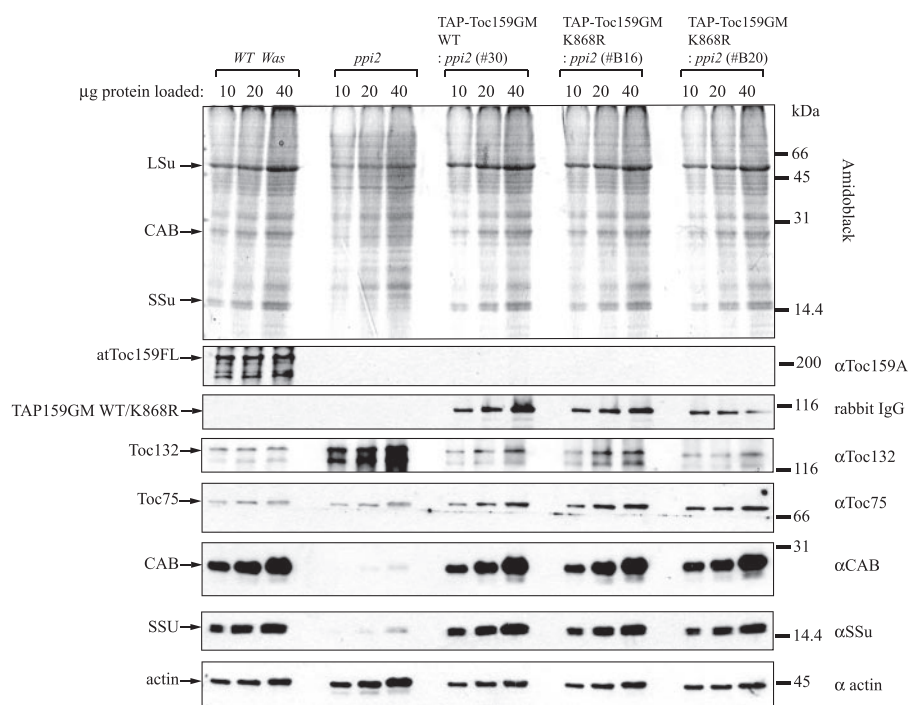
FIGURE 2. **Expression of wild-type or mutated TAP-Toc159GM remedies the deficiency of *ppi2* for Rubisco and chlorophyll a/b-binding protein.** Western blot analysis using antibodies as indicated of 10, 20, and 40 μg of total protein of wild-type *A. thaliana* (ecotype Wassilewskija), *ppi2* (*toc159*) mutant plants as well as *ppi2* plants transformed with TAP-Toc159GM WT or K868R. For TAP-Toc159GM K868R:*ppi2* plants of two different transgenic lines (B16) and (B20) were analyzed. Western blotting against actin was used to monitor gel loading.

Toc159GM:*ppi2* and TAP-Toc159-GM K868R:*ppi2* transgenic plants revealed wild-type levels of atToc132. This delivers further evidence for the full complementation of the *ppi2* import defect by wild-type and TAP-Toc159GM K868R.

Membrane Insertion and Toc Complex Assembly of the K868R Mutant—To test for the insertion of the atToc159 K868R mutant into the chloroplast membrane and its association with the components of the heterotrimeric Toc-complex membrane extraction and IgG pull-down experiments were carried out (Fig. 3).

Total extracts (L) were prepared from TAP-Toc159GM WT:*ppi2* and TAP-Toc159GM K868R:*ppi2* plants and separated into soluble (S) and membrane pellets (P) by centrifugation at 100,000 × *g*. The membrane pellets were extracted with either Tris buffer (Tris), 2 M NaCl (NaCl), or alkaline carbonate buffer (Na₂CO₃) and separated into supernatant (S) and pellet fractions (P) by centrifugation at 100,000 × *g*. The proteins in the fractions were separated by SDS-PAGE and transferred to nitrocellulose. TAP-tagged proteins were detected by Western blotting using nonspecific IgG. Both TAP-Toc159GM WT (Fig. 3*A*, *upper panel*) and TAP-Toc159GM K868R (Fig. 3*A*, *lower panel*), along with known membrane proteins Toc75 and CAB, were present exclusively in the 100,000 × *g* pellet and remained in the membrane fraction upon the various extraction conditions. When probing with nonspecific IgG a band at 28 kDa was detected in the supernatant fractions most likely corresponding to a short degradation product of TAP-Toc159GM WT/K868R still comprising the soluble TAP tag (Fig. 3*A*, TAP-tag). The predominant membrane localization of TAP-Toc159GM/TAP-Toc159GM K868R appears to be in contrast to the dual

