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Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane

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Two homologous GTP-binding proteins, atToc33 and atToc159, control access of cytosolic precursor proteins to the chloroplast. atToc33 is a constitutive outer chloroplast membrane protein, whereas the precursor receptor atToc159 also exists in a soluble, cytosolic form. This suggests that atToc159 may be able to switch between a soluble and an integral membrane form. By transient expression of GFP fusion proteins, mutant analysis, and biochemical experimentation, we demonstrate that the GTP-binding domain regulates the targeting of cytosolic atToc159 to the chloroplast and mediates the switch between cytosolic and integral membrane forms. Mutant atToc159, unable to bind GTP, does not reinstate a green phenotype in an albino mutant (*ppi2*) lacking endogenous atToc159, remaining trapped in the cytosol. Thus, the function of atToc159 in chloroplast biogenesis is dependent on an intrinsic GTP-regulated switch that controls localization of the receptor to the chloroplast envelope.

Introduction

Approximately 2,000 different nuclear-encoded proteins must be imported to acquire photosynthetic capacity during chloroplast biogenesis (Cline, 2000). These proteins are synthesized in the cytosol as precursors with an NH_2-terminal transit peptide, specifying targeting to the chloroplast. Both chloroplast envelope membranes contain translocon complexes to facilitate import of precursor proteins. These are termed the translocon at the outer chloroplast membrane (Toc)* and translocon at the inner chloroplast membrane complexes (Keegstra and Cline, 1999; Chen et al., 2000b; Schleiff and Soll, 2000; Bauer et al., 2001). The *Arabidopsis*–Toc complex consists of the three major components forming a trimeric complex (Hiltbrunner et al., 2001b). AtToc159 (the number indicates the molecular mass in kDs) and atToc33 are surface-exposed, integral membrane GTP-binding proteins sharing highly conserved GTP-binding domains (G-domains; Hiltbrunner et al., 2001a; Jackson-Constan and Keegstra, 2001). The pea homologue of atToc75, the third component of the Toc complex, forms at least part of a hydrophilic channel through which precursors are translocated across the outer membrane (Hinnah et al., 1997).

Evidence, obtained with isolated chloroplasts, indicates that atToc33 and atToc159 act concertedly in the recognition of the chloroplast-targeting signal (transit peptide). Based on direct cross-linking to transit sequences and inhibition of precursor binding by specific antibodies, pea Toc159 is thought to function as a primary precursor receptor at the chloroplast surface (Perry and Keegstra, 1994; Ma et al., 1996). The pea homologue of atToc33 (psToc34) has also been shown to specifically interact with the transit peptide of precursor proteins, and may therefore contribute to precursor receptor function (Sveshnikova et al., 2000; Schleiff et al., 2002). Moreover, GTP binding and hydrolysis at psToc34 regulate insertion of precursor proteins across the outer

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*Abbreviations used in this paper: A-domain, acidic domain; CaMV, cauliflower mosaic virus; G-domain, GTP-binding domain; M-domain, membrane domain; Ni-NTA, Ni-nitrilotriacetic acid; Toc, translocon at the outer chloroplast membrane.

Key words: *Arabidopsis*; chloroplast biogenesis; molecular switch; receptor targeting; GTP

membrane (Chen et al., 2000a). In *Arabidopsis*, atToc159 is essential for viability. The *Arabidopsis ppi2* mutant, disrupted in *TOC159*, has an albino phenotype coincident with the absence of differentiated chloroplasts. Furthermore, the failure of *ppi2* plastids to accumulate photosynthetic proteins in vivo is consistent with a defect in protein import (Bauer et al., 2000). Disruption of atToc33 has a less pronounced effect, leading to a pale green phenotype. The phenotype is indicative of a role in chloroplast biogenesis, but persists only during early seedling development (Jarvis et al., 1998).

AtToc159 has a tripartite structure, consisting of an NH_2-terminal acidic domain (A-domain), the G-domain, and the COOH-terminal membrane domain (M-domain; Bauer et al., 2000; Chen et al., 2000a). The function of the A-domain is unknown. The M-domain anchors atToc159 in the outer chloroplast membrane while lacking predicted membrane-spanning α-helices. The G-domain binds GTP specifically, but its functional significance is not yet understood. We have previously demonstrated that atToc159 exists in a complex with atToc33 and atToc75 (Hiltbrunner et al., 2001b). However, in contrast to atToc33 and atToc75, about half of the protein exists in a soluble, cytosolic form (Hiltbrunner et al., 2001b). Soluble atToc159 binds directly to $atToc33_{1–265}$, rendered soluble by deletion of the COOH-terminal transmembrane region. Furthermore, $atToc33_{1–265}$ competed for outer membrane insertion of atToc159 in isolated *Arabidopsis* chloroplasts. The results suggest that atToc33 functions as a receptor in the assembly of soluble atToc159 into the Toc complex (Hiltbrunner et al., 2001b). Support for this hypothesis stems from the crystal structure of pea Toc34 (Sun et al., 2002). Pea Toc34 forms homodimers when in the GDP-bound state. Conservation of the G-domains and the novel dimerization motifs in atToc159 as well as atToc33 suggests that the two proteins may form heterodimers via their G-domains, thus providing a structural basis for targeting of cytosolic atToc159 directly to the Toc complex (Kessler and Schnell, 2002; Sun et al., 2002). Here, we demonstrate that the functional G-domain is sufficient and necessary to target cytosolic atToc159 to the outer chloroplast membrane.

Results

Expression and targeting of atToc159 domain GFP fusions in living *Arabidopsis* cells

To determine the domains required for targeting of cytosolic atToc159 to the chloroplast, we engineered constructs encoding a series of NH_2-terminal GFP fusions under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The constructs were transformed into living *Arabidopsis* protoplasts and analyzed by confocal laser scanning microscopy (Fig. 1). Due to the length and instability of the full-length atToc159 cDNA (Bölter et al., 1998; Chen et al., 2000a), we were unable to obtain a construct encoding the corresponding GFP fusion. However, a T-DNA construct encoding the combined G- and M-domains (atToc159GM) complemented the *ppi2* mutant lacking atToc159, suggesting that atToc159GM is largely functional (unpublished data). Therefore, we fused GFP to the NH_2 terminus of a construct corresponding to the G- and M-domains in which the

