Nucleotide binding and dimerization at the chloroplast preprotein import receptor, atToc33, are not essential for receptor activity *in vivo*

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SUMMARY

The atToc33 protein is an import receptor in the outer envelope of Arabidopsis chloroplasts. It is a GTPase with motifs characteristic of such proteins, and its loss in the *plastid protein import 1* (*ppi1*) mutant interferes with the import of photosynthesis-related preproteins, causing a chlorotic phenotype in mutant plants. To assess the significance of GTPase cycling by atToc33, we generated several atToc33 point mutants with predicted effects on GTP binding (K49R, S50N, S50N/S51N), GTP hydrolysis (G45R, G45V, Q68A, N101A), both binding and hydrolysis (G45R/K49N/S50R), and dimerization or functional interaction between dimeric partners (R125A, R130A, R130K). First, a selection of these mutants was assessed *in vitro* or in yeast, to confirm that the mutations have the desired effects: in relation to nucleotide binding and dimerization, the mutants behaved as expected. Then, activities of selected mutants were tested *in vivo*, by assessing for complementation of *ppi1* in transgenic plants. Remarkably, all tested mutants mediated high levels of complementation: complemented plants were similar to wild type in growth rate, chlorophyll accumulation, photosynthetic performance, and chloroplast ultrastructure. Protein import into mutant chloroplasts was also complemented to a considerable degree. Overall, the data indicate that neither nucleotide binding nor dimerization at atToc33 is essential for the receptor's activity in import. Absence of atToc33 GTPase activity might somehow be compensated for by that of another receptor, Toc159. However, overexpression of atToc33 (or its close relative, atToc34) in Toc159-deficient plants did not mediate complementation, indicating that the receptors do not share functional redundancy in the conventional sense.

INTRODUCTION

Most chloroplast proteins are encoded in the nucleus and synthesized as preproteins in the cytosol. Each preprotein has an N-terminal transit peptide that is recognized by the TOC and TIC (Translocon at the Outer/Inner envelope membrane of Chloroplasts) complexes of the chloroplast envelope (Inaba and Schnell, 2008; Jarvis, 2008; Kessler and Schnell, 2006; Smith, 2006; Soll and Schleiff, 2004). These two multiprotein complexes cooperate to drive the post-translational import of preproteins across the envelope membranes. Preprotein import requires both ATP and GTP (Olsen and Keegstra, 1992; Olsen *et al.*, 1989; Young *et al.*, 1999). Most of the ATP is consumed at late stages of import, in the stroma, and this requirement is attributed to molecular chaperones within a putative import motor complex. By contrast, the GTP is required during early stages when preprotein recognition occurs, and is used by import receptors at the chloroplast surface. Once a preprotein arrives in the stroma, the transit peptide is proteolytically removed and the mature domain takes on its final conformation or engages intraorganellar sorting pathways (Gutensohn *et al.*, 2006; Schünemann, 2007).

Transit peptide recognition is mediated by the receptors, Toc34 and Toc159 (Hirsch *et al.*, 1994; Kessler *et al.*, 1994; Schnell *et al.*, 1994; Seedorf *et al.*, 1995). These are related GTPases, and they possess many of the guanine nucleotide binding and hydrolysis motifs that are characteristic of such proteins (Bourne *et al.*, 1991). Both receptors are anchored in the outer envelope by a C-terminal membrane domain, and both project a GTPase domain (G-domain) out into the cytosol. Although Toc159 additionally possesses an N-terminal acidic domain, it is thought that the G-domain plays a more important role in transit peptide binding (Bauer *et al.*, 2002; Bölter *et al.*, 1998; Chen *et al.*, 2000). Structural studies and *in vitro* interaction analyses have shown that the receptor G-domains are able to undergo homo- and heterodimerization (Koenig *et al.*, 2008; Reddick *et al.*, 2007; Sun *et al.*, 2002; Weibel *et al.*, 2003; Yeh *et al.*, 2007). There have been conflicting reports concerning the consequences of receptor dimerization for GTPase cycling. An early hypothesis was that each monomer acts as the GTPase activating protein (GAP) for its partner (Sun *et al.*, 2002). However, it was recently reported that receptor dimerization does not significantly affect GTPase activity, and that it instead serves to facilitate the transfer of preproteins to the translocation channel (Lee *et al.*, 2009). While it is generally accepted that the two receptors cooperate closely to mediate preprotein recognition and early stages of import, the mechanisms have not been clearly established (Becker *et al.*, 2004; Kessler and Schnell, 2004; Li *et al.*, 2007).

In Arabidopsis, both receptors are encoded by a small gene family: Toc34 is encoded by two genes (*atTOC33*, *atTOC34*), whereas Toc159 is encoded by four genes (*atTOC159*, *atTOC132*, *atTOC120*, *atTOC90*) (Bauer *et al.*, 2000; Gutensohn *et al.*, 2000; Hiltbrunner *et al.*, 2001; Jarvis *et al.*, 1998; Jelic *et al.*, 2003); the different numbers indicate the sizes (in kDa) of the encoded proteins (Schnell *et al.*, 1997). Genetic and biochemical studies led to the hypothesis that the different receptor isoforms have distinct substrate specificities: the dominant isoform in each family (atToc159,

atToc33) is thought to have recognition preference for highly-abundant components of the photosynthetic apparatus (Bauer *et al.*, 2000; Kubis *et al.*, 2003; Smith *et al.*, 2004), whereas other isoforms (atToc132, atToc120, atToc34) act preferentially in the import of less abundant, housekeeping preproteins (Hiltbrunner *et al.*, 2004; Ivanova *et al.*, 2004; Kubis *et al.*, 2004). The Arabidopsis *plastid protein import 1* (*ppi1*) and *ppi2* mutants, which lack atToc33 and atToc159, respectively, have striking chlorotic or albino phenotypes due to deficiencies in the import of photosynthesis-related preproteins (Bauer *et al.*, 2000; Jarvis *et al.*, 1998; Kubis *et al.*, 2003; Smith *et al.*, 2004).

It has been proposed that preprotein binding by the receptors is strongly influenced by their nucleotide status (Becker *et al.*, 2004; Jelic *et al.*, 2002; Schleiff *et al.*, 2002; Sveshnikova *et al.*, 2000). One possibility is that the receptors bind to transit peptides when in the GTP-bound state, and that transit peptide binding stimulates GTP hydrolysis to yield a lower-affinity, GDP-bound form of the receptor, allowing the preprotein to disassociate and progress to the next stage of the translocation pathway (Soll and Schleiff, 2004). Other models suggest that the stimulation of GTP hydrolysis is mediated by dimerization between the receptors (as already mentioned) (Kessler and Schnell, 2004), or that the transit peptide acts as a guanine nucleotide exchange factor (GEF) rather than as a GAP (Li *et al.*, 2007). One thing that all models share is a key role for nucleotide cycling at the receptors. However, two recent studies revealed that transgenes expressing atToc159 mutants with defects in GTP binding and/or hydrolysis can still mediate efficient complementation of the *ppi2* phenotype (Agne *et al.*, 2009; Wang *et al.*, 2008). This is surprising, since it indicates that GTPase cycling at atToc159 is not strictly required for efficient protein import. To assess the significance of GTP cycling (and dimerization) at the other main receptor in Arabidopsis, we generated a series of atToc33 G-domain mutants and tested them for activity, both *in vitro* and *in vivo*.

RESULTS

Generation of the atToc33 mutants

The atToc33 protein possesses many of the conserved motifs found in other GTP-binding proteins (Bourne *et al.*, 1991). Numerous studies on various GTPases have shown that the mutation of certain residues can interfere selectively with nucleotide binding or hydrolysis. We generated the following mutations within the *atTOC33* cDNA, with predicted effects on GTP binding or hydrolysis: K49R, S50N, S50N/S51N (binding); G45R, G45V, Q68A, N101A (hydrolysis). Several of these (S50N, G45V, G45R) are equivalent to standard G1-motif mutations that have been used to induce dominant-negative or constitutively-active effects in many GTPases (Li *et al.*, 2001); the S50N/S51N double mutant was used to eliminate the possible contribution of an adjacent serine at position 51. Mutations equivalent to K49R, S50N and G45R have been studied in the context of atToc159, and were shown to interfere with functionality as expected (Lee *et al.*, 2003; Smith *et al.*, 2002). The Q68A and N101A mutations were designed based on pea Toc34 (psToc34) structural data, which suggested that these residues may act in GTPase catalysis (Sun *et al.*, 2002). Additionally, we generated a G-domain triple mutant (G45R/K49N/S50R), which is equivalent to previously-studied mutants in psToc34, atToc159 and atToc33 (Bauer *et al.*, 2002; Chen and Schnell, 1997; Wallas *et al.*, 2003). In atToc159, this triple mutation essentially abolished nucleotide binding and hydrolysis (Bauer *et al.*, 2002), and so it is thought to severely disrupt the G-domain. Finally, we generated three mutations with predicted effects on dimerization or dimerization-mediated stimulation of GTPase activity (R125A, R130A, R130K) (Reddick *et al.*, 2007; Sun *et al.*, 2001). Arginine 130 was suggested to act as an arginine finger, such that each subunit within an atToc33 dimer functions as a GAP for the opposing monomer (Yeh *et al.*, 2007); an alternative possibility is that this residue simply mediates dimerization (Weibel *et al.*, 2003; Lee *et al.*, 2009). All of these mutations and their predicted effects are summarized in Figure 1.