localization of atToc159 in the membrane and in the cytosol described in earlier reports (13, 14). Therefore we performed an identical experiment but with Na₂CO₃ extraction only with untransformed wild-type plants (Fig. 3*B*). Western blotting of the fractions obtained with antibodies against the A-domain of atToc159 revealed the presence of full-length atToc159 in the 100,000 × *g* pellet fraction. Like the TAP-Toc159GM transgenic proteins this form is resistant toward alkaline carbonate extraction. In addition to full-length atToc159 a shorter form of atToc159 (Fig. 3*B*, *Toc159**) could be detected. This shorter form was present in the same fractions as full-length atToc159, but in addition was also detected in the 100,000 × *g* supernatant fraction. In summary, TAP-Toc159GM and TAP-Toc159GM K868R generally showed the same cellular distribution as endogenous wild-type atToc159, with the full-length form of the protein being located in the membrane fraction and carbonate-resistant and a shorter form located in the soluble fraction. In both cases the soluble shorter form comprises the N-terminal region of the respective protein as it could be detected with antibodies against the N-terminal TAP-tag or the N-terminal A-domain of Toc159, respectively. The shorter forms observed are most likely due to proteolytic cleavage. However, it is difficult to rule out if degradation is due to a specific event occurring before cell disruption or an unspecific one occurring during experimental manipulation. As to endogenous wild-type Toc159 the aforementioned shorter form/degradation product is commonly observed when using antibodies against the A-domain (13) even in samples with minor and only short experimental manipulation like plant extracts (Figs. 1*D* and 2 and supplemental Fig. S1). The absence of full-length TAP-Toc159GM or full-length endogenous Toc159 in the soluble fraction as observed in Fig. 3*B* challenges a "targeting model" of Toc159 function assuming repeated receptor cycles between the cytosol and the outer chloroplast membrane (see "Discussion").

To determine whether TAP-Toc159GM WT and TAP-Toc159GM K868R associated with the atToc33 and atToc75 components of the Toc-core complex total protein extracts were prepared and solubilized using Triton X-100. The solutes were cleared by centrifugation and subjected to immunopurification using nonspecific IgG to capture the TAP-tagged proteins. The load, unbound, and eluate fractions were separated by SDS-PAGE and transferred to nitrocellulose and analyzed by Western blotting (Fig. 3*C*). Nonspecific IgG to detect TAP-tagged Toc159GM, anti-atToc75, and anti-atToc33 antibodies indicated the presence of the three Toc core proteins in the
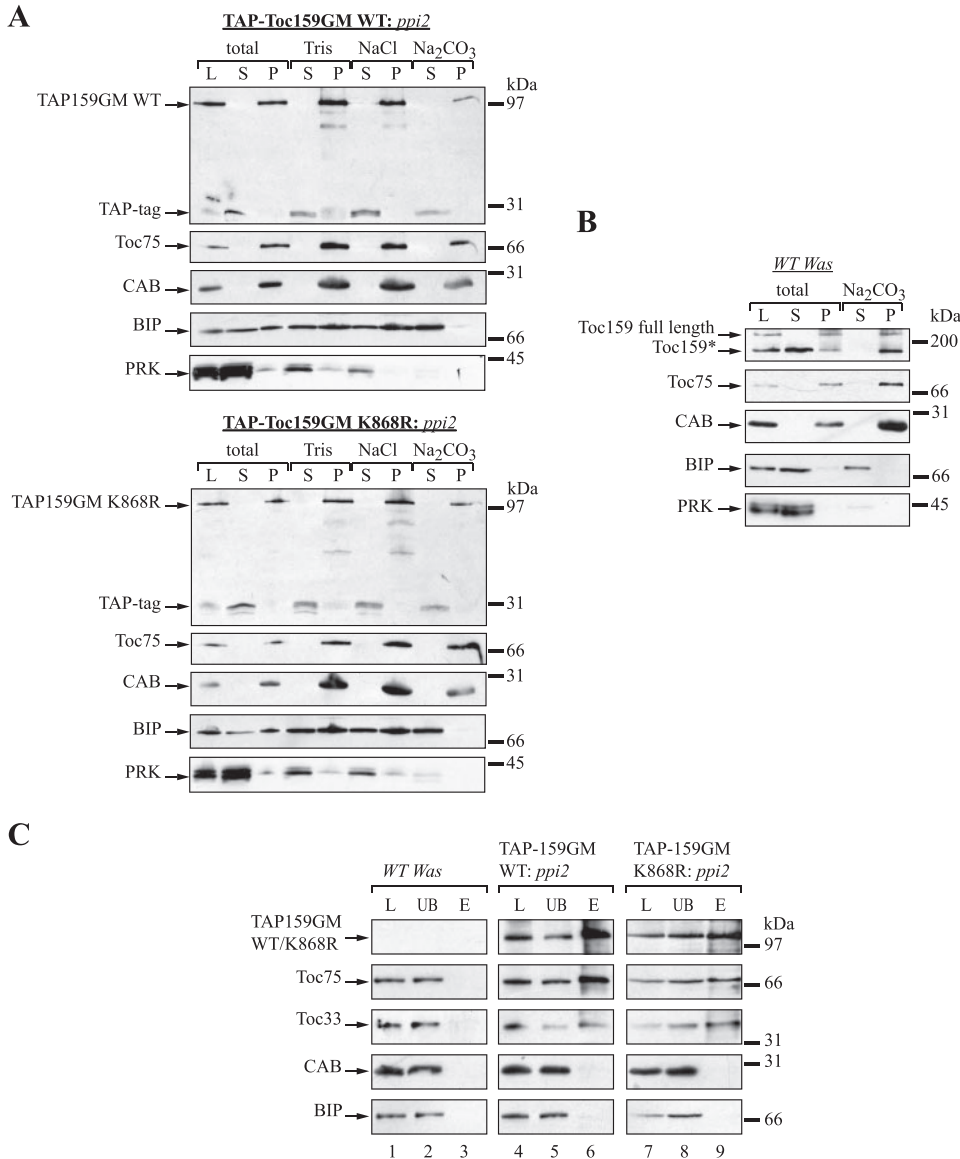
A