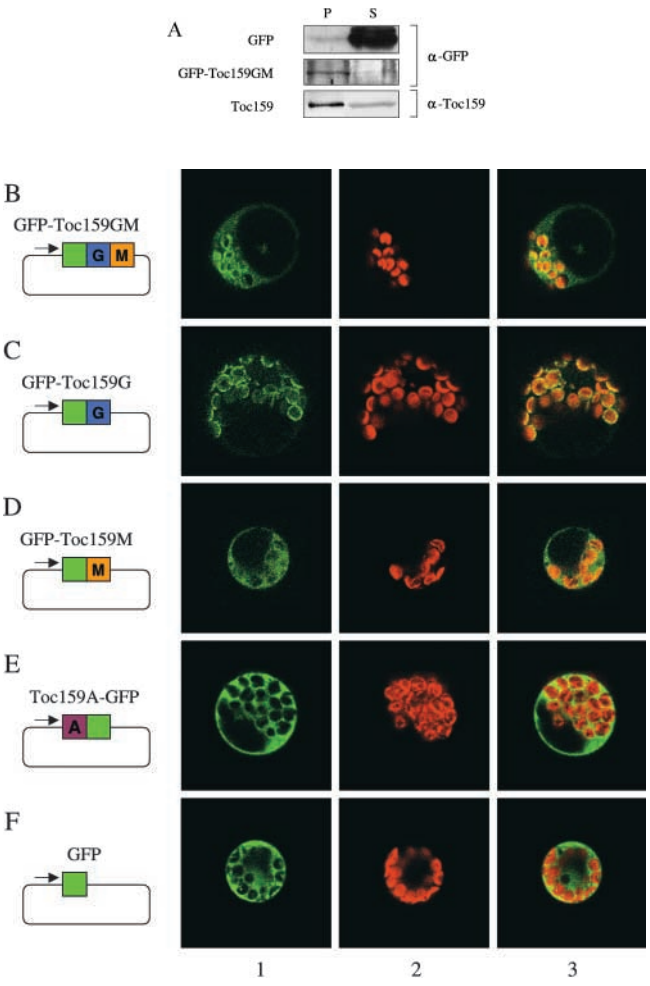


Figure 1. **Transient expression of atToc159 domains fused to GFP.** GFP fusion constructs were transformed into isolated *Arabidopsis* protoplasts using the PEG method. Expression of GFP fusion proteins was monitored by Western blotting to confirm functionality of a GFP fusion to the NH_2 terminus of the G- and M-domains of atToc159 (Fig. 1 A; GFP-Toc159GM) or confocal laser scanning microscopy using a Leica DM IRBE microscope and a Leica TCS SP laser (Fig. 1, B–F). (A) Western blotting of the carbonate-extracted total membrane fraction (P) and soluble fraction (S) of protoplasts expressing either GFP or GFP-Toc159GM. Proteins were detected using either antibodies against GFP (α-GFP) or the A-domain of atToc159 (α-Toc159) to detect full-length endogenous atToc159. (B) GFP-Toc159GM. (C) GFP-Toc159G, NH_2-terminal GFP fusion to the G-domain. (D) GFP-Toc159M, NH_2-terminal GFP fusion to the M-domain. (E) Toc159A-GFP, COOH-terminal GFP fusion to the A-domain. (F) GFP control. Lane 1, GFP fluorescence; lane 2, autofluorescence; lane 3, merge of GFP and autofluorescence.

A-domain is replaced by GFP (GFP-atToc159GM). To confirm that GFP-atToc159GM behaves like full-length endogenous atToc159, we performed a Western blotting experiment on fractions of transiently transformed protoplasts using mAbs against GFP (α-GFP), as well as antibodies against the A-domain to detect endogenous atToc159 (Fig. 1 A; α-atToc159). Both GFP-atToc159GM (detected by α-GFP) and endogenous atToc159 (detected by α-atToc159) were present in the carbonate-extracted total membrane fraction and to a lesser degree in the soluble fraction. In contrast, GFP was present almost exclusively in the soluble frac-

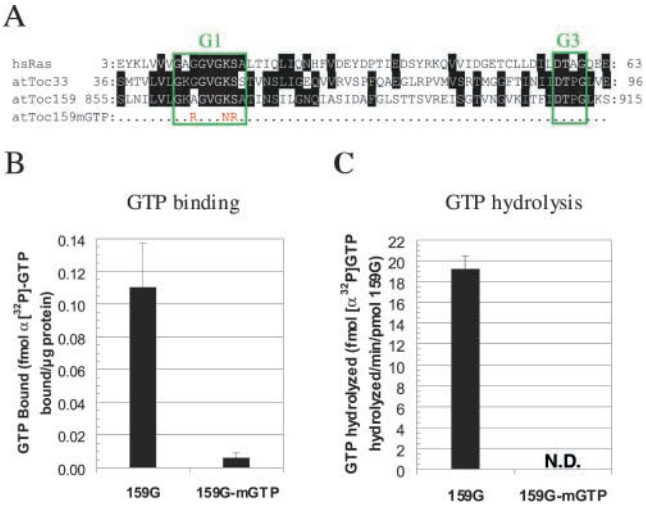


Figure 2. **Alignment of the GTP-binding domains of hsRas, atToc33, and atToc159 encompassing the G1 and G3 motifs.** (A) Triple-point mutations in the G1 motif of atToc159mGTP are indicated in red. GenBank/EMBL/DDBJ accession nos.: hsRas, P01112; atToc159, AF069298; atToc33, U89959. (B) GTP binding to wild-type and mutant atToc159 G-domains (159G). Purified 159G and 159G-mGTP were bound to nitrocellulose and incubated with 50 nM α-[^{32}P]GTP (3,000 Ci/mmol) in the presence of 1 μM ATP. Bound α-[^{32}P]GTP was quantitated using a phosphorimager. Error bars indicate SD. (C) GTP hydrolysis by wild-type and mutant atToc159 G-domains. 1 μM α-[^{32}P]GTP (150 mCi/μmol) was incubated with 0.5 μM 159G or 159G-mGTP for 60 min at 25°C. Radiolabeled GTP and GDP were resolved by TLC on PEI-cellulose F plates using 1 M LiCl as the solvent, and radioactivity was quantitated using a phosphorimager. Error bars indicate SD. N.D., not detectable above background.

tion. By confocal microscopy, GFP-atToc159GM, transiently expressed in *Arabidopsis* protoplasts, gave strong fluorescence at the chloroplast periphery, but was also present in the cytosol, indicating partitioning between the cytosol and the chloroplast outer membrane (Fig. 1 A). Thus, GFP-atToc159GM is apparently able to recapitulate the properties of wild-type atToc159. A fusion between GFP and the G-domain of atToc159 alone (GFP-atToc159G), was present predominantly at the outer membrane and to a lesser degree in the cytosol (Fig. 1 B). A fusion between GFP and the M-domain of atToc159 alone (GFP-atToc159M), was present predominantly in the cytosol (Fig. 1 C) and to a lesser degree at the chloroplast surface. Finally, a fusion between the A-domain and GFP (Fig. 1 D) was localized in the cytosol, similar to GFP (Fig. 1 E), suggesting that the A-domain does not play a role in chloroplast targeting of atToc159. The results of these transient expression experiments indicate that the G-domain alone is sufficient to target GFP to the chloroplast periphery, and likely constitutes the targeting domain of atToc159.

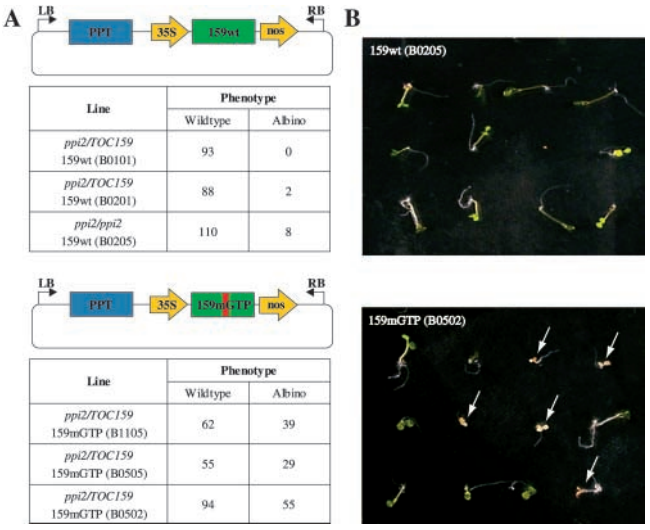
GTP-binding and -hydrolysis measurements of atToc159mGTP