In vitro *analyses of GTP binding and hydrolysis by the atToc33 mutants*

A subset of the mutations were selected for *in vitro* analysis (K49R, G45R, S50N, S50N/S51N, Q68A, N101A, G45R/K49N/S50R, R125A, R130K), to confirm that they exert the expected effects on GTP binding and hydrolysis. Thus, the mutations were introduced into a truncated cDNA lacking the transmembrane region, enabling the preparation of soluble proteins for analysis. The proteins were expressed in bacteria and were purified using affinity chromatography (Figure 2). Two of the mutants, S50N and S50N/S51N, were almost completely insoluble and could not be purified to homogeneity, despite repeated attempts (data not shown); this suggested that impairment of magnesium cofactor binding within the nucleotide binding site (which is the predicted effect of these mutations;

Farnsworth *et al.*, 1991) had a severely destabilizing effect on the whole G-domain. The G45R/K49N/S50R triple mutant, and to a lesser extent R125A and K49R, were similarly problematic, although preparation of reasonably pure samples was eventually achieved in these cases (Figure 2). Two independent G45R/K49N/S50R samples are shown to give an indication of the persistent contaminating proteins in the ~50-75 kDa range.

Nucleotide binding was assessed using two different procedures: a filter-binding assay using immobilized protein and radiolabelled nucleotide ([α-^{32}P]GTP) (Figure 3a); and, a liquid-phase assay using fluorophore-modified nucleotides (*mant*-GTP, *mant*-GDP, *mant*-ATP) that respond with different intensities and wavelengths of fluorescence upon protein binding (Figure 3b). The results indicated that most of the mutations were behaving as expected. Essentially normal nucleotide binding was detected for those mutants predicted to be specifically hydrolysis-defective (G45R, Q68A, N101A); consistent with previous work (Aronsson *et al.*, 2003), all binding-competent proteins bound GTP and GDP but not ATP (Figure 3b). By contrast, strongly reduced nucleotide binding was seen for those mutants predicted to be binding-defective (K49R, G45R/K49N/S50R). The results for R125A were somewhat unexpected, as this mutant was reported to disrupt dimerization and, thus, hydrolysis (Sun *et al.*, 2002). However, our data show clearly that this mutant is also binding-defective, indicating that the mutation has more wide-ranging consequences than previously predicted.

Next, GTP hydrolysis assays were conducted using those mutants predicted to have hydrolytic defects (G45R, Q68A, N101A). Assays were conducted in liquid-phase using [α-^{32}P]GTP, and were analysed by thin-layer chromatography (TLC). Normalized activity detected using wild-type protein was ~0.2-0.5 nmol GTP/min/µmol atToc33, which is broadly consistent with what has been reported previously at similar nucleotide concentrations (Reddick *et al.*, 2007). Surprisingly, none of the putative hydrolytic mutants showed significantly reduced activity (Figure 4); an atToc159 mutant (K868R; equivalent to atToc33 K49R) that was previously shown to be both binding- and hydrolysis-defective *in vitro* was used as a negative control in these assays, and it behaved as expected (Agne *et al.*, 2009; Smith *et al.*, 2002). The Q68A and N101A results can be explained by structural data that were published recently (Koenig *et al.*, 2008), after we initiated this study based on the earlier proposal that the Gln 71 and Asn 104 of psToc34 are candidate catalytic residues (Sun *et al.*, 2002); these are equivalent to Gln 68 and Asn 101 of atToc33, respectively. The results of Koenig *et al.* (2008) suggest that neither residue is likely to perform a catalytic role, since their side-chains are oriented away from the γ-phosphate of the nucleotide; instead, it was suggested that the main-chain carbonyl group of Gly 74 (in psToc34) is responsible for the protein's intrinsic hydrolytic activity, by positioning a water molecule for nucleophilic attack on the γ-phosphate of bound GTP. In relation to G45R, the results are more difficult to explain, since Gly 45 is a core residue of the highly-conserved, G1 P-loop motif, and its mutation is known to affect hydrolytic activity in the context of other GTPases, including atToc159 (Smith *et al.*, 2002; Wang *et al.*, 2008). To help understand the hydrolysis results, we utilized the recent structural data (Koenig *et al.*, 2008) to model the equivalent

Gly-to-Arg mutation of psToc34 (G46R) (Supplementary Figure S1). Despite extensive primary sequence conservation in the P-loop region, we found that the Arg 46 side-group points away from the γ-phosphate of the nucleotide, such that it cannot interfere with any of the proposed catalytic residues (as equivalent mutations do in other GTPases, such as H-Ras; Supplementary Figure S1). Significant structural differences between Toc34 and Toc159 led to the suggestion that the two receptor GTPases employ distinct hydrolytic mechanisms (Koenig *et al.*, 2008), which may account for the differences between our results (Figure 4) and those reported for atToc159 (Smith *et al.*, 2002; Wang *et al.*, 2008).

Analyses of dimerization of the atToc33 mutants

Some of the mutations were predicted to exert a primary effect on receptor dimerization, or on dimerization mediated GAP functionality. To assess this possibility, we analysed homodimerization by conducting pull-down experiments using recombinant atToc33 G-domain as bait and *in vitro* translated, [^{35}S]methionine-labelled atToc33 as prey (Weibel *et al.*, 2003). A principal aim here was to quantitatively compare the effects of the R130A and R130K mutations. In accordance with earlier results (Weibel *et al.*, 2003), R130A had a strong effect on homodimerization: the amount of bound prey was reduced to <10% of that observed using wild-type prey (Figure 5a,b). The R130K mutation also reduced dimerization efficiency, but not to the same extent as R130A: prey binding was ~30% of the wild-type level (Figure 5a,b). The reduced severity of R130K presumably reflects the fact that it is a more conservative mutation than R130A. These results are more consistent with a physical role for arginine 130 at the dimer interface, than with one as a GAP arginine finger. The R125A mutation was not included in these assays since an equivalent mutation was previously shown to abolish homodimerization in the context of psToc34 (Sun *et al.*, 2001).

Next, we investigated the consequences of selected mutations in relation to atToc33 heterodimerization with atToc159. For these experiments, we used a yeast two-hybrid assay which allows for the assessment of protein-protein interactions in intact cells. Because such assays are relatively straightforward, we were additionally able to test some of the other mutants that have primary effects on GTP binding/hydrolysis. As shown in Figure 5c, both R125A and R130A strongly interfered with G-domain heterodimerization, as expected. By contrast, the binding/hydrolysis mutations (K49R, G45R, Q68A) did not interfere with heterodimerization. Interestingly, the G45R/K49N/S50R triple mutant was found to be heterodimerization-defective, which is consistent with the notion that this mutation causes severe disruption of the G-domain. Similarly, the S50N mutant did not heterodimerize, supporting the hypothesis (discussed earlier) that impairment of magnesium binding in this mutant severely destabilizes of the whole G-domain. In no instance could our failure to detect heterodimerization be attributed to a lack of hybrid protein expression (Figure 5d).

Overall, data in Figure 5 provide clear evidence that R125A, R130A and R130K do interfere

with receptor dimerization, as expected. Considering the negative heterodimerization results for G45R/K49N/S50R and S50N (Figure 5c,d), and the failure of R125A to exhibit detectable nucleotide binding (Figure 3), it is conceivable that the dimerization defect of R125A is also due to a general disruption of the G-domain.

Generation of ppi1 *transgenic lines expressing various atToc33 mutants*

The *in vitro* and yeast studies above led us to conclude that most of the mutants were behaving as expected (the putative hydrolytic mutants being one notable exception; Figure 4). Thus, we proceeded to assess the behaviour of selected mutations *in vivo*. Mutations were introduced into the full-length *atTOC33* cDNA in an expression construct, and then transformed into homozygous *ppi1* plants using standard procedures. A large number of T_1 transformants were identified for each mutant (as well as for an equivalent wild-type *atTOC33* construct, termed WT), and these were allowed to grow to maturity and produce seed.