B

C

FIGURE 3. Membrane association and integration of TAP-Toc159GM K868R into the Toc complex. *A*, Western blot analysis of protein distribution in 100,000 × *g* supernatant (S) and pellet (P) fractions of plant protein extracts (L). The pellet fraction was treated in parallel with extraction buffer, 2 M NaCl or Na$_2$CO$_3$, and re-centrifuged. 50 μg of soluble or pelletable protein were analyzed by immunoblotting with antibodies as indicated. *B*, as control the same experiment as described in *A* but with Na$_2$CO$_3$ extraction only was performed with untransformed wild-type plants. *C*, co-isolation of Toc core complex components with TAP-Toc159GM WT or K868R. Western blot of the fractions obtained from affinity purification of TAP-tagged proteins on IgG-Sepharose. *L*, load; *UB*, unbound; *E*, eluate; *PRK*, phosphoribulokinase; *BIP*, luminal-binding protein.

immunoisolates. CAB, an abundant thylakoid protein, and BIP, an abundant soluble ER-resident protein, were used as negative controls and were not detectable in the immunoisolates by Western blotting. As an additional negative control a mock purification procedure was performed with a detergent extract from untransformed wild-type plants (Fig. 3*C*, *WT Was*) and yielded no Toc core components in the eluate.

Comparison of in Vitro Outer Membrane Insertion of the K868R and A864R/K868N/S869R Mutants—Earlier work demonstrated that a triple point mutation (mGTP; A864R, K868N, S869R) in the G-domain of atToc159 was lethal and blocked its insertion into the outer chloroplast membrane (14). The K868R mutation alone inhibited the *in vitro* insertion of atToc159 into the outer chloroplast membrane (12). Here (Fig.

4), we directly compare outer membrane binding and insertion of the wild type, the K868R, and mGTP mutant in the Toc159GM background to determine the relative effects of the mutations on outer membrane insertion (Fig. 4*A*). Binding (−TL) is defined as the radioactive Toc159GM protein associated with chloroplasts upon incubation whereas insertion is defined as the 52-kDa M-domain fragment of Toc159 retained upon thermolysin treatment after the incubation (+TL). We observed a stronger effect of the mGTP triple mutation on Toc159GM binding and insertion when compared with the wild type and the K868R mutation. This is in agreement with the observed proper *in vivo* targeting and insertion of TAP-Toc159GM K868R (Fig. 3) and the *in vivo* mistargeting of Toc159mGTP reported in an earlier study (14).