Targeting of the fusion between GFP and the G-domain to the chloroplast (Fig. 1 B) suggests that the G-domain directs atToc159 to the organelle. Therefore, it also appears probable that the targeting and distribution of atToc159 is regulated by guanosine nucleotide binding and/or hydrolysis. To determine the role of GTP binding to atToc159, a mutant protein (atToc159mGTP) containing three-point mutations in the G1-motif (A864R, K868N, and S869R) was designed (Fig. 2 A). The mutations are predicted to strongly reduce both guanosine nucleotide binding and hydrolysis (Chen and Schnell, 1997). To determine GTP-binding and -hydrolysis activities, we expressed the G-domain of wild-type atToc159 (159G) as well as that of atToc159mGTP (159G-mGTP) as hexahistidinyl-tagged proteins in *Escherichia coli*. Isolated G-domains were used because functional expression of full-length atToc159 in *E. coli* was not feasible due to the instability of the protein and low levels of expression. However, we expect the mutations to have the same relative effects on the isolated G-domain as on the full-length protein. The recombinant G-domains were purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography. GTP binding to the isolated G-domains was measured using a solid-phase GTP overlay assay. In this assay, the purified proteins were spotted onto nitrocellulose and incubated in buffer containing α-[^{32}P]GTP. The blots were washed and binding was measured using a phosphorimager (Fig. 2 B). GTP binding to 159G-mGTP was ~15-fold lower than that of 159G (Fig. 2 B), confirming the predicted effect of the three-point mutations on atToc159.

GTPase activity was measured in the presence of α-[^{32}P]GTP in a soluble phase assay. At the 0- and 60-min time points, samples were removed, inactivated by heating in the presence of SDS and EDTA, as well as excess GDP and GTP. The samples were then resolved by TLC to separate α-[^{32}P]GTP from α-[^{32}P]GDP, and were quantitated using a phosphorimager (Fig. 2 C). Although 159G hydrolyzed GTP, 159G-mGTP had no detectable activity, providing additional evidence for the inactivation of the G-domain in atToc159mGTP. The rate of GTP hydrolysis by 159G under substrate saturation (unpublished data) is similar to the rate of hydrolysis by isolated SRP receptor (Connolly and Gilmore, 1993).

Functional dissection of atToc159 in vivo

AtToc159 is essential for the onset of photoautotrophic growth, and hence, plant viability (Bauer et al., 2000). To determine whether the G-domain contributes to the essential function, an in vivo complementation assay was designed. The assay relies on the restoration of the wild-type green phenotype in the albino *ppi2* mutant (lacking wild-type atToc159 due to T-DNA insertion) as the criteria for complementation. We engineered T-DNA constructs (conferring phosphinothricine resistance) encoding either full-length wild-type atToc159 (atToc159wt) or atToc159mGTP under the control of the CaMV 35S promoter. The constructs were introduced into plants heterozygous for the *ppi2* mutation (conferring kanamycine resistance) by agrobacterium-mediated transformation (Bechtold and Pelletier, 1998). *ppi2* plants transgenic for either atToc159wt or atToc159mGTP were identified by dual phosphinothricine and kanamycine selection. We determined the *ppi2* background of plants carrying either of the transgenic constructs by PCR. Three lines, each carrying either heterozygous (B0101, B0201, B0502, B1105, or



Line	Phenotype	
	Wildtype	Albino
ppi2/TOC159 159wt (B0101)	93	0
ppi2/TOC159 159wt (B0201)	88	2
ppi2/ppi2 159wt (B0205)	110	8

Line	Phenotype	
	Wildtype	Albino
ppi2/TOC159 159mGTP (B1105)	62	39
ppi2/TOC159 159mGTP (B0505)	55	29
ppi2/TOC159 159mGTP (B0502)	94	55

Figure 3. **Segregation analysis of *ppi2* plants expressing atToc159wt (159wt) or atToc159mGTP (159mGTP).** (A) Complementation of the *ppi2* mutant. Three *Arabidopsis* lines each, expressing either of the constructs, were analyzed. Note that lines B0101, B0201, B1105, B0505, and B0502 are heterozygous for *ppi2* (*ppi2/TOC159*), whereas line B0205 expressing atToc159wt is homozygous for *ppi2* (*ppi2/ppi2*). PPT, phosphinothricine resistance gene; 35S, cauliflower mosaic virus 35S promoter; nos, nopaline synthase terminator; LB, left border of T-DNA; RB, right border of T-DNA. (B) Phenotypes of complemented plants. *ppi2* plants expressing atToc159wt (top panel; line B0205, homozygous for *ppi2*) or atToc159mGTP (bottom panel; line B0502, heterozygous for *ppi2*). Plants were grown on selection media containing kanamycine and phosphinothricine.

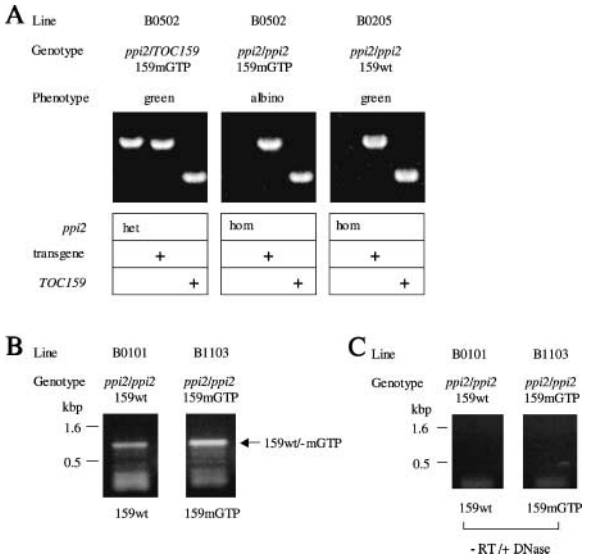


Figure 4. **Genetic background of complementation lines.** (A) PCR analysis of heterozygous (*ppi2/TOC159*) and homozygous (*ppi2/ppi2*) *ppi2* plants expressing atToc159mGTP (159mGTP; line B0502) and homozygous *ppi2* plants expressing full-length wild-type atToc159 (159wt, line B0205). The first reaction indicates the presence of undisrupted *TOC159*, the second the presence of the respective transgene, and the third indicates the presence of the 3′ end of TOC159 (control reaction). (B) Expression analysis of atToc159 (159wt) and atToc159mGTP (159mGTP) in a homozygous *ppi2* background using RT-PCR. (C) Control PCR on total RNA after DNase treatment, but without reverse transcription.

B0505) or homozygous (B0205) *ppi2* mutations as well as either the atToc159wt or the atToc159mGTP transgene, were chosen for segregation analysis (Fig. 3 A). Complementation was scored by determining the ability of transgenic atToc159wt or atToc159mGTP to restore a wild-type, green phenotype in *ppi2* homozygous plants. The construct encoding atToc159wt resulted in a percentage of green plants close to 100% among the surviving seedlings of the three lines analyzed, indicating that the construct had successfully complemented the *ppi2* mutation (Fig. 3, A and B, upper panels). In contrast, the construct encoding atToc159mGTP resulted in a percentage of green plants close to 66% among the surviving seedlings of the lines analyzed (Fig. 3, A and B, bottom panels). This result is consistent with the inability of the construct to complement the *ppi2* mutant and restore the wild-type, green phenotype. Thus, the data suggest that the G-domain of atToc159 has an essential function.