Segregation analysis was used to select homozygous, single-locus insertion lines for analysis, and led to the surprising conclusion that all of the mutants are able to complement *ppi1* to some degree (Supplementary Results; Supplementary Table S1). To confirm that the transgenic lines were of the expected genotypes, we amplified the transgenic copy of *atTOC33* in each case, and sequenced the resultant PCR products: all transformants were found to carry the appropriate G-domain mutation (data not shown). During the identification of transgenic lines, we observed considerable variability between different transformants. This variability was not linked to effects of the mutations, and was instead attributed to transgene silencing or genomic position effects, necessitating careful selection of stable lines for analysis (see Supplementary Results).

Basic characterization of the atToc33 transgenic lines

Seedlings of the selected transgenic lines were indistinguishable from untransformed Columbia-0 wild type (Figure 6a). Moreover, chlorophyll accumulation was normal in the transgenic lines (Figure 6b), and this phenotypic normality extended throughout development (Supplementary Figure S2; data not shown). Photosynthetic efficiency in the transgenic lines was also indistinguishable from that in wild type (Supplementary Figure S3; data not shown). Slight variations between the transgenic lines and wild type (e.g., Figure 6b) were not due to effects of the mutations on atToc33 functionality, since even within the carefully-selected, stable lines (and even for the WT construct) we occasionally observed individual plants that were not fully complemented (Supplementary Figure S4). This variation between individuals of the same transgenic line is most likely due to the same transgene

silencing effects mentioned earlier in relation to the much greater variation that exists between different transgenic lines.

Immunoblot analyses revealed that each one of the complemented lines exhibits restored accumulation of atToc33 protein, at levels broadly consistent with those in wild type (Figure 7). Equivalent Coomassie-stained gels served as loading controls, and additionally revealed substantially recovered levels of the three main photosynthetic proteins in the complemented plants (Figure 7). The different *atTOC33* constructs did not significantly affect the expression of the native *atTOC34* gene (a functionally similar homologue that is expressed at ~20% of the level of *atTOC33* in green tissues; Jarvis *et al.*, 1998), supporting the notion that the transgenes are directly responsible for the complementation observed (Supplementary Figure S5).

Organellar and molecular level characterization of the atToc33 transgenic lines

That the mutants were able to mediate efficient *ppi1* complementation was surprising, especially in relation to the G45R/K49N/S50R triple mutant, which is predicted to have a strongly disrupted G-domain. We therefore conducted experiments to test for consequences of the mutations at the organellar or molecular levels. First, we analysed chloroplast ultrastructure in two independent G45R/K49N/S50R transgenic lines. However, there were no obvious differences between the organelles in these lines and those in untransformed wild-type plants: all three genotypes had large chloroplasts with more developed thylakoidal networks than those in *ppi1*(Figure 8).

Recent reports indicated that transgenes encoding mutant forms of atToc159 can efficiently complement the *ppi2* albino phenotype, but that the mutations nonetheless have consequences in relation preprotein import efficiency (Agne *et al.*, 2009; Wang *et al.*, 2008). To assess whether atToc33 mutations are similarly associated with altered import efficiency, we conducted import assays with chloroplasts isolated from selected transgenic lines. As expected (based on phenotypic data in Figures 6 and 8), we observed significantly improved import efficiency in all transgenic lines tested, relative to *ppi1* (Figure 9). Import into chloroplasts from *ppi1* plants carrying the WT construct was almost as efficient as that into chloroplasts from untransformed Columbia-0 wild type. By contrast, import into chloroplasts carrying the G45R/K49N/S50R and R130A mutants was of intermediate efficiency. Whether these results are a direct consequence of the atToc33 mutations, or due to the transgene silencing effects discussed earlier, is difficult to determine. However, because the plants used for the import experiments were indistinguishable from Columbia-0 wild type, the former explanation seems most likely.

Overall, these results indicate that GTP binding and dimerization at atToc33 are not strictly required for the receptor to fulfil its role in the import mechanism.

Overexpression of atToc33 and atToc34 in Toc159-deficient mutants

The ability of the atToc33 mutants to complement *ppi1* suggested that another TOC GTPase might be able to compensate for the defective atToc33 G-domain. The other main receptor of the TOC complex is Toc159, which has three principal isoforms in Arabidopsis: atToc159 acts in the import of photosynthetic preproteins, whereas the closely-related atToc132 and atToc120 isoforms act redundantly in the import of housekeeping preproteins. To test for possible redundancy between atToc33 and either atToc159 or atToc132/120, we overexpressed atToc33 in *ppi2* and *toc132 toc120* double-mutant plants. Additionally, we conducted similar overexpression studies using the atToc33 homologue, atToc34.

Two independent transformants exhibiting high levels of overexpression for each receptor/mutant combination were selected, and analysed carefully. However, overexpression of neither *atTOC33* nor *atTOC34* could mediate significant complementation of *ppi2* or *toc132 toc120*: transgenic plants accumulated chlorophyll to the same extent as untransformed mutants (Figure 10), and were visibly indistinguishable from the controls (data not shown). This indicates that the two main TOC receptor families in Arabidopsis (atToc33/34 and atToc159/132/120) do not share significant functional redundancy in the conventional sense. One possible explanation of the atToc33 mutant complementation results (Figures 6-9) is that the presence of both receptor types is required to maintain structural integrity of the complex, but that only one of the two receptors need have normal GTPase functionality in order for import to proceed. In other words, although the receptors as a whole do not share redundancy, it is possible that their G-domains do.

DISCUSSION

Our finding that none of a diversity of point mutations within the atToc33 G-domain interferes significantly with the protein's ability to complement *ppi1* was a great surprise. Early stages of the preprotein import mechanism require GTP (Kessler *et al.*, 1994; Ma *et al.*, 1996; Olsen and Keegstra, 1992; Young *et al.*, 1999), and this requirement is attributed to the receptors, Toc34 and Toc159, since both proteins possess sequence motifs characteristic of GTPases, and both can bind and hydrolyse GTP (Becker *et al.*, 2004; Kessler *et al.*, 1994; Reddick *et al.*, 2007; Seedorf *et al.*, 1995). These observations led to the formulation of models for preprotein recognition and early-stage translocation, in which the two receptors act co-ordinately as molecular switches and/or translocation motors (Kessler and Schnell, 2004; Li *et al.*, 2007). A key component of these models is a GTPase cycle at each receptor. The fact that it is possible to disrupt this cycle at the principal Toc34 isoform in Arabidopsis, atToc33, and yet retain *in vivo* functionality, is inconsistent with all models thus far proposed.

Similar results were reported for the Toc159 receptor by Wang *et al.* (2008) and Agne *et al.* (2009). The former study revealed that an A864R mutation within the G-domain of the principal Arabidopsis Toc159 isoform, atToc159, does not impair the protein's ability to complement the albino atToc159 knockout mutant, *ppi2*. The A864R mutant was reported to exhibit normal GTP binding, but strongly reduced hydrolytic activity (Smith *et al.*, 2002). The *in vivo* functionality of this mutant protein led the authors to conclude that atToc159 plays a switching role (Wang *et al.*, 2008), since their results were inconsistent with the alternative proposal that it functions as a GTP-powered translocation motor (Schleiff *et al.*, 2003). Interestingly, although A864R-complemented *ppi2* plants were visibly indistinguishable from wild-type plants, preprotein binding and translocation efficiencies were slightly elevated in chloroplasts carrying the mutant protein. The equivalent atToc33 mutant in our study was G45R. Consistent with the earlier report, we found that atToc33-G45R can complement the *ppi1* visible phenotype, as can the similar mutant, G45V (Figure 6). However, we observed no effect of G45R on hydrolytic activity *in vitro* (Figure 4), most likely due to structural differences between atToc33 and other GTPases (Supplementary Figure S1; Koenig *et al.*, 2008), and so did not select G45R for detailed *in vivo* analysis. Thus, we are unable to comment on whether it has a stimulatory effect on import similar to atToc159-A864R.

Agne *et al.* (2009) reported that an atToc159 K868R mutant is also able to complement the *ppi2* visible phenotype. In contrast with A864R, this mutation interferes strongly with GTP binding (Smith *et al.*, 2002). Whereas *ppi2* chloroplasts harbouring the A864R mutant exhibited elevated levels of preprotein binding and import, those carrying K868R displayed reduced binding and import, relative to wild-type organelles. This further supported the notion that GTP-binding/hydrolysis cycles at atToc159 are not strictly linked with individual preprotein translocation events, and that Toc159 functions as a molecular switch; in fact, the data suggest that the GTP-bound form promotes import,

while the GDP-bound form inhibits import. The atToc33 mutation equivalent to K868R in our study was K49R. This mutant was not selected for *ppi1* complementation analysis, as we felt that it would likely behave in the same way as G45R/K49N/S50R, which also carries a mutation at Lys 49. Accordingly, while G45R/K49N/S50R complemented the *ppi1* visible phenotype efficiently (Figure 6), we observed reduced import efficiency in *ppi1* chloroplasts carrying the G45R/K49N/S50R protein (Figure 9).