The Complementation of ppi2 by Toc159 K868R Is Independent of the A-domain and the TAP Tag—The A-domain of Toc159 is known to be sensitive to proteolytic degradation and dispensable for Toc159 function. We therefore omitted it from our constructs in order to circumvent proteolytic loss of the TAP tag. However, complementation of the *ppi2* mutant by TAP-Toc159GM carrying the K868R mutation was surprising and unexpected. We therefore determined whether the ability of mutated Toc159 to complement was influenced by the presence of the A-domain or the N-terminal TAP-tag. We engineered T-DNA constructs encoding full-length atToc159 with and without N-terminal TAP-tag and performed *in vivo* complementation experiments by transient (Fig. 5) or stable transformation of the *ppi2* mutant (supplemental Table S1). In the transient expression system protoplasts derived from leaf tissue of homozygous *ppi2* plants were cotransformed with a construct encoding GFP fused to the transit peptide of the small subunit of Rubisco (RbcS-nt:GFP) together with various atToc159 constructs or the empty vector as a negative control (Fig. 5). In this system complementation of the *ppi2* import defect can be assessed by comparing the precursor and mature forms of RbcS-nt:GFP. Because of the import defect in *ppi2* protoplasts transformed with RbcS-nt:GFP around 40% of the total expressed RbcS-nt:GFP accumulates in the precursor form (26). In the transient expression
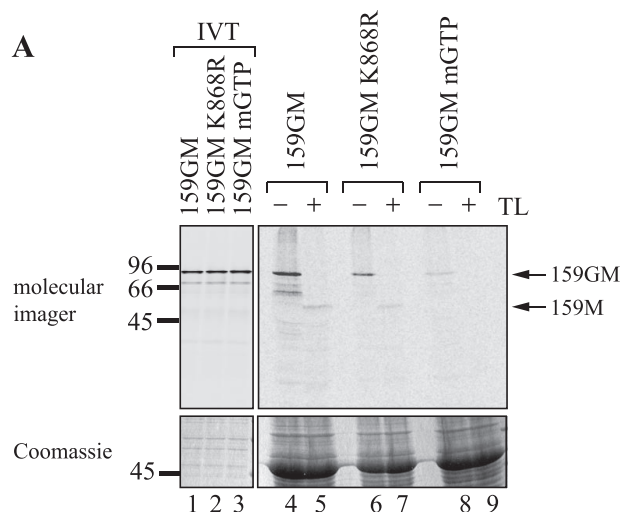
A

B

FIGURE 4. **The effects of the GTPase mutations K868R and mGTP (A864R, K868N, S869R) on the *in vitro* membrane insertion of Toc159GM into chloroplasts.** *A*, *in vitro* translated, [35S]methionine-labeled Toc159 lacking the A-domain (Toc159GM) with or without the K868R or mGTP mutation were incubated with isolated *Arabidopsis* chloroplasts. Chloroplasts were reisolated and incubated in the absence (−) or presence (+) of 50 μg/ml of thermolysin (*TL*) for 30 min on ice. Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control. *B*, quantitative analysis of data from three replicate experiments using the Quantity One® software (BioRad). The experiments were calibrated to the amount of *in vitro* translated radioactive protein added to the chloroplasts (is 100%). For the quantification of insertion of Toc159GM, the data were normalized based on the methionine content of Toc159GM (15) and Toc159M (8).

system TAP-Toc159GM (Fig. 4, *lane 3*), TAP-Toc159GM K868R (Fig. 4, *lane 4*), TAP-Toc159 full-length (Fig. 4, *lane 6*) as well as TAP-Toc159 full-length K868R (Fig. 4, *lane 7*) reduced the precursor form and conferred efficient chloroplast import capacity to the *ppi2* protoplasts as was observed for Toc159GM or Toc159 full-length with a only a small epitope tag (T7) at the N terminus (Fig. 5, *lanes 2* and *5*). These results confirm complementation of the *ppi2* import defect by atToc159 carrying the K868R mutation and demonstrate that the ability of mutated Toc159 to complement is independent of the presence or absence of the A-domain. Moreover, stable transformation yielded homozygous *ppi2* plants expressing TAP-Toc159 K868R or untagged atToc159 K868R (supplemental Table S1). These plants revealed the same green phenotype indistinguishable from wild-type as TAP-Toc159GM:*ppi2* and TAP-Toc159GM K868R:*ppi2* plants (data not shown and supplemental Fig. S1). In addition, Western blot analysis revealed that the expression level of untagged atToc159 K868R under the 35S
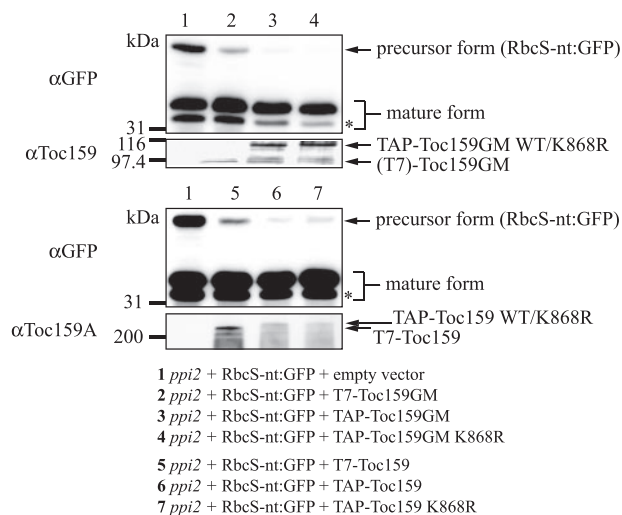