To substantiate the results of the segregation analysis, we molecularly characterized the complementation lines (Fig. 4). PCR analysis using transgene-specific primers demonstrated that the T-DNA constructs encoding atToc159wt or atToc159mGTP were indeed present in the lines analyzed (Fig. 4 A). PCR analysis of the *ppi2* background showed that albino plants of line B0502 carrying the atToc159mGTP construct were homozygous for *ppi2* (Fig. 4 A, middle), whereas green plants were heterozygous (Fig. 4 A, left). Therefore, the PCR analysis confirms the inability of the atToc159mGTP construct to complement *ppi2*. On the other hand, green plants of line B0205 carrying the atToc159wt construct were homozygous for *ppi2* (Fig. 4 A, right), confirming complementation by the construct.

To rule out that the failure of the atToc159mGTP construct to complement *ppi2* was due to a lack of gene expression, RT-PCR experiments were done on transgenic plants homozygous for *ppi2* (Fig. 4 B and C). The RT-PCR analysis using transgene-specific primers indicate that in albino *ppi2* plants carrying the atToc159mGTP construct, atToc159mGTP (Fig. 4 B, 159mGTP) was at least as strongly expressed as atToc159wt (Fig. 4 B, 159wt) in green *ppi2* plants carrying the atToc159wt construct (Fig. 4 B). In a control experiment (Fig. 4 C) in which the RNA was pretreated with DNase and not reverse transcribed, PCR failed to yield any products, indicating that the RT-PCR products (Fig. 4 B) were not due to amplification of genomic DNA. The RT-PCR experiments demonstrate that the inability of atToc159mGTP construct to complement *ppi2* is not due to a lack of transgene expression.

The presence of transgene-encoded proteins was determined by Western blotting using affinity-purified antibodies specific to the A-domain of atToc159 (Fig. 5). Albino *ppi2* plants (line B0502) carrying the atToc159mGTP construct expressed the corresponding protein (Fig. 5 A, lane 1). However, atToc159mGTP was present at somewhat lower levels than atToc159wt in homozygous *ppi2* plants (Fig. 5 A, lane 3) or than total atToc159 in heterozygous *ppi2* plants expressing atToc159mGTP (Fig. 5 A, lane 2). Nevertheless, the data suggest that the inability of atToc159mGTP to complement the *ppi2* phenotype is

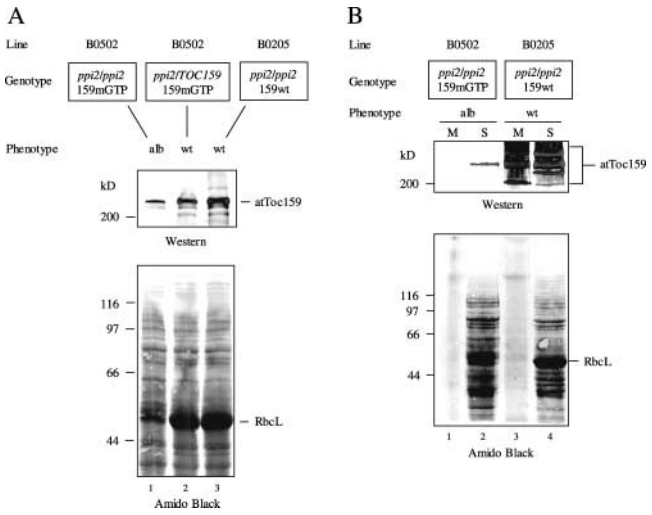


Figure 5. **AtToc159mGTP (159mGTP) and atToc159wt (159wt) protein expression.** (A) Total protein extracts were prepared from homozygous (*ppi2/ppi2*, lane 1) and heterozygous (*ppi2/TOC159*, lane 2) *ppi2* plants expressing atToc159mGTP as well as homozygous (*ppi2/ppi2*) *ppi2* plants expressing atToc159wt (lane 3). 50 μg of protein each were separated by SDS-PAGE, blotted onto nitrocellulose and stained with amido black (bottom). The blot was probed with affinity-purified antibodies raised against the A-domain of Toc159 (top). RbcL, large subunit of Rubisco. (B) Distribution of atToc159mGTP (159mGTP) and atToc159wt (159wt) between an alkaline-extracted membrane fraction (M, lanes 1 and 3) and a soluble fraction (S, lanes 2 and 4) prepared from homozygous *ppi2* plants (*ppi2/ppi2*) expressing atToc159mGTP (lanes 1 and 2) or atToc159wt (lanes 3 and 4), respectively. 20 μg of protein each were separated by SDS-PAGE, blotted onto nitrocellulose, and stained with amido black (bottom). The blot was probed with affinity-purified antibodies raised against the A-domain of Toc159 (top). RbcL, large subunit of Rubisco.

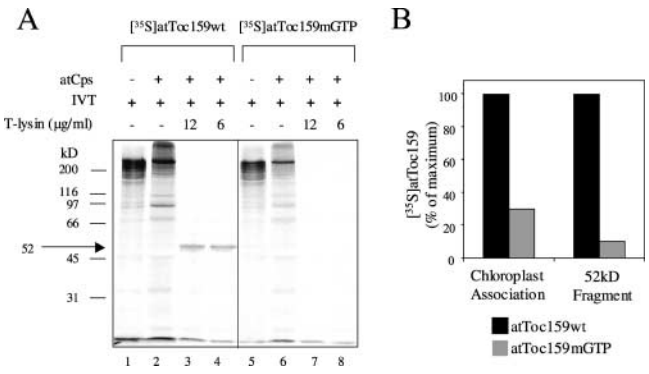


Figure 6. **Association of atToc159 with isolated chloroplasts and integration into the outer chloroplast membrane.** (A) In vitro synthesized [^{35}S]atToc159wt and [^{35}S]atToc159mGTP, respectively, were incubated with isolated Arabidopsis chloroplasts. Chloroplasts were subsequently reisolated and either directly analyzed by SDS-PAGE followed by autoradiography (lanes 2 and 6) or subjected to protease treatment with thermolysin at the concentrations indicated (T-lysin, lanes 3, 4, 7, and 8) before analysis. (B) Phosphorimager quantitation of the chloroplast association experiment. The relative amounts of [^{35}S]atToc159wt and [^{35}S]atToc159mGTP, respectively, associated with isolated chloroplasts (Chloroplast Association) as well as the 52-kD proteolytic fragments formed on treatment with thermolysin (52-kD Fragment) are indicated.

probably not due to a lack of protein production or protein stability, but is rather due to the inability of the mutant G-domain to bind and hydrolyze guanosine nucleotides.

The functional significance of the atToc159–GTPase cycle is currently not known. However, the transient expression of GFP fusion proteins suggests that the G-domain is required for targeting of cytosolic atToc159 to the chloroplast outer membrane and may regulate partitioning between subcellular compartments (Fig. 1). To determine whether the subcellular distribution of atToc159mGTP is affected when compared with atToc159wt, we separated homozygous *ppi2* plants expressing either atToc159mGTP (Fig. 5 B, lanes 1 and 2) or atToc159wt (Fig. 5 B, lanes 3 and 4) into a soluble and a total membrane fraction (Fig. 5 B). The total membrane fraction was then extracted twice with alkaline carbonate buffer to remove peripheral proteins. Soluble and membrane proteins were analyzed by Western blotting using affinity-purified antibodies against the A-domain of atToc159. Although atToc159wt was present both in the soluble and membrane fractions of homozygous *ppi2* plants expressing atToc159wt (Fig. 5 B, lanes 3 and 4), atToc159mGTP was present exclusively in the soluble fraction of homozygous *ppi2* plants expressing atToc159mGTP, albeit at reduced levels (Fig. 5 B, lane 2) when compared with atToc159wt. The reduced levels of atToc159mGTP may reflect sensitivity of the protein to the extraction procedure. Nonetheless, these data strongly suggest that atToc159mGTP is not properly targeted to plastids, and is therefore unable to integrate into the outer chloroplast membrane in vivo.

