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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 NH₂-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 chloroplast membrane.

*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* pp2 mutant, disrupted in *TOC159*, has an albino phenotype coincident with the absence of differentiated chloroplasts. Furthermore, the failure of pp2 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 NH2-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 GDP 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 atToc33L–265, rendered soluble by deletion of the COOH-terminal transmembrane region. Furthermore, atToc33L–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 NH2-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 pp2 mutant lacking atToc159, suggesting that atToc159GM is largely functional (unpublished data). Therefore, we fused GFP to the NH2 terminus of a construct corresponding to the G- and M-domains in which the 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 1. Transient expression of atToc159 domains fused to GFP.](image-url)
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-nitrotriacetic 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 $\alpha-[^32P]GTP$. The blots were washed and binding was measured using a phosphorimager (Fig. 2 B). GTP binding to 159G-mGTP was $\sim$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 $\alpha-[^32P]GTP$ 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 $\alpha-[^32P]GTP$ from $\alpha-[^32P]GDP$, 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 (confering 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 (confering 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

![Alignment of the GTP-binding domains of hsRas, atToc33, and atToc159 encompassing the G1 and G3 motifs.](image)
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 B0505 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 were 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
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/ppi2 plants expressing atToc159mGTP (Fig. 5 B, lane 1) and heterozygous ppi2/TOC159, lane 2) ppi2 plants expressing atToc159mGTP as well as homozygous 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 (ppi2/2) 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 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 (ppi2/2) 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.

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 [35S]atToc159wt or [35S]atToc159mGTP as the substrate. Association of [35S]atToc159wt or [35S]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 [35S]atToc159, whereas the 52-kD fragment reflects only the membrane-inserted protein. Both [35S]atToc159wt (Fig. 6 A, lane 1) and [35S]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 [35S]atToc159wt (Fig. 6 A, lane 2) or [35S]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). [35S]atToc159wt associated with isolated chloroplasts, and on treatment with the protease thermolysin yielded a 52-kD fragment, indicating the expected func-

![Figure 6](https://example.com/figure6.png)

Figure 6. Association of atToc159 with isolated chloroplasts and integration into the outer chloroplast membrane. (A) In vitro synthesized [35S]atToc159wt and [35S]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 (lanes 3, 4, 7, and 8) before analysis. (B) Phosphorimager quantitation of the chloroplast association experiment. The relative amounts of [35S]atToc159wt and [35S]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.
tionality of the wild-type protein. In contrast, mutant [35S]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 [35S]atToc159wt control (Fig. 6 B, 52-kD Fragment). Thus, both targeting to the chloroplast and insertion into the outer membrane may be affected in [35S]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 [35S]atToc159wt by incubation in the presence of EDTA and subsequent gel filtration before the in vitro targeting experiment. In a control experiment, [35S]atToc159wt was incubated and subjected to gel filtration in the presence of Mg2+ and GTP. Both association and formation of the protease-protected M-domain fragments of EDTA-treated [35S]atToc159wt were strongly reduced when compared with the Mg2+- and GTP-treated sample. Therefore, the results obtained with EDTA-treated [35S]atToc159wt are in agreement with the data obtained for [35S]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 (atToc331–265-H6) 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 [35S]atToc159mGTP or [35S]atToc159wt were incubated with increasing concentrations of a recombinant soluble version of atToc33 (atToc331–265-H6) lacking a short COOH-terminal transmembrane region (Fig. 7 A; Hiltbrunner et al., 2001b). As a control, we used the in vitro synthesized 35S-labeled A-domain (35S)atToc1591–731. 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. atToc331–265-H6, 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 [35S]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 atToc331–265-H6, confirming that both mutant and wild-type atToc159 had directly bound to atToc33 (Fig. 7 A, lanes 1 and 5). Furthermore, [35S]atToc1591–731 gave no detectable binding to either the Ni-NTA resin alone or to atToc331–265-H6, 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 [35S]atToc159wt and [35S]atToc159mGTP to atToc331–265-H6 is indeed specific. The reduced binding of atToc159mGTP to atToc331–265-H6 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.