FIGURE 5. **Complementation of the *ppi2* import deficiency in a transient expression system using protoplasts.** Protoplasts derived from leaf cells of homozygous *ppi2* plants were transformed with a plasmid encoding the N-terminal transit peptide of the small subunit of Rubisco fused to GFP (RbcS-nt:GFP) together with the empty vector (1) or together with different Toc159 constructs as indicated (2–7). Total protein extracts were prepared from protoplasts 12 h after transformation and subjected to Western blot analysis with anti-GFP and anti-Toc159 antibodies (for the *upper panel*, anti-atTOC159 serum was used, for the *lower panel*, affinity-purified anti-atTOC159 A-domain). *, proteolytic product of the mature form (26).

promoter in *ppi2* is only slightly higher than the level of endogenous atToc159 in the wild type (supplemental Fig. S1). This excludes that complementation by the Toc159 K868R constructs is achieved by overexpression.

In Vitro Preprotein Binding and Import into Isolated Toc159GM K868R Chloroplasts—While the K868R mutant proteins complemented both the albino defect in *ppi2* and the import defect in *ppi2* protoplasts, this does not exclude kinetic effects on *in vitro* preprotein import into isolated chloroplasts. The TAP-Toc159GM K868R complementing line allowed us to isolate intact chloroplasts with a properly inserted Toc159 receptor lacking GTPase hydrolysis activity and thus to study the role of hydrolysis in preprotein binding and import experiments (Fig. 6). The preprotein binding experiment was carried out using chloroplasts isolated from either TAP-159GM WT:*ppi2* or TAP-Toc159GM K868R:*ppi2* plants. The isolated chloroplasts were incubated with 35S-labeled small subunit preprotein (pSSu) in the absence of added nucleotides (−) or in the presence of 0.1 mM ATP and GTP (+ATP+GTP). Subsequently the chloroplasts were reisolated, subjected to SDS-PAGE, and analyzed using a PhosphorImager. Under both nucleotide conditions a reduction of 20−25% of pSSu binding to TAP-Toc159GM K868R chloroplasts was observed (Fig. 6*A*). To determine effects of the K868R mutation on protein import, chloroplasts isolated from either wild type, TAP-Toc159GM WT:*ppi2*, or TAP-Toc159GM K868R:*ppi2* plants