Insertion of atToc159 into the outer chloroplast membrane requires the intact GTP-binding site

The presence of atToc159mGTP in the soluble fraction, as well as its absence from carbonate-extracted membranes, suggests that the GTP binding and hydrolysis at the G-domain may be required not only for chloroplast targeting, but also for membrane insertion. Therefore, in vitro chloroplast-targeting experiments were performed using either synthetic [^{35}S]atToc159wt or [^{35}S]atToc159mGTP as the substrate. Association of [^{35}S]atToc159wt or [^{35}S]atToc159mGTP with isolated *Arabidopsis* chloroplasts and formation of the 52-kD protease-resistant fragments on membrane insertion (Hirsch et al., 1994; Kessler et al., 1994; Bauer et al., 2000) were used as criteria for correct targeting and insertion of atToc159, respectively (Bauer et al., 2000). Here, association is defined as the sum of peripherally bound and inserted [^{35}S]atToc159, whereas the 52-kD fragment reflects only the membrane-inserted protein. Both [^{35}S]atToc159wt (Fig. 6 A, lane 1) and [^{35}S]atToc159mGTP (Fig. 6 A, lane 5) were synthesized in a cell-free reticulocyte lysate and subsequently incubated with isolated *Arabidopsis* chloroplasts. Aliquots of reisolated chloroplasts that had been incubated with either [^{35}S]atToc159wt (Fig. 6 A, lane 2) or [^{35}S]atToc159mGTP (Fig. 6 B, lane 6) were either directly analyzed by SDS-PAGE and autoradiography (to determine chloroplast association) or treated with protease (thermolysin; Fig. 6 A, lanes 3, 4, 7, and 8) before analysis (to determine membrane integration). [^{35}S]atToc159wt associated with isolated chloroplasts, and on treatment with the protease thermolysin yielded a 52-kD fragment, indicating the expected func-

tionality of the wild-type protein. In contrast, mutant [^{35}S]atToc159mGTP associated with chloroplasts to a much lower degree (~30% of wild type; Fig. 6 B, Chloroplast Association), and formation of the 52-kD fragment was reduced to ~10% of the [^{35}S]atToc159wt control (Fig. 6 B, 52-kD Fragment). Thus, both targeting to the chloroplast and insertion into the outer membrane may be affected in [^{35}S]atToc159mGTP.

It can likely be ruled out that the targeting and insertion defects of atToc159mGTP were due to other reasons than the guanosine nucleotide–binding deficiency. Smith et al. (2002) report additional experiments in the accompanying paper in which GTP was removed from [^{35}S]atToc159wt by incubation in the presence of EDTA and subsequent gel filtration before the in vitro targeting experiment. In a control experiment, [^{35}S]atToc159wt was incubated and subjected to gel filtration in the presence of Mg^{2+} and GTP. Both association and formation of the protease-protected M-domain fragments of EDTA-treated [^{35}S]atToc159wt were strongly reduced when compared with the Mg^{2+}- and GTP-treated sample. Therefore, the results obtained with EDTA-treated [^{35}S]atToc159wt are in agreement with the data obtained for [^{35}S]atToc159mGTP, suggesting that its targeting and insertion defect correlates with the guanosine nucleotide–binding deficiency.

Interaction between atToc159 and atToc33 may be regulated by GTP binding at atToc159

We have previously demonstrated that the soluble G-domain of atToc33 (atToc33$_{1–265}$-H$_6$) binds directly to atToc159 and prevents its association with the chloroplast (Hiltbrunner et al., 2001b). Thus, it appears that targeting of atToc159 to the chloroplast outer membrane involves its interaction with atToc33. The presence of dimerization motifs in the G-domains of both atToc159 and atToc33 (Sun et al., 2002) suggests that the interaction between the proteins involves the G-domains and may possibly be regulated by GTP binding and hydrolysis. To determine whether atToc159mGTP is affected in its ability to interact with atToc33, equivalent concentrations of either [^{35}S]atToc159mGTP or [^{35}S]atToc159wt were incubated with increasing concentrations of a recombinant soluble version of atToc33 (atToc33$_{1–265}$-H$_6$) lacking a short COOH-terminal transmembrane region (Fig. 7 A; Hiltbrunner et al., 2001b). As a control, we used the in vitro synthesized ^{35}S-labeled A-domain ([^{35}S]atToc159$_{1–731}$). The transient expression experiments (Fig. 1 D) indicated that the fusion between the A-domain and GFP remained cytosolic, suggesting that the A-domain may not directly interact with atToc33. atToc33$_{1–265}$-H$_6$, together with any bound atToc159 or A-domain, was reisolated by Ni-NTA agarose chromatography. The eluates were analyzed by SDS-PAGE followed by autoradiography and phosphorimager quantitation (Fig. 7 B). The data show that dose-dependent binding of [^{35}S]atToc159mGTP to atToc33 is reduced by up to threefold when compared with that of the wild type. Neither of the proteins bound to Ni-NTA resin significantly in the absence of atToc33$_{1–265}$-H$_6$, confirming that both mutant and wild-type atToc159 had directly bound to atToc33 (Fig. 7 A, lanes 1 and 5). Furthermore, [^{35}S]atToc159$_{1–731}$ gave no detectable binding to either the Ni-NTA resin alone or to atToc33$_{1–265}$-H$_6$, supporting the notion that the A-domain does not play a role in chloroplast targeting of atToc159. Moreover, these results suggest that the binding of [^{35}S]atToc159wt and [^{35}S]atToc159mGTP to at atToc33$_{1–265}$-H$_6$ is indeed specific. The reduced binding of atToc159mGTP to atToc33$_{1–265}$-H$_6$ suggests that the interaction of the two proteins is indeed regulated by GTP binding and hydrolysis. The M-domain is also likely to participate in targeting of the receptor to the Toc complex. In addition, other Toc components that are not included in this in vitro binding assay, such as Toc75, may be involved in the targeting reaction.