Overall, our data indicate that GTPase cycling at atToc33 is not strictly linked to individual preprotein translocation events. In this sense, the results are similar to those reported for atToc159 (Agne *et al.*, 2009; Wang *et al.*, 2008), and suggest that atToc33 also plays a switching role in the import mechanism. Moreover, the reduced import efficiency seen in lines carrying the G45R/K49N/S50R mutant implies that GTP-binding similarly converts atToc33 into a form that promotes import. However, as with atToc159, the conversion of this putative atToc33 switch into the "active" form is not a strict requirement for import; it is possible that nucleotide changes at the receptor cause affinity or structural changes that promote import progression, but that import can nonetheless proceed in the absence of GTPase cycling, albeit with reduced efficiency. Similarly, receptor dimerization is also not an absolute prerequisite for translocation, as several mutants with directly or indirectly impaired dimerization (Figure 5) were able to efficiently complement *ppi1* (Figure 6). Nonetheless, an impairment in preprotein import was observed in *ppi1* chloroplasts carrying the R130A mutant (Figure 9), suggesting that dimerization acts to optimize the efficiency of transport. In this regard, our results are entirely consistent with those of Lee *et al.* (2009), who reported that *ppi1* plants complemented with dimerization-defective atToc33 mutants (R130A and F67A) exhibit reduced chloroplast protein import efficiency. It was suggested that receptor dimerization helps to promote the transition of the preprotein from initial binding at the receptors to the TOC channel (Lee *et al.*, 2009).

The atToc159 receptor is different from atToc33 in that it possesses two additional domains: an N-terminal acidic domain and a large C-terminal membrane domain (M-domain) (Chen *et al.*, 2000). It was previously demonstrated that the M-domain, in isolation, is able to mediate partial complementation of *ppi2* (Lee *et al.*, 2003). Together with other results from transient assays using atToc159 G-domain mutants (which were found to be non-functional), this result led Lee *et al.* (2003) to propose that the M-domain is the minimal structure required to support preprotein import, and that the G-domain plays a regulatory role. This hypothesis is consistent with the aforementioned "switching" hypothesis (Agne *et al.*, 2009; Wang *et al.*, 2008), although it remains to be determined why there is a need for two GTPase switches. A lack of redundancy between the two receptor families (Figure 10) suggests that they do perform distinct roles. The greater structural complexity of atToc159, which distinguishes it from atToc33, may account for the negative *ppi2* complementation results reported for the atToc159 A864R/K868N/S869R mutant (Bauer *et al.*, 2002), which is equivalent to the atToc33-G45R/K49N/S50R mutant reported here.

Finally, the data have significance in relation to earlier *in vivo* studies on atToc33 phosphorylation (Aronsson *et al.*, 2006). Phosphorylation of atToc33 was reported to impair GTP binding, and this was proposed to have a knock-on effect on preprotein binding (Jelic *et al.*, 2003; Sveshnikova *et al.*, 2000). We reported that various mutations at the phosphorylation site (Ser 181) do not interfere with *in vivo* activity of the protein, and concluded that phosphorylation is not an essential or stringent regulator of import. While this conclusion remains valid, it should be considered in the context of the present results (indicating that nucleotide binding is also not essential for *in vivo* activity). Thus, Ser 181 mutations may well interfere with GTP binding by atToc33 *in planta*, but, as the present results suggest, this may be of limited consequence in relation to organelle or plant development.

EXPERIMENTAL PROCEDURES

Mutations and vector construction

For expression in *E. coli* of atToc33 G-domain (for use in Figures 2-4), a truncated cDNA encoding residues 1-258 of the wild type was PCR amplified and cloned as an NcoI/SalI fragment into NcoI/XhoI-cut pET23d (Novagen), which adds a C-terminal hexahistidine tag. A series of similar constructs, containing mutations in the *atTOC33* cDNA, were created by overlap extension PCR using mutagenic primers; see Supplementary Methods for further details, and for information on the atToc159 constructs employed in Figure 4b.

For *ppi1* complementation (Figures 6-9), full-length wild-type *atTOC33* cDNA was PCR amplified and cloned as an NcoI/XbaI fragment into a derivative of pRT2LGUS containing a CaMV 35S promoter cassette (Bate *et al.*, 1996). Mutant *atTOC33* fragments (generated as above) were sub-cloned as NcoI/BstXI fragments into the resulting 35S-*atTOC33* construct, displacing the wild-type sequence; each mutation was confirmed by sequencing. The 35S-*atTOC33* cassettes were inserted into binary vector, pPZP221, as HindIII fragments for plant transformation (Hajdukiewicz *et al.*, 1994).

Recombinant protein expression and in vitro *analysis*

Soluble atToc33 and atToc159 G-domains (residues 1-258 and 727-1093, respectively) were overexpressed in *Escherichia coli* BL21-DE3 (Novagen) and purified using affinity resin as described (Aronsson *et al.*, 2003; Aronsson *et al.*, 2006; Rahim *et al.*, 2009; Smith *et al.*, 2002); see Supplementary Methods. Filter-binding assays (Figure 3a) were conducted as reported by Weibel *et al.* (2003). The liquid-phase binding assay (Figure 3b) was described previously (Aronsson *et al.*, 2003), and utilized nucleotide analogues carrying the *N*-methyl-3'-*O*-anthraniloyl (*mant*) fluorophore (John *et al.*, 1990). The GTP hydrolysis assays (Figure 4) were conducted as reported by Aronsson *et al.* (2003). Minor modifications to these procedures are described in the Supplementary Methods.

Receptor G-domain interactions in vitro *and in yeast*

In vitro homodimerization experiments (Figure 5a,b) were conducted as described (Weibel *et al.*, 2003). Hexahistidine-tagged atToc33 recombinant protein (residues 1-265), and the 265-residue wild-type and R130A *in vitro* translation products, were generated according to Weibel *et al.* (2003). The R130K mutant was generated in the context of a slightly shorter protein (residues 1-258), as was the corresponding wild-type control, as described in "Mutations and vector construction".

Heterodimerization experiments (Figure 5c,d) were conducted using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech Laboratories, Palo Alto, USA), as described previously (Rahim *et al.*, 2009); see Supplementary Methods.

Plant growth, transformation, and analysis of transgenics

All *Arabidopsis thaliana* plants were Columbia-0 ecotype. Growth conditions were as described by Aronsson and Jarvis (2002). The *ppi1* mutant, Columbia-0-introgressed *ppi2*, and the *toc132-2 toc120-2* double mutant have all been described (Jarvis *et al.*, 1998; Kubis *et al.*, 2004).

Plant transformation employed *Agrobacterium* GV3101 (pMP90) and the floral dipping method (Clough and Bent, 1998). Transgenic plants were selected and analysed using standard procedures (see Supplementary Methods).

Total-protein extracts were analysed by staining with Coomassie Brilliant Blue R250 (Fisher Scientific), or by immunoblotting (Aronsson *et al.*, 2003). Rabbit antibodies were against an atToc33-specific peptide (Aronsson *et al.*, 2003) or the atTic110 stromal domain (mature residues 93-966) (Inaba *et al.*, 2003).

Chlorophyll content and fluorescence measurements

Chlorophyll content was determined photometrically as described (Aronsson *et al.*, 2003; Porra *et al.*, 1989). Chlorophyll fluorescence measurements were made using a continuous excitation fluorimeter (Handy PEA; Hansatech, King's Lynn, UK), according to the manufacturer's instructions and as described (Strasser *et al.*, 2004).

Electron microscopy

Transmission electron microscopy was conducted as described previously (Aronsson *et al.*, 2007; Aronsson *et al.*, 2006) with minor modifications (see Supplementary Methods). Mid-lamina cross-sections of cotyledons from plants grown *in vitro* for ten days were analysed. These procedures were carried out at the Electron Microscopy Laboratory, University of Leicester.

Protein import assays

Isolation of chloroplasts from Arabidopsis seedlings, the synthesis by *in vitro* translation of [^{35}S]methionine-labelled preSSU (Arabidopsis Rubisco small subunit, ats1A), and protein import were all conducted as described (Aronsson *et al.*, 2006; Aronsson and Jarvis, 2002; Kubis *et al.*, 2008).