were incubated with the radioactive small subunit preprotein (pSSu) in the presence of 5 mM ATP in time course experiments (Fig. 6*B*). Samples were removed after 0, 2, 5, 10, and 15 min. Chloroplasts were reisolated, subjected to SDS-PAGE and analyzed using a PhosphorImager. The accumulation rates of mature small subunit (SSu) of wild type and TAP-Toc159GM chloroplasts were almost identical. In comparison, the accumulation
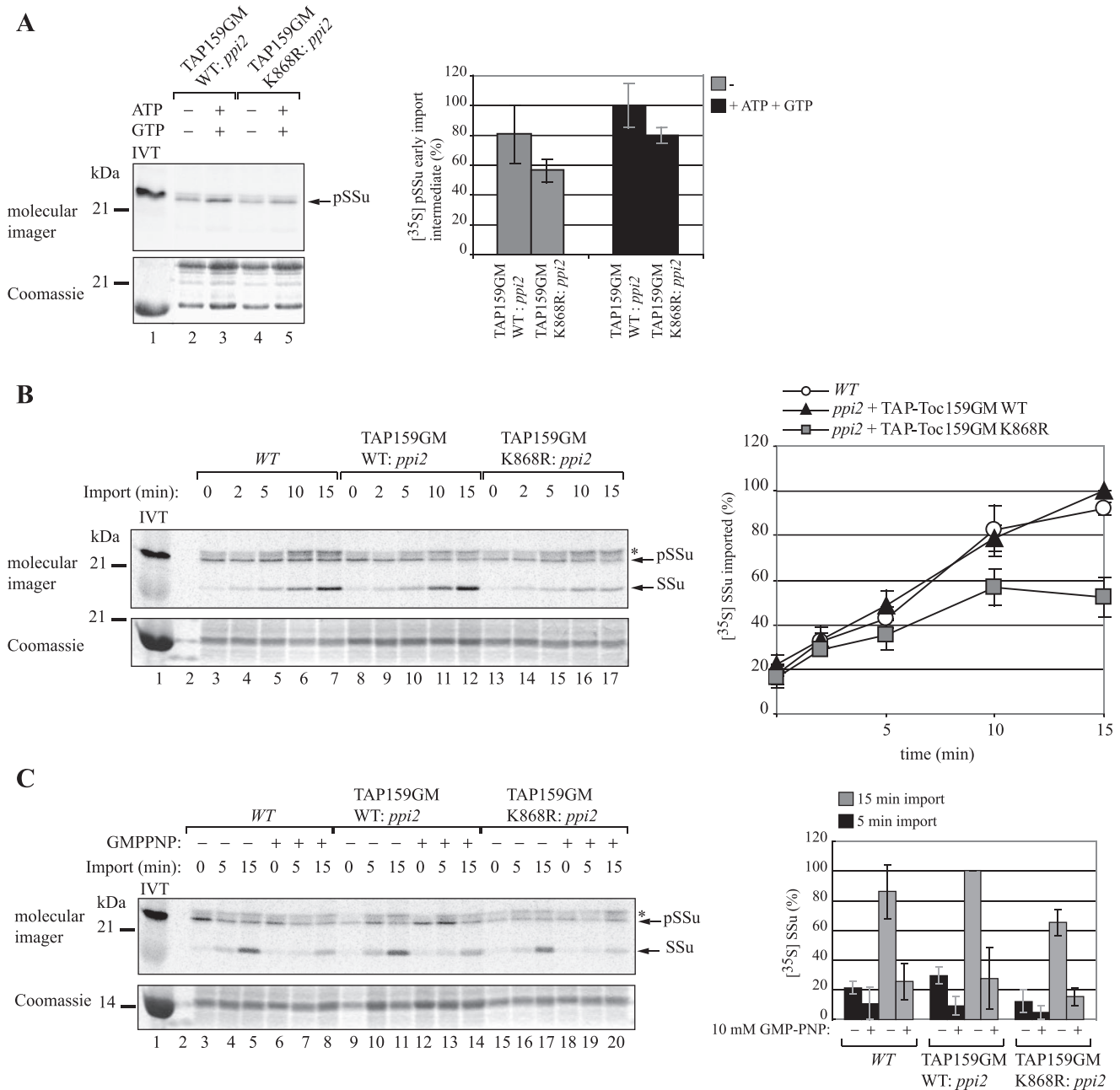
FIGURE 6. **Import deficiency in TAP-Toc159GM K868R chloroplasts.** *A*, *in vitro* translated, [^{35}S]methionine-labeled preprotein of the small subunit of Rubisco (pSSu) was incubated with isolated chloroplasts in the absence or presence of 0.1 mM ATP and GTP. The low concentrations of ATP and GTP allow for formation of the early import intermediate but not for complete translocation. Chloroplasts were reisolated and analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control (*Coomassie*). The graph shows quantitative data of three experiments. The pSSu preprotein bound to TAP-Toc159GM WT chloroplasts in the presence of ATP and GTP was set to 100%. *B*, chloroplasts were isolated from plants of the indicated genotypes, and used in protein import assays with the radioactive pSSu. Import was allowed to proceed for 2, 5, 10, and 15 min. Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. The bands corresponding to mature SSu were quantified from triplicate experiments. The amount of imported SSu after 15 min of import into TAP-Toc159GM WT chloroplasts, was set to 100%. A section of the Coomassie Blue-stained gel is shown as a loading control (*Coomassie*). *C*, protein import into isolated chloroplasts was allowed to proceed for 5 and 15 min in the absence (−) or presence (+) of 10 mM of the non-hydrolyzable GTP analog GMP-PNP. The graph shows the quantification of mature SSu from three independent experiments. The amount of SSu after 15 min of import into TAP-Toc159GM WT chloroplasts in the absence of GMP-PNP was set to 100%. *, pSSu modified in the course of the import reactions.

of mature small subunit in TAP-Toc159GM K868R chloroplast after 15 min (Fig. 6*B*, *lane 17*) was only around 50% of that in the wild-type and TAP-159GM chloroplasts.