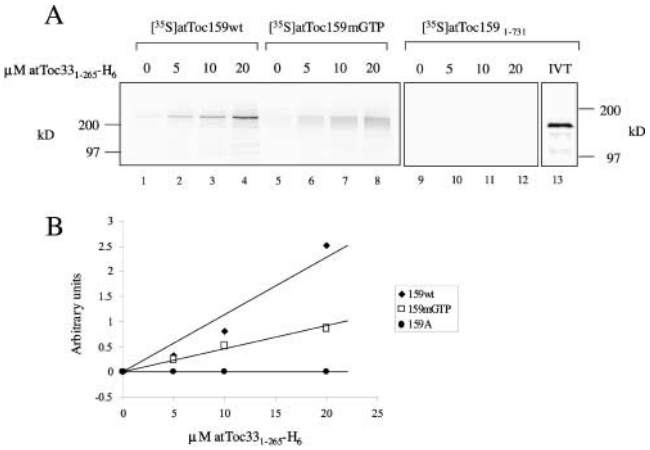


Figure 7. **Binding of [^{35}S]atToc159wt, [^{35}S]atToc159mGTP, and [^{35}S]atToc159$_{1–731}$ to atToc33$_{1–265}$-H$_6$.** (A) Increasing concentrations of atToc33$_{1–265}$-H$_6$ were incubated with in vitro synthesized [^{35}S]atToc159wt, [^{35}S]atToc159mGTP, and [^{35}S]atToc159$_{1–731}$, respectively, reisolated using Ni-NTA chromatography and eluted with imidazole. The imidazole eluates were analyzed by SDS-PAGE followed by autoradiography. (B) Phosphorimager quantitation of the binding experiment. Binding of [^{35}S]atToc159wt (159wt), [^{35}S]atToc159mGTP (159mGTP), and [^{35}S]atToc159$_{1-731}$ (159A) is given in arbitrary units. Binding in the absence of atToc33$_{1-265}$-H$_6$ was adjusted to zero.

Discussion

The trimeric Toc complex contains two homologous GTPases (atToc33 and atToc159) in addition to atToc75, a protein-conducting channel component. In contrast to atToc33 and atToc75, which exclusively behave as integral outer membrane proteins, atToc159 also exists in a cytosolic form (Hiltbrunner et al., 2001b). Targeting of soluble, cytosolic atToc159 involves an interaction with atToc33 at the chloroplast surface. The presence of novel dimerization motifs in the G-domains of both atToc159 and atToc33 supports a role for atToc33 as an atToc159 receptor, and potentially provides a structural basis for targeting of cytosolic atToc159 to the outer chloroplast membrane via heterodimer formation between the G-domains (Sun et al., 2002). In this paper, we demonstrate that the G-domain of atToc159 mediates and likely regulates chloroplast targeting by GTP binding and hydrolysis.

To determine which of the domains of atToc159 are required for targeting to the chloroplast, a series of GFP fu-

sions was engineered and transiently expressed in isolated *Arabidopsis* protoplasts. Technical difficulties prohibited the construction of a fusion between full-length atToc159 and GFP. However, a T-DNA construct encoding the combined G- and M-domains complements the *ppi2* mutant lacking atToc159, suggesting that the truncated protein without the A-domain is largely functional (unpublished data). Therefore, not surprisingly, GFP, fused NH_2-terminally to the combined G- and M-domains (GFP-atToc159GM), was present both at the chloroplast periphery and in the cytosol (Fig. 1 B), recapitulating the partitioning of endogenous atToc159 observed in earlier fractionation experiments and immunofluorescence (Hiltbrunner et al., 2001b). Therefore, these data provide additional evidence for the functionality of the truncated protein lacking the A-domain. Moreover, GFP-atToc159GM corresponds to 'Toc86', the proteolytic fragment atToc159 originally described in Hirsch et al. (1994), Kessler et al. (1994), and Schnell et al. (1994). The data shown here confirm that Toc86 may represent a targeting-competent form of atToc159 (Muckel and Soll, 1996). A fusion protein between the G-domain and GFP was also efficiently targeted to the chloroplast periphery of isolated *Arabidopsis* protoplasts (Fig. 1 C). This finding indicates that the G-domain is sufficient for targeting to the chloroplast. In contrast, GFP fusions to the A- and M-domains, respectively, were predominantly present in the cytosol on transient expression in protoplasts, suggesting that neither the A- nor the M-domain are sufficient for targeting. In the case of the A-domain, a role in targeting was not expected; pea Toc159 lacking the A-domain has been previously shown to be targeted and inserted into the chloroplast outer membrane in vitro (Muckel and Soll, 1996). The M-domain GFP fusion protein was evenly distributed in the cytosol, suggesting that it may behave as a soluble protein, although it was originally identified as the integral M-domain (Hirsch et al., 1994). This appears possible, as the primary sequence of the M-domain consists mostly of hydrophilic residues and does not contain predicted hydrophobic transmembrane helices. In the integral membrane form of atToc159, however, the M-domain is resistant both to alkaline extraction and protease treatment. Therefore, the M-domain may conformationally switch to expose a hydrophobic region. Conceivably, this switch may underlie membrane insertion, and may ultimately be coupled to a GTPase-regulated conformational switch taking place at the G-domain.

Transient expression of GFP fusion proteins in isolated *Arabidopsis* protoplasts suggest that the G-domain controls targeting of cytosolic atToc159 to the chloroplast. To analyze the role of GTP binding to atToc159, we engineered a mutant of atToc159 (Fig. 2 A, atToc159mGTP) that is almost completely unable to bind and hydrolyze GTP (Fig. 2 B) when compared with the wild type. AtToc159mGTP was inactive in an in vivo complementation assay in which the albino *ppi2* mutant lacking atToc159 was transformed with constructs encoding atToc159mGTP or wild-type atToc159 (atToc159wt), respectively (Fig. 3). Though homozygous *ppi2* plants expressing the atToc159mGTP protein were obtained, the mutant protein, in contrast to the wild type, failed to restore the normal green phenotype (Fig. 4 A). Thus, the functional G-domain of atToc159 is essential for chloroplast biogenesis.

To further characterize the defect of atToc159mGTP, *ppi2* plants expressing either atToc159mGTP or atToc159wt were fractionated into soluble and integral membrane fractions. Although atToc159wt partitioned between the two fractions, atToc159mGTP was present exclusively in the soluble fraction of *ppi2* plants. The result suggests that atToc159mGTP is neither properly targeted nor inserted into the outer membrane (Fig. 4 B), thereby providing further evidence for the role of the G-domain. Furthermore, the nature of the mutations in atToc159mGTP suggests that GTP binding and hydrolysis by the G-domain may regulate targeting and membrane insertion. In agreement with the in vivo fractionation experiment, wild-type atToc159, but not synthetic atToc159mGTP, was able to insert into the outer membrane of isolated chloroplasts (Fig. 6 A). A marked reduction of association with the chloroplast outer membrane was also observed (Fig. 6, A and B). Moreover, synthetic atToc159wt, from which GTP had been biochemically removed, also did not associate with isolated chloroplasts efficiently and did not insert into the outer membrane in vitro (Smith et al., 2002). The reduced association may reflect a decreased affinity for either atToc33 or other outer membrane components. This conclusion is supported by in vitro binding data, demonstrating that atToc159mGTP binds to the soluble G-domain of atToc33 less efficiently than the wild type (Fig. 7). In this experiment, the A-domain showed no detectable interaction with the soluble G-domain of atToc33, suggesting that the A-domain does not contribute to the interaction between atToc159 and atToc33. In summary, the results suggest that the G-domain of atToc159 controls targeting of the cytosolic form to the outer membrane in a two-step reaction in which binding to outer membrane receptors precedes membrane insertion.