Overexpression of* atTOC33/34 *in the* ppi2 *and* toc132 toc120 *mutants

The *atTOC33* and *atTOC34* cDNAs were expressed from a double-enhancer version of the 35S promoter using the pCHF2 vector (Jarvis *et al.*, 1998). Constructs were used to transform *ppi2* heterozygotes or *toc132 toc120* heterozygotes (genotype: *toc132*/*toc132*; +/*toc120*). Transgene overexpression was estimated by semi-quantitative RT-PCR using published procedures (Constan *et al.*, 2004); see Supplementary Methods. Expression data for the *TOC* genes were normalized using equivalent data for *eIF4E1* (Rodriguez *et al.*, 1998).

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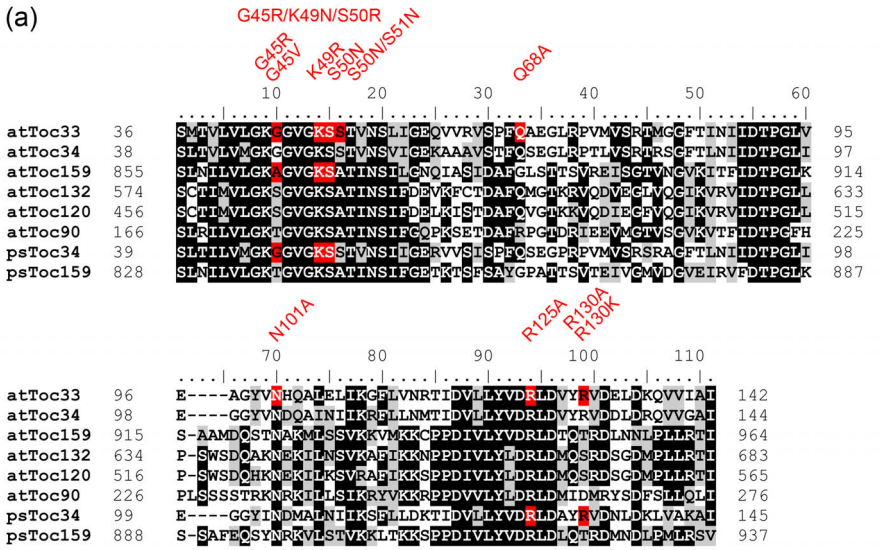
FIGURE 1



(b)

Mutation	Predicted or known effect	Reference(s)
K49R	GTP binding	Smith *et al.* , 2002; Agne *et al.* , 2009
S50N	GTP binding	Lee *et al.* , 2003
S50N/S51N	GTP binding	Lee *et al.* , 2003
G45R	GTP hydrolysis	Smith *et al.* , 2002; Wang *et al.* , 2008
G45V	GTP hydrolysis	-
Q68A	GTP hydrolysis	Sun *et al.* , 2002
N101A	GTP hydrolysis	Sun *et al.* , 2002
G45R/K49N/S50R	GTP binding & hydrolysis	Chen & Schnell, 1997; Bauer *et al.* , 2002
R125A	Dimerization	Sun *et al.* , 2002
R130A	Dimerization / GAP function	Weibel *et al.* , 2003; Yeh *et al.* , 2007
R130K	Dimerization / GAP function	-

Figure 1. Details of the atToc33 mutations used in this study.

(a) Alignment of the central region of the atToc33 G-domain (At1g02280) with the corresponding regions of the other five TOC receptors in *Arabidopsis* (atToc34, At5g05000; atToc159, At4g02510; atToc132, At2g16640; atToc120, At3g16620; atToc90, At5g20300), as well as those of the two pea receptors (psToc34, accession Q41009; psToc159, accession AAF75761). The alignment was generated using ClustalW within BioEdit (Hall, 1999; Thompson *et al.*, 1994). Numbers to the left and right of the alignment indicate positions within the corresponding full-length proteins. Residues identical in at least four sequences are shaded black, whereas similar residues are shaded grey. Residues in the atToc33 sequence that have been mutated in this study, or in previous studies, are shaded red; equivalent residues in the other GTPases that have also been subjected to mutational analysis are similarly shaded. Mutations used here are indicated above the alignment in red text.

(b) List of the mutations used in this study. The predicted or known effects of the mutations are indicated; some are known since the relevant mutations have already been studied in the context of atToc33. References are for relevant reports on mutational studies conducted in the context of atToc33 or one of the other GTPases.

FIGURE 2

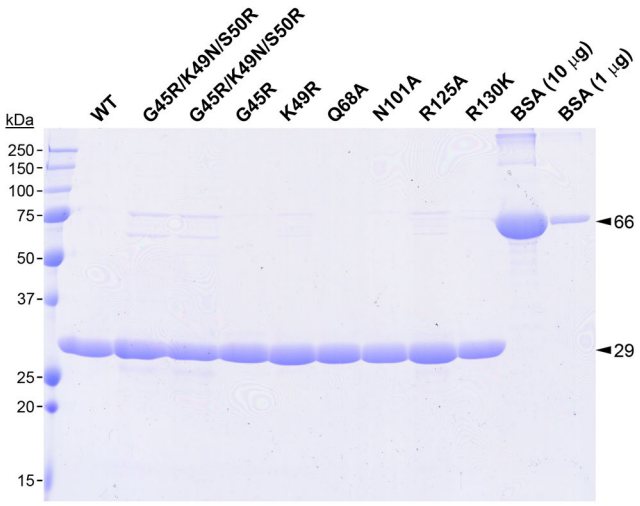
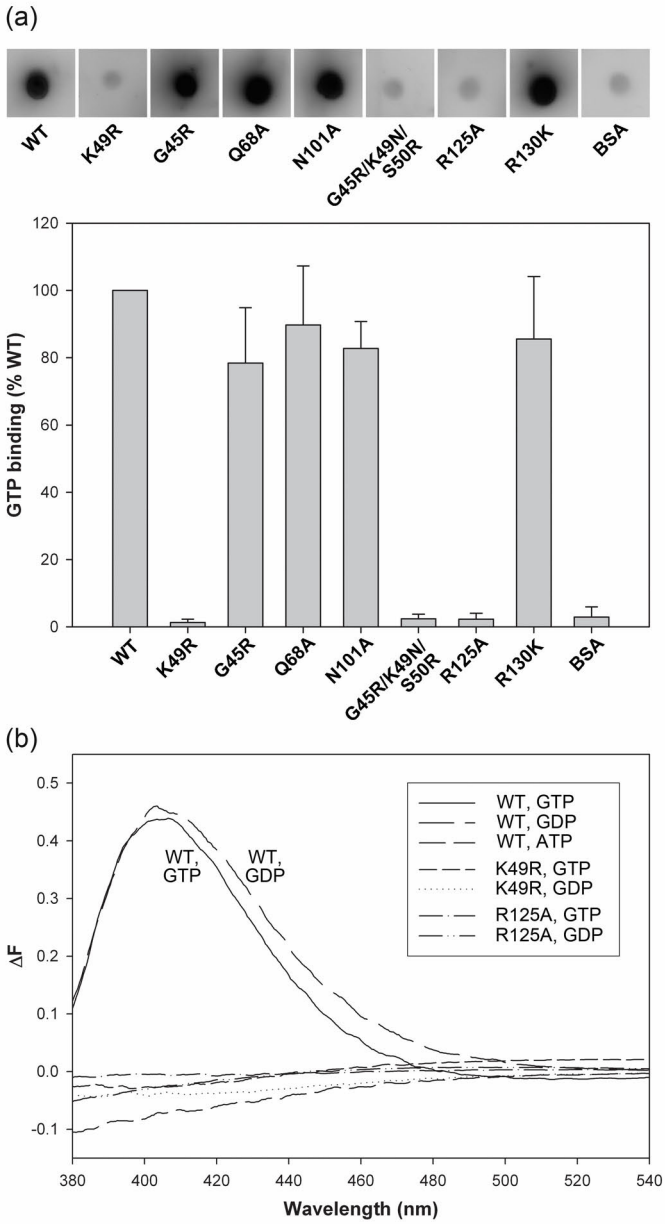


Figure 2. Recombinant atToc33 proteins for *in vitro* studies.
Recombinant wild-type (WT) and mutant (G45R/K49N/S50R, G45R, K49R, Q68A, N101A, R125A, R130K) atToc33 proteins lacking the C-terminal transmembrane domain were resolved by SDS-PAGE and stained with Coomassie Blue. Each lane contains ~5 μg of recombinant protein. To confirm sample concentrations, 10 μg and 1 μg aliquots of BSA were also loaded. Molecular weight standards are indicated in kDa.