We tested whether other GTPases than atToc159 may be involved in supporting preprotein import in the background of TAP-Toc159GM K868R. To do so we added the non-hydrolyz-

able GTP analog, GMP-PNP, to *in vitro* import assays to determine whether it would increase inhibition of import in TAP-Toc159GM K868R:*ppi2* chloroplasts. Wild type, TAP-Toc159GM WT:*ppi2*, and TAP-Toc159GM K868R:*ppi2* chloroplasts were incubated in import reactions with the radioactive small subunit pSSu in the additional presence or absence of

10 mM GMP-PNP (Fig. 6C). Time course experiments were carried out, and samples were removed after 0, 5, and 15 min. Chloroplasts were reisolated, subjected to SDS-PAGE, and analyzed using a PhosphorImager. The results obtained in the absence of GMP-PNP largely confirmed those presented in Fig. 6B and showed a reduction of mature small subunit accumulation in TAP-Toc159GM K868R chloroplasts of around 40% after 15 min of incubation. Addition of GMP-PNP reduced mature small subunit accumulation not only in wild-type and TAP-Toc159GM WT chloroplasts, but interestingly also in TAP-Toc159GM K868R chloroplasts by about 70%.

DISCUSSION

Earlier studies dissected the role of Toc159 domains to examine their roles in protein import. Independent *in vivo* and *in vitro* experimentation suggested that the G-domain controls insertion of Toc159 into the outer chloroplast membrane, its assembly into the Toc complex (12–14, 26) as well as preprotein binding (11). The atToc159 GTPase mutants (S869N, D909L in Ref. 26; A864R/K868N/S869R in Ref. 14) did not result in complementation of *ppi2*. Seedling lethality of atToc159 GTPase mutants has with the exception of A864R (20) precluded isolation of plastids on a large enough scale to allow for classical biochemical studies such as *in vitro* preprotein binding and import experiments or for isolation of mutant Toc complexes.

In this study, we identify the atToc159 K868R as a non-lethal GTP-hydrolysis mutant and determine its effects on the various aspects of Toc159 function (*i.e.* membrane insertion, complex assembly, preprotein binding, and import). We have earlier characterized the K868R mutant of atToc159 biochemically (GTP binding reduced by 80%, no detectable hydrolytic activity in the recombinant mutant G-domain) and demonstrated *in vitro* defects in outer membrane insertion and preprotein binding in the full-length mutant protein (10–12, 14). In direct comparison to the triple point mutant A864R/K868N/S869R (termed Toc159 mGTP), however, we detected a measurable capacity of the K868R mutant to insert into the outer chloroplast membrane (Fig. 4). TAP-Toc159GM K868R reinstated the green phenotype in homozygous *ppi2* plants (Fig. 1A) as well as the accumulation of wild type levels of the Rubisco subunits and chlorophyll a/b-binding protein (Fig. 2). Complementation of *ppi2* by the receptor carrying the K868R mutation occurs at levels comparable to endogenous atToc159 in wild-type plants (supplemental Fig. S1) and is not affected by the absence of the A-domain or the presence of the TAP tag (Fig. 5 and supplemental Table S1 and Fig. S1). These results indicated that the K868R mutation despite reduced GTP binding and the absence of measurable hydrolytic activity is still sufficiently functional *in vivo* to support chloroplast biogenesis. Moreover, TAP-Toc159GM K868R behaves as an integral membrane protein *in vivo* and as a component of a Toc core complex, which is not discernibly different from the wild type.