Questions regarding the function of cytosolic atToc159 remain open. However, targeting of cytosolic atToc159 to the chloroplast outer membrane most likely does not reflect the maturation of an immature cytosolic precursor version of the protein, as is the case during the biogenesis of other known chloroplast outer membrane proteins (Schleiff and Klösgen, 2001). Our results suggest that atToc159 must be in an active form in the cytosol, as its targeting requires the correctly folded protein to be able to bind GTP. GTP-regulated targeting of atToc159 to the outer membrane represents a novel, possibly reversible targeting mechanism to the outer chloroplast membrane. This is in contrast to other studied proteins that spontaneously insert into the outer membrane (Schleiff and Klösgen, 2001) or are targeted to the outer membrane by a mechanism involving a bipartite transit sequence, as is the case for pea Toc75 (Tranel and Keegstra, 1996). As cytosolic atToc159 is likely a functional protein, it is tempting to speculate that it binds to newly synthesized precursors, escorting them to the Toc complex and promoting their subsequent import into the stroma. After precursor delivery, atToc159 may deinsert from the outer membrane and undergo further precursor targeting cycles. Current research is directed at the elucidation of the function of soluble atToc159.

Materials and methods

DNA constructs used in protein synthesis

pET21d-159G and pET21d-159G-mGTP used for the production of recombinant 159G and 159G-mGTP, respectively, were obtained as follows: using a forward primer (5′-CATGCCATGGAATTCTCATGAGGGGATCGCATCACCATCACCATCACACTAGTCAGGATGGTACGAAACTTTTCTCTATGGATCG-3′) including a SpeI site and a reverse primer including a HindIII site as well as a His_6 tag (5′-CCCAAGCTTTTAGTGATGGTGATGGTGATGCAAGTAAGGGAGAGGCGGGGATCTA-3′), PCR fragments corresponding to $Toc159_{727–1101}$ and $Toc159_{727–1101}$ A865R/K868N/S869R were amplified from pET21d-atToc159 and pET21d-atToc159mGTP. The PCR fragments were then ligated into the SpeI-HindIII site of pET21d derivative containing a start codon followed by a SpeI site instead of the NcoI site. pET21d-Toc159G and pET21d-Toc159G mGTP were transformed into *E. coli* BL21(DE3). Expression and purification under native conditions using Ni-NTA agarose was done for $atToc33_{1–265}$-H_6 as described previously (Hiltbrunner et al., 2001b). The cDNA encoding the A-domain of atToc159 ($atToc159_{1–731}$) was amplified from pET21d-atToc159 using a forward primer (5′-ACGCGTCGACGGATGGACTCAAAGTCGGTTACTC-3′) and a reverse primer (5′-AAGGAAAAAAGCGGCCGCTACCATCCTGAGATGTTATG-3′). The resulting PCR product was blunt-end ligated into the PCR-Script® (Stratagene) vector, resulting in the coding sequence under the control of the T7 promoter.

DNA constructs used in the complementation assay

The 3.9-kb NcoI fragments of pET21d-atToc159 or pET21d-atToc159mGTP were ligated into the NcoI site of pTD31 (Tinland et al., 1995), resulting in the constructs pTD31-atToc159NcoI and pTD31-atToc159mGTPNcoI, respectively. By ligating a StuI-XbaI fragment of pET21d-atToc159 into the corresponding sites of pTD31-atToc159NcoI/-atToc159mGTPNcoI, the complete sequences were obtained. The resulting constructs were named pTD31-atToc159 and pTD31-atToc159mGTP. From the two pTD31 constructs, the promoter (CaMV 35S) gene–terminator (nos) cassettes were excised by EcoRI-HindIII and ligated into identical sites in pCAMBIA3300 (CAMBIA). The resulting constructs (p3300-atToc159 and -atToc159mGTP) were transformed into *Agrobacterium tumefaciens* C58.

Arabidopsis thaliana for complementation assay

A. thaliana plants were grown under long-day conditions (16 h light, 100 μmol/m^2/s, 21°C, 60% humidity) on soil. Plants heterozygous for a T-DNA insertion in *TOC159* (*ppi2*, line CS11072; Bauer et al., 2000) were transformed using the infiltration method (Bechtold and Pelletier, 1998). Seeds of T0 generation were grown on soil and sprayed with phosphinothricine (150 mg/l) for selection. Seeds (T1 generation) of resistant plants were further selected on medium containing 0.5 × Murashige-Skoog medium, 1% sucrose, 50 mg/l kanamycine, and 30 mg/l phosphinothricine. Resistant plants were transferred to soil. Seeds of these plants (T2 generation) were grown on medium containing kanamycine and phosphinothricine and subsequently used for segregation and PCR/Western analysis.

In vitro translation

^{35}S-labeled proteins were produced by coupled in vitro transcription and translation. ^{35}S-labeled proteins used for insertion assays in absence of nucleotides were incubated in import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 7.5, 40 mM potassium acetate, 2 mM magnesium $acetate_2$, and 25 μM DTT) containing 5 mM EDTA for 5 min at 25°C to remove bound nucleotides. For proteins used in control assays with GTP, 5 mM GTP was used instead of EDTA. Proteins were then passed over spin columns (Sephadex G-25; Amersham Biosciences) equilibrated with import buffer containing 5 mM EDTA or 5 mM GTP, respectively, and used for insertion assays. The in vitro binding assay was performed as described previously (Hiltbrunner et al., 2001b).

GTP binding and hydrolysis assays

GTP binding to 159G and 159GmGTP was measured using a solid phase GTP overlay assay. Purified 159G and 159GmGTP were diluted to 16 μg/ml in 50 mM Hepes-KOH, pH 7.5, and 40 mM potassium acetate (HK buffer), and 250 μl of each sample was spotted onto nitrocellulose membrane. An equivalent sample corresponding to the eluate from Ni-NTA chromatography of an *E. coli* strain expressing RbcS (Ma et al., 1996) was used as a background reference. The nitrocellulose filter was incubated for 30 min at RT in 20 mM Tris-HCl, pH 7.5, 50 μM $MgCl_2$, and 0.3% vol/vol Tween 20 (GTP-binding buffer). The blot was transferred to GTP-binding buffer containing 50 nM α-[^{32}P]GTP (3,000 Ci/mmol; PerkinElmer Life Sciences) and 1 μM ATP, and incubated for 1 h at 4°C. The blot was washed five times with 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 0.3% vol/vol Tween 20, and binding of α-[^{32}P]GTP was measured using a phosphorimager (Storm 840; Amersham Biosciences) and ImageQuant v.1.2 software. Data are presented as fmol of α-[^{32}P]GTP bound per μg of protein.

GTP hydrolysis was measured using a method adapted from Liang et al. (2000). In brief, purified 159G and 159GmGTP were diluted to 0.5 μM with HK buffer containing 2 mM $MgCl_2$. The hydrolysis reaction was initiated by the addition of α-[^{32}P]GTP to a final concentration of 1 μM (150 mCi/μmol) in a 25-μl reaction, and incubation at 25°C. GTP hydrolysis was linear over a 60-min incubation period. Therefore, samples were removed after 0 min and 60 min of hydrolysis, and the reactions were immediately stopped by heating at 65°C for 5 min in 0.2% wt/vol SDS, 10 mM EDTA, 4 mM GTP, and 4 mM GDP. The samples were spotted onto PEI cellulose-F TLC plates (EM Science), and GTP and GDP were resolved using 1 M LiCl solvent. The plates were dried and radiolabeled spots of GTP and GDP were quantified using a Storm 840 phosphorimager. Data are presented as fmol α-[^{32}P]GTP per hydrolyzed pmol protein per min^{-1}. Hydrolysis of GTP by soluble *E. coli* proteins that nonspecifically bound to Ni-NTA resin was used as a background reference.