FIGURE 3



Protein / Mutation	GTP binding (n = 3)	GDP binding (n = 3)	ATP binding (n = 3)
WT	+	+	–
K49R	–	–	–
G45R	+	+	–
Q68A	+	+	–
N101A	+	+	–
G45R/K49N/S50R	–	–	–
R125A	–	–	–
R130K	+	+	–

Figure 3. *In vitro* analyses of nucleotide binding to the atToc33 mutants.

(a) Filter-binding assay using radiolabelled GTP. Recombinant atToc33 proteins were incubated with [α -^{32}P]GTP on ice, and then transferred to PVDF membrane. Unbound material was removed by washing, and then bound nucleotide was visualized by autoradiography. Amounts of bound [α-

^{32}P]GTP were quantified using ImageQuant software (GE Healthcare). The upper part of the panel shows a representative binding experiment. The chart in the lower part of the panel presents means (±SE) derived from three, four or five independent experiments (each individual experiment comprised three separate measurements per protein sample). BSA was used as a negative control. (b) Liquid-phase binding assay using *mant* nucleotides. Excitation at 350 nm gives a fluorescence emission peak at ~450 nm with unbound *mant*-nucleotide, and a more intense peak nearer ~400 nm with bound nucleotide. Increases in *mant* fluorescence and a blue-shift in the emission maximum after addition of atToc33 protein are indicative of the degree of protein-nucleotide binding. Fluorescence spectra were recorded 0 min (T_0) and 7 min (T_7) after the initiation of each assay, normalized, and then used to derive fluorescence difference (ΔF) spectra by subtracting T_0 values from corresponding T_7 values. Representative spectra for the WT protein and for two of the non-binding mutants (K49R, R125A) are shown in the chart. A summary of the whole dataset is given below the chart in tabular form: “+” indicates a positive binding result; “–” indicates a negative result.

FIGURE 4

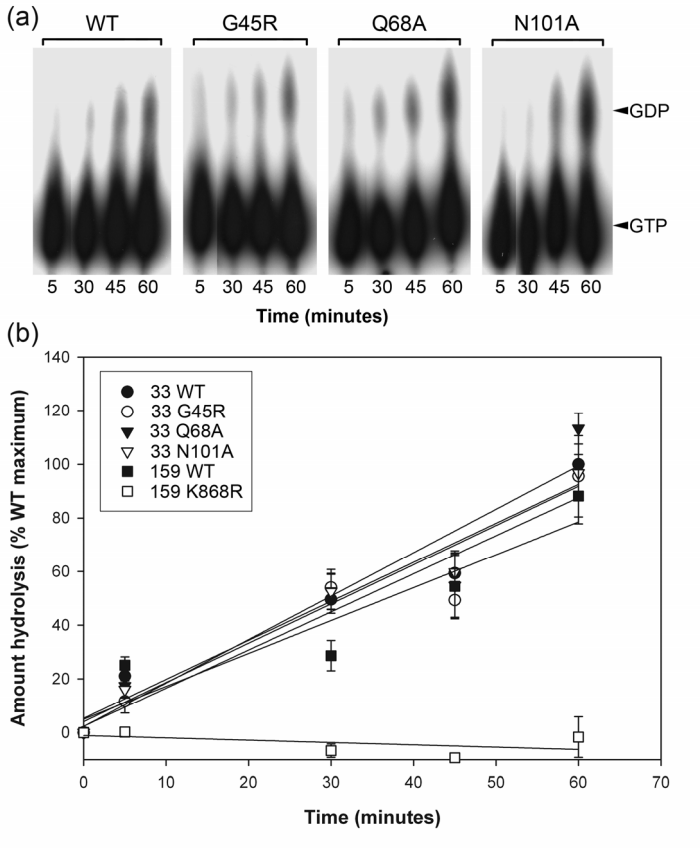


Figure 4. *In vitro* analyses of nucleotide hydrolysis by the atToc33 mutants.

(a) Recombinant atToc33 proteins were incubated with [α-^{32}P]GTP for 5, 30, 45 and 60 min. Samples were then separated by TLC and visualized by autoradiography. The lower band in each case represents non-hydrolysed GTP, and the upper band represents GDP.

(b) Radioactivity associated with the bands in panel (a), and with those from several similar experiments, was quantified and used to calculate the degree of GTP hydrolysis at each time-point. One of the non-binding atToc33 mutants, K49R, was analysed as a negative control; the K49R mutant was selected as it was more efficiently purified than any of the other binding-defective mutants (Figure 2). Trace levels of GTPase activity detected using the K49R preparation (not shown) were attributed to small amounts of contaminating protein, and so the data for this mutant were used to normalize those for the putative hydrolytic mutants. The atToc159 proteins were expressed and purified as GST fusions, and so the corresponding data were normalized using a control GST preparation. The data presented are means (±SE) derived from five or six independent experiments, and all are expressed as a percentage of the wild-type atToc33 60 min value.

FIGURE 5

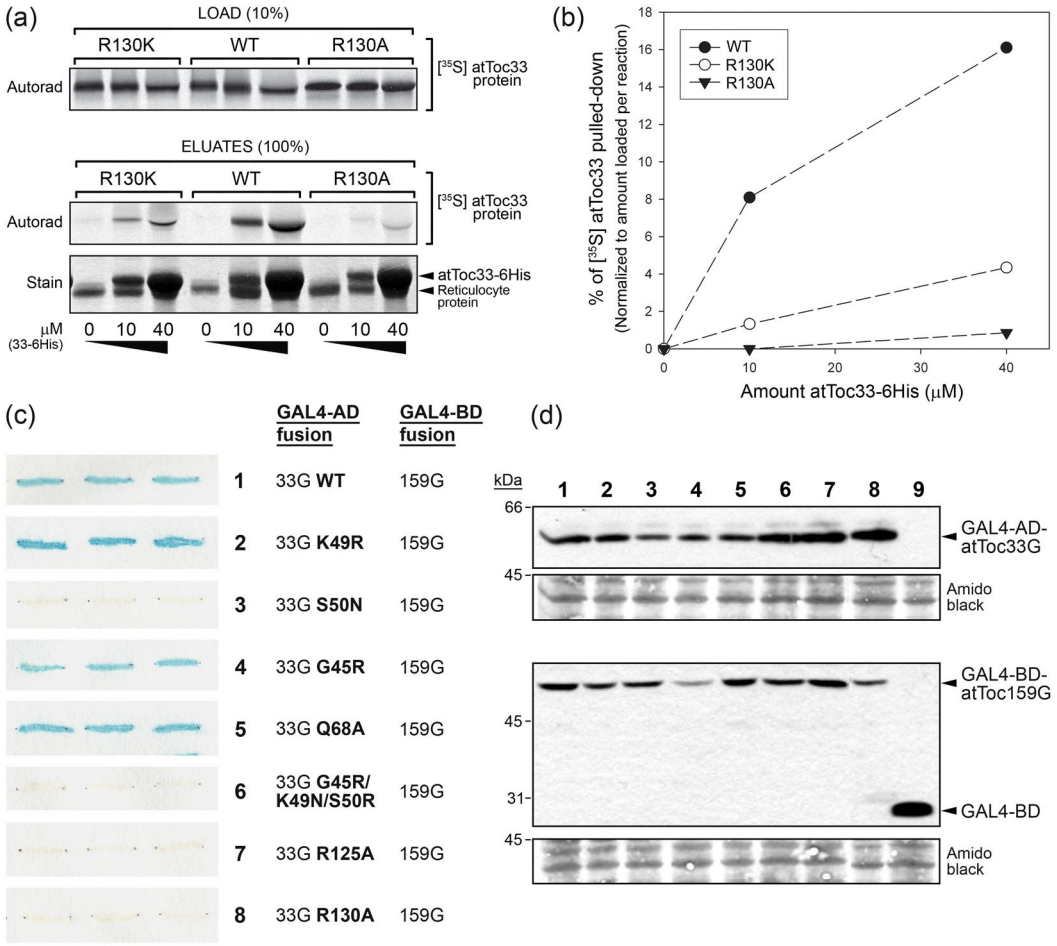


Figure 5. Homodimerization and heterodimerization of selected atToc33 mutants.

(a,b) Analysis of homodimerization *in vitro*. (a) Increasing concentrations of recombinant, hexahistidine-tagged wild-type atToc33 (atToc33-6His; 0, 10 and 40 µM) were incubated with *in vitro* translation reactions containing ^{35}S-labelled atToc33 G-domain; radiolabelled proteins were either wild-type or mutant (R130K, R130A). Recombinant protein together with any bound radioactive protein was captured using Ni-NTA chromatography, washed, and eluted with imidazole. Aliquots of the pre-pull-down binding mixtures (Load, 10%) and the post-pull-down eluates (100%) were analyzed by SDS-PAGE and/or Coomassie Blue staining followed by autoradiography. Because the R130K and R130A mutants were generated in the context of G-domains with slightly different truncation points (residues 1-258 and 1-265, respectively), two different, similarly-truncated wild-type G-domains were analysed as controls (only the latter is shown). (b) Amounts of bound radiolabelled protein were quantified and normalized relative to the amount of radiolabelled protein loaded in each reaction; binding in the absence of recombinant protein was adjusted to zero. Average wild-type data are shown.