As mentioned above, complementation studies with GTPase defective atToc159 mutants gave different results: mistargeting of the receptor and non-complementation in case of atToc159mGTP (14) and atToc159 S869N or atToc159 D909L (26) and complementation in case of atToc159 A864R (20) and

atToc159 K868R (this study). Likely, the different outcome of the complementation studies reflects the nature of the respective mutations. Considering the biochemical data available on atToc159mGTP (15 times lower affinity for GTP, no measurable GTP hydrolysis activity) (14) and atToc159 K868R (about 4 times lower affinity for GTP, likewise no measurable GTP hydrolysis activity) (12), we conclude that complementation ability depends on the ability of atToc159 to bind GTP. This is supported by the findings of Wang *et al.* (20) on the atToc159 A864R mutant. This mutant is preferentially in its GTP-bound state as it has a greatly reduced GTP hydrolytic activity. AtToc159 A864R not only complements the *ppi2* mutant but increases preprotein binding and import of isolated chloroplasts beyond wild-type levels. Unfortunately no biochemical data are available on the atToc159 S869N or atToc159 D909L GTPase mutants. As the two residues mutated are predicted to be involved in catalytic Mg2+ binding and no complementation was observed, a strong GTP binding defect for both mutants is likely.

The residual GTP binding activity by atToc159 K868R turned out to be sufficient to support its integration into the Toc complex and complementation of the visual *ppi2* phenotype. However, the import rates of isolated TAP-Toc159 K868R chloroplasts were affected (Fig. 6). In isolated chloroplasts the K868R mutation reduced energy-independent preprotein binding and early import intermediate formation by around 20% (Fig. 6A). Preprotein import was reduced to around 50% (Fig. 6B). These results are consistent with the function of atToc159 as a preprotein receptor. The reduction of preprotein binding to TAP-Toc159GM K868R chloroplasts is less than that observed for preprotein binding to synthetic full-length Toc159 K868R protein (11). This suggests the possible involvement of additional Toc proteins such as atToc33 in isolated chloroplasts. Also, direct interaction of preprotein transit sequences with the outer membrane lipid bilayer has been reported and may also contribute to preprotein binding (35). The high import activity in the GTPase compromised TAP-Toc159GM K868R chloroplasts suggests that individual Toc159 GTPase binding and hydrolysis cycles may not directly correlate to individual translocation events at the Toc complex. This conclusion is supported by the results obtained for the mutant atToc159 A864R (20). Despite reduced GTP hydrolytic activity atToc159 A864R chloroplasts show increased preprotein binding and import.

In the TAP-Toc159GM K868R plants Toc159 GTP hydrolysis is inactivated. We took advantage of this to detect a requirement for additional GTPases and carried out preprotein import experiments into isolated chloroplasts in the presence of the non-hydrolyzable GTP analog, GMP-PNP (Fig. 6C). We observed an increased preprotein import inhibition by GMP-PNP over that already incurred by the K868R mutation (Fig. 6C). These data suggest that at least two independent GTPase activities are required at the Toc complex to achieve optimal import activity in isolated *Arabidopsis* chloroplasts. As the Toc core complex containing atToc159 prevailingly contains atToc33 (25), it appears likely that the latter may represent an additional GTPase. However, our results also do not exclude atToc132, -120, and -90 (the homologs of atToc159) or atToc34

(the homolog of atToc33) as the source of additional GTP requirement. With regard to a sequential action of the GTPases, our results do not allow any conclusions.

The results obtained in this study are informative with respect to the two current models of Toc complex function, the targeting, and motor hypotheses. Both models propose a direct correlation between Toc159 GTP- binding and hydrolysis cycles and preprotein translocation events. In the targeting model one cycle of GTP binding and hydrolysis at Toc159 is required to supply Toc34 and Toc75 with a preprotein molecule. In the motor model, one or more binding and hydrolysis cycles at Toc159 are necessary to drive the preprotein through the Toc75 channel. Our results showing that a non-hydrolyzing GTPase supports protein translocation appear inconsistent with both models requiring one or more GTPase hydrolysis events per translocated preprotein. Moreover, the apparent requirement for GTP binding and not GTP hydrolysis for Toc159 function, indicates that Toc159 functions as a molecular switch rather than in generation of the driving force for translocation.

In addition, another finding of our study, absence of intact TAP-Toc159GM or full-length endogenous Toc159 from the soluble fraction (Fig. 3), raises questions regarding some aspects of the targeting hypothesis. The findings, as presented, do neither support a role of Toc159 as a soluble preprotein import receptor nor repeated receptor cycles between the cytosol and the chloroplast envelope. In conclusion, it appears that both the targeting and motor models of Toc159 function need to be refined. To do so and to resolve the nature and origin of the shorter form of TAP-Toc159GM/Toc159 observed in the soluble fraction (Fig. 3) requires further experimentation, which is currently underway.

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