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 NH₂-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 (Hilbrunner 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 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 desinsert 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: upstream forward primer (5'-CATGCGTGGGAATTCCTTACGAGGGGTGATCGATC-3') and reverse primer corresponding to Throc159-256 (5'-CTATGCAACCTCACTAACTTAGTCGACGTATGAC-3') were amplified from pET21d insertion site of Arabidopsis thaliana (ATCC, Manassas, VA). The resulting PCR product was blunt-end ligated into the pCR2.1 vector (Stratagene). Sequences of the resulting plasmid were confirmed by DNA sequencing.

DNA constructs used in the complementation assay

The 3.9-kb Ncol fragments of pET21d-atToc159 or pET21d-atToc159GTP were ligated into the Ncol site of pTD31 (Tianland et al., 1994), resulting in the constructs pTD31-atToc159Ncol and pTD31-atToc159GTPNcol, respectively. In vivo translocation assays were done as described elsewhere (Rensink et al., 1994).

In vitro translation

35S-labeled proteins were produced by coupled in vitro transcription and translation. 35S-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, 25 mM 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 (Hiltnbrunner 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 MgCl2, and 0.3% vol/vol Tween 20 (GTP-binding buffer). The blot was transferred to GTP-binding buffer containing 50 mM α-[32P]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 MgCl2, and 0.3% vol/vol Tween 20, and binding of α-[32P]GTP was measured using a phosphorimager (Storm 840; Amersham Biosciences) and ImageQuant v.1.2 software. Data are presented as fmol of α-[32P]GTP bound per µg of protein.

Chloroplast isolation and insertion assay

Chloroplasts used for thermolysin treatment were washed in HS. Thermolysin digest was performed in HS supplemented with 30 µM CaCl2 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). Briefly, protoplasts were incubated in darkness for 20 min with 3 µg of plasmid DNA. The presence of GFP under the control of the CAMV 35S promoter and the nos terminator, psh111 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-

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 grown 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 30 min. The supernatant was removed and the pellet subjected to a second round of 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 CHCl3/MeOH precipitation (Wessel and Flugge, 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 assays were performed as described previously (Jin et al., 2001) using the following modification: Instead of 4% polyethylene glycol (PEG) for selection, 1% (v/vol) PEG2000 was used. In the presence of the nos terminator, psh111 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-

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ing 5′ and 3′ primers including a NotI site (5′ primer for atToc159G and atToc159GM: 5′-AAGGAAAAAACGGCCGACCATCAGTTGCTCCCAAACTCTTCAAA-3′; 5′ primer for atToc159M, 5′-AAGGAAAAAACGGCCGACCATCAGTTGCTCCCAAACTCTTCAAA-3′) and a 3′ primer (5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′) for atToc159 and atToc159GM. The following PCR products were then ligated into the NotI site of pH11, resulting in pH11-atToc159, pH11-atToc159GM, and pH11-atToc159GM, respectively. A DNA fragment coding for Toc159A was amplified using a 5′ primer (5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′) and a 3′ primer (5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′) incorporating Ncol sites and ligated into the NotI site of pcDL6, resulting in pC6L6-atToc159A. For Western blot analysis, protoplasts transiently expressing GFP or the GFP-atToc159GM fusion protein were collected by centrifugation at 100,000 g for 20 min. After inactivation of DNase by heating, first-strand synthesis was performed using 5 μg Tris-HCl, pH 7.5, 2 mM MgCl2 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 Na2CO3, 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 CHCl3-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).

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 following primers were used to identify individual Toc159 knock-out plants: (pup2; 5′-GAATTCATGTCATGAACTCAGCTTACCCGATACCG-3′ and 5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′) and plants containing the transgene (Toc159 and Toc159GTP, respectively) under the control of the 335 CaMV promoter and nos terminator (5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′ and 5′-CATGTCATGAACTCAGCTTACCCGATACCG-3′) and 5′-CCATGACGTGTTGATATGACG-3′) were used as positive control.

References