Preparation of plant protein extracts and subfractions

Isolation of total protein was done as described elsewhere (Rensink et al., 1998). For isolation of total soluble and membrane protein, plants were ground in liquid nitrogen. TE (50 mM Tris-HCl, pH 7.5, and 2 mM EDTA) was added and the homogenate was filtered and centrifuged at 100,000 *g* at 4°C for 30 min. The supernatant was considered total soluble protein. To obtain total membrane protein samples, the pellet was resuspended in 50 mM Tris-HCl, pH 7.5. For carbonate extraction, the pellet was resuspended in 0.2 M Na_2CO_3 and centrifuged at 100,000 *g* at 4°C for 30 min. The supernatant was removed and the pellet subjected to a second round of carbonate extraction. The final pellet was considered total integral membrane protein and resuspended in 50 mM Tris-HCl, pH 7.5. All samples were concentrated by $CHCl_3$/MeOH precipitation (Wessel and Flügge, 1984) and used for SDS-PAGE and immunoblotting according to standard protocols. HRP-coupled secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reaction (Roche) were used for immunodetection.

Chloroplast isolation and insertion assay

A. thaliana plants var. Columbia 2 were grown under long day conditions (16 h light, 8 h dark) on agarose plates (0.8% wt/vol) containing 0.5 × Murashige-Skoog medium supplemented with 1% wt/vol sucrose, and were used for isolation of intact chloroplast as described previously (Fitzpatrick and Keegstra, 2001), but with minor modifications. 1% wt/vol cellulase (Serva) was used instead of 4%, and 0.25% wt/vol macerozyme (Yakult Honsha Co., Ltd.) instead of 1%. For purification of intact chloroplasts, a 40%:85% wt/vol Percoll step gradient was used rather than a linear gradient.

Chloroplasts corresponding to 15 μg chlorophyll were used for insertion assays. Insertion assays contained import buffer, 2.5 mM ATP, 1 mM GTP, and 8 μl in vitro translate in a final volume of 100 μl. The insertion reaction was started by adding the in vitro translate, incubated for 15 min at 25°C, and stopped by adding 1 ml HS (330 mM sorbitol and 50 mM Hepes/KOH, pH 7.5). Chloroplasts for insertion assays in the absence of GTP were depleted of endogenous nucleotides for 20 min at RT in darkness. The insertion reaction was done in darkness, too. ATP and GTP were omitted from these reactions; instead, 5 mM EDTA was added.

Chloroplasts used for thermolysin treatment were washed in HS. Thermolysin digest was performed in HS supplemented with 30 μM $CaCl_2$ for 15 min on ice.

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 elsewhere (Jin et al., 2001) using the following modification: Instead of 4% and 1%, respectively, we used only 1% wt/vol cellulase (Serva) and 0.25% wt/vol macerozyme (Yakult Honsha Co., Ltd.). For transient expression of Toc159G, Toc159M, and Toc159GM NH_2-terminally fused to the COOH terminus of GFP, we used pSH11, a derivative of pCL60 (Dr. C. Lupi, Institute of Plant Sciences, ETH Zürich). pCL60 is a pBluescript-based construct containing GFP under the control of the CaMV 35S promoter and the nos terminator. pSH11 was derived from pCL60 by omitting the stop codon of GFP and inserting a NotI site at the 3′ end of GFP. cDNA fragments coding for atToc159G, atToc159M, and atToc159GM were obtained by PCR us-

ing 5′ and 3′ primers including a NotI site (5′ primer for atToc159G and atToc159GM: 5′-AAGGAAAAAAGCGGCCGCACCACGTGCAAACCGTTCCAA-3′; 5′ primer for atToc159M, 5′-AAGGAAAAAAGCGGCCGCACCATTGGACCATCGTAAAG-3′; 3′ primer for atToc159G, 5′-AAGGAAAAAAGCGGCCGCTTAAACTCGGAAACCAAATACTTTA-3′; 3′ primer for atToc159M and atToc159GM, 5′-AAGGAAAAAAGCGGCCGCTAGTACATGCTGTACTTGTCG-3′). The respective PCR products were then ligated into the NotI site of pSH11, resulting in pSH11-atToc159G, pSH11-atToc159M, and pSH11-atToc159GM, respectively. A DNA fragment coding for Toc159A was amplified using a 5′ primer (5′-CATGCCATGGACTCAAAGTCGGTTA-3′) and a 3′ primer (5′-CATGCCATGGACAAACCAGCAGGTCGATCCA-3′) incorporating NcoI sites and ligated into the NcoI site of pCL60, resulting in pCL60-atToc159A. For Western blot analysis, protoplasts transiently expressing GFP or the GFP-atToc159GM fusion protein were collected by centrifugation at 100 *g* for 1 min, and lysed by resuspending in lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$) containing a protease inhibitor cocktail (0.5% vol/vol; no. 9599; Sigma-Aldrich) followed by one cycle of freezing and thawing. The lysate was centrifuged at 100,000 *g* for 20 min, and the resulting supernatant was considered total soluble protein. The pellet was carbonate-extracted twice by resuspending in 0.2 M Na_2CO_3, pH 11.5, and centrifuging at 100,000 *g* for 20 min. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.5. All samples were concentrated by $CHCl_3$-MeOH precipitation and used for SDS-PAGE and immunoblotting. Blots were probed with anti-GFP mAb (CLONTECH Laboratories, Inc.) or anti-atToc159 (Bauer et al., 2000).

DNA extraction and diagnostic PCR

DNA for PCR on *Arabidopsis* plants was isolated from single leaves according the following procedure: one leaf was ground in 500 μl extraction buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% wt/vol SDS). After 5′ centrifugation at 16,000 *g* at 4°C, the supernatant was transferred to an equal volume of isopropanol and centrifuged for 30 min at 16,000 *g* at RT. The supernatant was discarded and the pellet washed with 80% vol/vol ethanol, air dried, and resuspended in 50 μl water. 1μl was used for PCR reactions according to standard protocols. The following primers were used to identify individual Toc159 knock-out plants: (*ppi2*; 5′-GAATAGGGTTTTAATCGGAAG-3′ and 5′-CATCCCCAATTTGATCAAC-3′) and plants carrying the transgene (Toc159 and Toc159mGTP, respectively) under the control of the 35S CaMV promoter and nos terminator (5′-CAATTCCGAGGCTGTAGCCGAC-3′ and 5′-CCATGACTGTGGATATGACG-3′). The primers 5′-CCATGACTGTGGATATGACG-3′ and 5′-CCTCGAGGTTCGCACCATAG-3′ were used as positive control.

RT-PCR experiments

Total RNA was isolated from *Arabidopsis* plants using the RNeasy Plant Mini Kit (QIAGEN). Preceding reverse transcription RNA was treated with 5 U RQ1 DNase (Promega) in presence of 40 U RNasin RNase inhibitor (Promega). After inactivation of DNase by heating, first-strand synthesis was performed as described elsewhere (Gubler and Hoffmann, 1983). The cDNA was directly used for PCR according to standard protocols. The primers 5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′ and 5′-CAATTCCGAGGCTGTAGCCGAC-3′ were used for detection of the transgene and 5′-CATGTCATGAACTCCTCTATGCTCTCCTCTGC-3′ and 5′-ACTGATGCATTGAGCATCAGTGAAGCTTGG-3′ were used in a control reaction to amplify part of ats1B (GenBank/EMBL/DDBJ accession no. X14564).

This work is dedicated to professor Nikolaus Amrhein on the occasion of his 60th birthday.

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