(c,d) Analysis of heterodimerization in yeast by two-hybrid analysis. (c) Three independent yeast

colonies carrying the indicated Gal4 activation domain (AD) and DNA-binding domain (BD) constructs were subjected to X-Gal filter assays. Development of a strong blue colouration in cells from all three colonies indicated *LacZ* reporter gene activation and a positive interaction result. All AD constructs contained the atToc33 G-domain (residues 1-265), which was either wild type or mutant. All BD constructs contained the wild-type atToc159 G-domain (residues 728-1093). (d) Expression levels of the yeast two-hybrid fusion proteins. Trichloroacetic-acid-extracted yeast protein samples were analysed by immunoblotting using anti-atToc33 for the detection of the Gal4-AD-atToc33 fusion proteins (upper panel), and anti-myc for the detection of Gal4-BD-atToc159 fusion proteins (lower panel; the proteins contain a myc epitope tag between the Gal4-BD domain and the fused protein). Samples are identified by the numbering system shown in panel (c); sample 9 is from yeast cells carrying empty AD and BD vectors. Each lane contains protein derived from 0.05 OD (600 nm) units of yeast cells. Sample loading was controlled by staining with amidoblack, and a section of stained membrane is shown in each case.

FIGURE 6

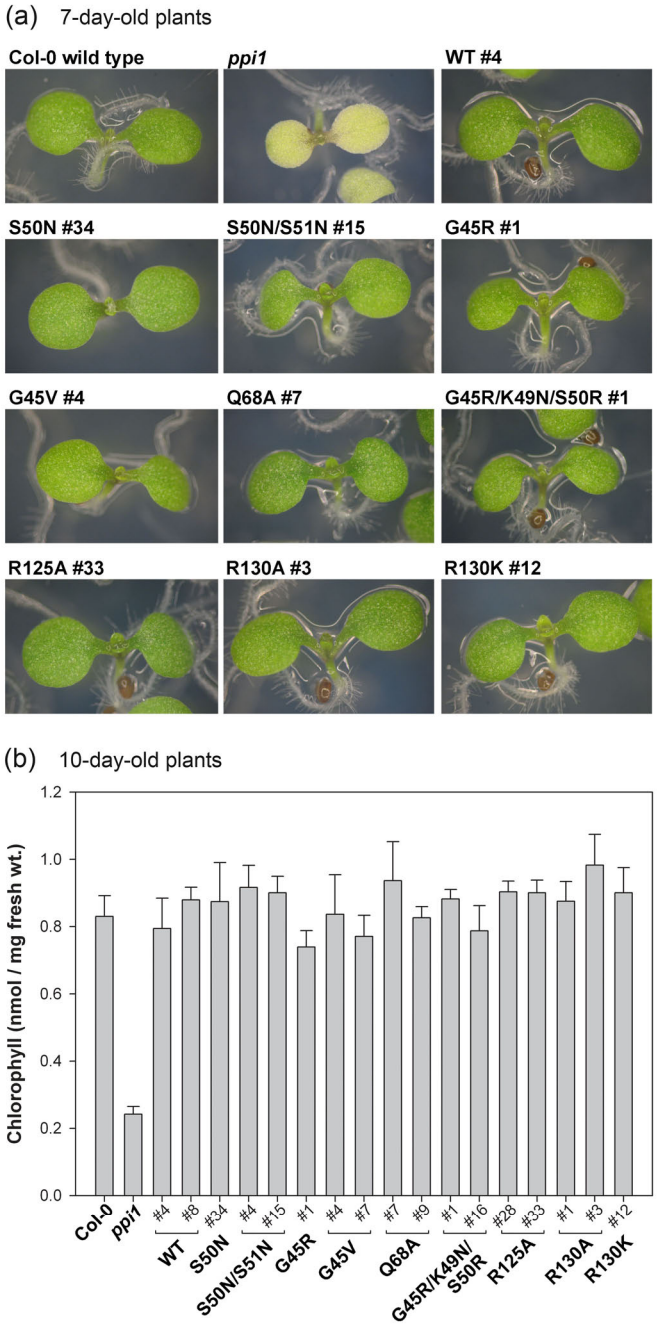


Figure 6. Basic characterization of transgenic *ppi1* plants expressing atToc33 mutant constructs.
(a) Plants of the indicated genotypes were grown side-by-side, *in vitro*, for seven days, and then photographed. Representative plants are shown in each case.
(b) Chlorophyll concentrations (nmol chlorophyll *a*+*b* per mg fresh weight) in ten-day-old, *in vitro*-grown T_4- or T_5-generation plants, and in Columbia-0 wild-type and *ppi1* controls, were determined photometrically. Values are means (±SD) derived from five independent samples, each one containing eight plants.

FIGURE 7

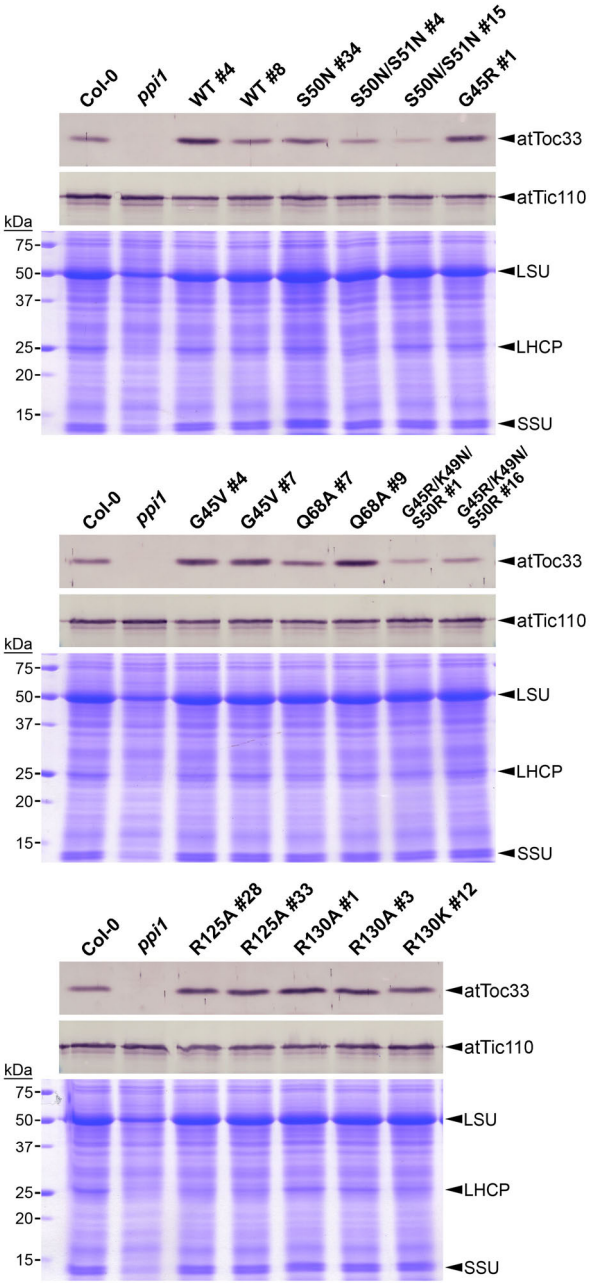


Figure 7. Immunoblots showing expression of atToc33 proteins in the transgenic *ppi1* plants. Total-protein samples (40 μg each) isolated from ten-day-old, *in vitro*-grown plants of the indicated genotypes were separated by SDS-PAGE and used to produce blots. Each blot was cut into two parts: one was probed with an atToc33-specific antibody; the other with an atTic110 antibody to control sample loading. Similarly loaded gels (20 μg per lane) were stained with Coomassie Blue to control loading and visualize the main photosynthetic proteins: LSU, Rubisco large subunit; LHCP, light harvesting chlorophyll protein; SSU, Rubisco small subunit. Molecular weight standards are indicated in kDa. Transgenic plants were homozygous and of the T_4- or T_5-generation.

FIGURE 8

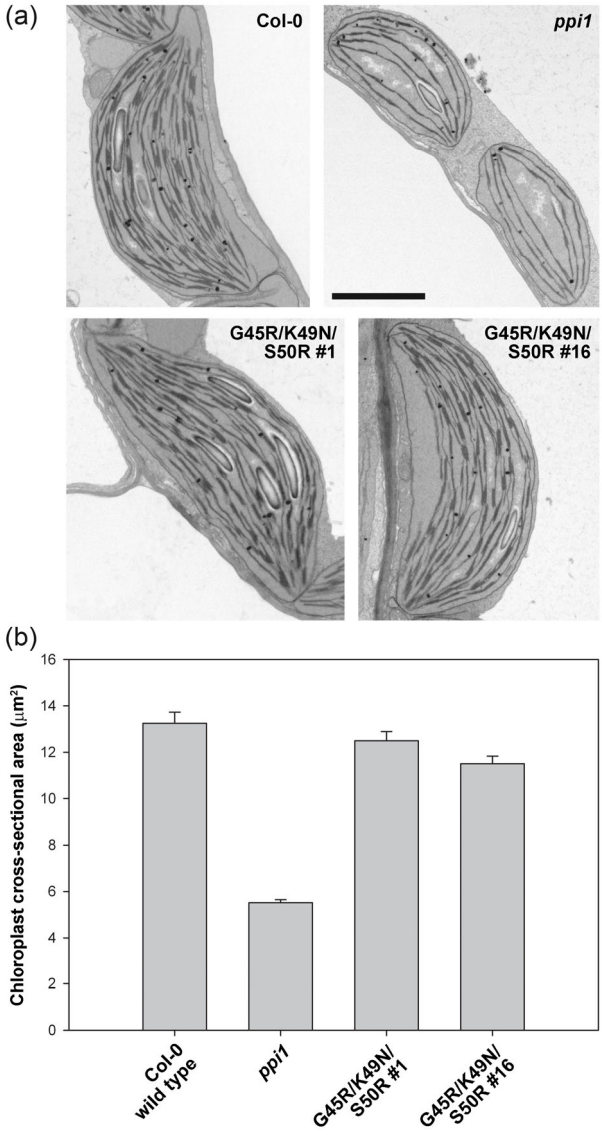


Figure 8. Ultrastructural analysis of chloroplasts in *ppi1* plants expressing the G45R/K49N/S50R construct.

(a) Mid-lamina cross sections of cotyledons from ten-day-old, *in vitro*-grown plants of the indicated genotypes were analysed by transmission electron microscopy. At least ten whole-chloroplast micrographs from each of three independent plants per genotype (i.e., a minimum of 30 chloroplasts per genotype) were analysed carefully, and used to select the representative images shown. Size bar indicates 2.0 μm.

(b) Length and width of each chloroplast in each of the micrographs described in (a) was measured. These values were used to estimate chloroplast cross-sectional area using the formula: $\pi \times 0.25 \times \text{length} \times \text{width}$. Values are means (±SE) derived from measurements of 37-68 different chloroplasts per genotype.

FIGURE 9

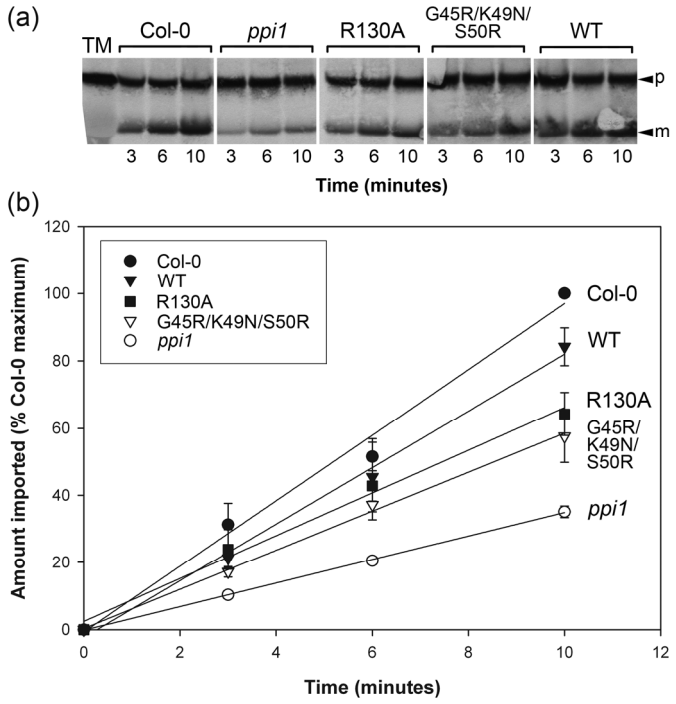


Figure 9. Analysis of the efficiency of preprotein import into chloroplasts isolated from *ppi1* plants expressing mutant atToc33 proteins.

(a) Chloroplasts were isolated from 14-day-old, *in vitro*-grown plants of the indicated genotypes, and used in protein import assays with [^{35}S]-methionine-labelled preSSU. Import was allowed to proceed for 3, 6 and 10 minutes, as indicated, and then samples were analysed by SDS-PAGE and fluorography. TM indicates an aliquot of the preSSU translation mixture equivalent to 10% of the amount added to each assay; p and m indicate the precursor and mature forms of the preprotein, respectively.

(b) Mature protein bands observed in (a) were quantified using ImageQuant software, and then the data were expressed as percentages of the value for the final, wild-type time-point. These data, together with those from up to three additional, similar experiments, were used to calculate the mean (±SE) values shown. Results shown for R130A and G45R/K49N/S50R are combined data derived from two independent transformants each.

FIGURE 10

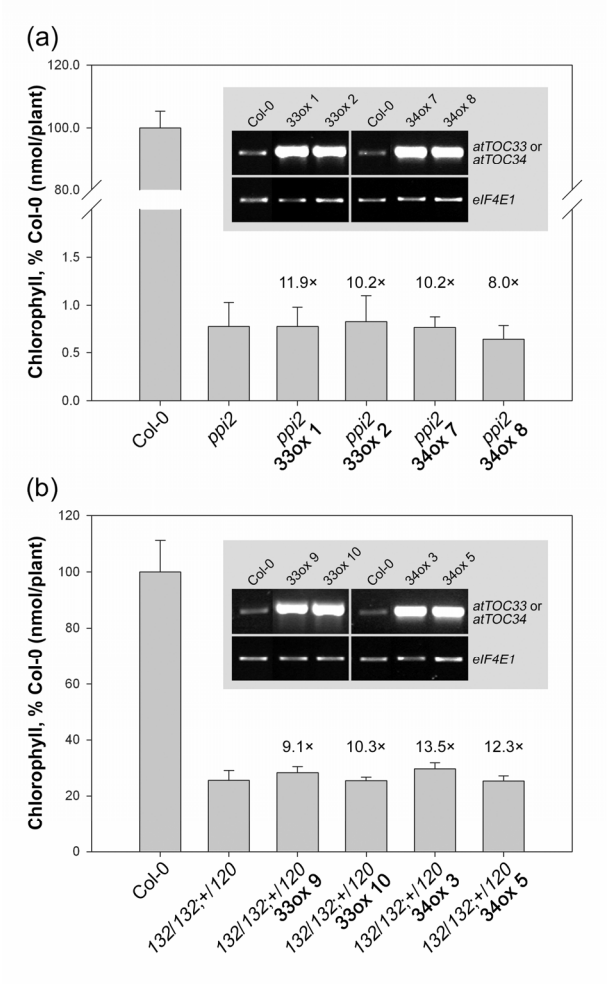


Figure 10. Overexpression of *atTOC33* and *atTOC34* in *ppi2* and *toc132 toc120* mutant plants. Plants carrying the *ppi2* (a) or *toc132 toc120* (b) mutations were stably transformed with different T-DNA constructs: 35S-*atTOC33* ("33ox"); 35S-*atTOC34* ("34ox"). Possible complementation was tested for in the T_4 generation by measuring chlorophyll in plants with appropriate mutant genotypes [i.e., *ppi2*/*ppi2* in (a), and *toc132*/*toc132*; +/*toc120* in (b)], and which were homozygous for the relevant transgene. Data for two independent transformants of each construct are shown, for each mutant background; these were representative of 4-6 independent transformants in each case. Chlorophyll values are means (±SD) derived from five independent measurements per genotype; values are expressed as a percentage of the wild-type chlorophyll concentration. The extent of transgene overexpression in each transformant was estimated by semi-quantitative RT-PCR analysis of T_2 plants that were either heterozygous or homozygous for the relevant transgene. Values for fold change in expression, relative to wild type, for each transgene/mutant combination are given textually above the corresponding chlorophyll data bars, and representative gel images are shown in the insets. Normalization of the *atTOC33*/*34* RT-PCR data was done relative to equivalent *eIF4E1* data.