

RESEARCH PAPER

# Phosphorylation regulates the assembly of chloroplast import machinery

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## Abstract

**Chloroplast function depends on the translocation of cytosolically synthesized precursor proteins into the organelle. The recognition and transfer of most precursor proteins across the outer membrane depend on a membrane inserted complex. Two receptor components of this complex, Toc34 and Toc159, are GTPases, which can be phosphorylated by kinases present in the hosting membrane. However, the physiological function of phosphorylation is not yet understood in detail. It is demonstrated that both receptors are phosphorylated within their G-domains. *In vitro*, the phosphorylation of Toc34 disrupts both homo- and heterodimerization of the G-domains as determined using a phosphomimicking mutant. In endogenous membranes this mutation or phosphorylation of the wild-type receptor disturbs the association of Toc34, but not of Toc159 with the translocation pore. Therefore, phosphorylation serves as an inhibitor for the association of Toc34 with other components of the complex and phosphorylation can now be discussed as a mechanism to exchange different isoforms of Toc34 within this ensemble.**

Key words: GTPase, membrane complex dynamics, phosphorylation, plastids, protein complex assembly, protein translocation, TOC.

## Introduction

Plastids are complex organelles undergoing transitions depending on the developmental or metabolic context (Lopez-Juez, 2007), which requires massive changes of

the organelle proteome. This process is regulated at multiple levels (Kessler and Schnell, 2006; Oreb *et al.*, 2008) including (i) transcription of nuclear genes encoding plastid destined proteins, (ii) targeting of precursor proteins to the chloroplast, (iii) their recognition and translocation across the chloroplast double membrane, and (iv) protein turnover within the organelle. The translocation process requires functional hetero-oligomeric protein complexes Toc (Translocon at the outer chloroplast envelope membrane) and Tic (Translocon at the inner chloroplast envelope membrane). The Toc core complex is formed by multiple copies of the translocation pore Toc75, the GTP-dependent receptor Toc34 and one tripartite receptor Toc159 (Schleiff *et al.*, 2003a; Kikuchi *et al.*, 2006). The latter protein contains a large membrane-anchored (M) domain, a cytosolic GTPase (G) domain highly homologous to that of Toc34, and an N-terminal acidic (A) domain of unknown function (Chen *et al.*, 2000). Toc34 is anchored in the membrane by a short tail at its carboxy-terminus (Chen and Schnell, 1997). In *Arabidopsis*, two paralogues of Toc34 exist, namely *atToc33* and *atToc34*, as well as four members of the Toc159 family, *atToc159*, *atToc132*, *atToc120*, and *atToc90* (Jackson-Constan and Keegstra, 2001; Oreb *et al.*, 2006). It is proposed that members of both families differentially associate to form translocons specialized for different classes of precursor proteins (Gutensohn *et al.*, 2006; Kessler and Schnell, 2006). According to the current model, *atToc33* preferably interacts with *atToc159* forming the translocon for photosynthetic proteins whereas *atToc34* forms a complex recognizing house-keeping proteins together with *atToc132* and/or *atToc120*. However, according to coimmunoprecipitation experiments

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Abbreviations: *at/psToc33/34*, the homologous GTPases *atToc33* from *Arabidopsis thaliana* and *psToc34* from *Pisum sativum* that have been studied. The first two italicized letters indicate source organism, followed by the GTPase name. Toc34 without denominator refers to both, *atToc33* and *psToc34*; OE(V), outer envelope (vesicle); ppi1, plastid protein import mutant 1; LHCP11, light harvesting complex protein 2.

(Ivanova *et al.*, 2004), other interaction patterns are also possible. The ability of *atToc33* and *atToc34* to overtake the function of each other (Jarvis *et al.*, 1998; Constan *et al.*, 2004) in knock-out mutants supports this possibility. It is not yet understood in detail what mechanisms drive the assembly of the Toc complex, but there is evidence that interactions of Toc159 and Toc34 G-domains are important for this process (Kessler and Schnell, 2006; Oreb *et al.*, 2008).

Phosphorylation of the G-domains is discussed as a second circuit for the regulation of complex assembly in this study. For both GTPases Toc159 and Toc34 phosphorylation was demonstrated *in vitro* (Sveshnikova *et al.*, 2000; Fulgosi and Soll, 2002). In the past, the properties of the phosphorylated Toc34 were explored in some detail (Jelic *et al.*, 2002). Phosphorylation leads to a negative regulation of precursor protein recognition (Sveshnikova *et al.*, 2000) and the GTPase cycle (Jelic *et al.*, 2002, 2003), albeit the phosphorylation sites are located in different areas of the protein from different sources (e.g. *atS181*, Jelic *et al.*, 2003; *psS113*, Jelic *et al.*, 2002). Although the phosphorylation sites of *atToc33* and *psToc34* have been mapped, the *in vivo* significance and the mechanistic effect are uncertain (Aronsson *et al.*, 2006; Oreb *et al.*, 2007). Here, the aim was to investigate the influence of the post translational modification of the GTPases on the complex formation in endogenous membranes. Evidence is provided here for a regulatory mechanism involving post-translational phosphorylation, which is suggested to change the state of association of the GTPases within the Toc complex.

## Materials and methods

### Protein production

Mutants of *atToc33*, *atToc33<sub>S/A</sub>*, *atToc33<sub>S/E</sub>* (aa 1–251), and *psToc159* (aa 658–1048) were generated by conventional PCR (Jelic *et al.*, 2003) and cloned into pET21d (Novagen, Madison, WI, USA) or pGEX6P-1 (GE Healthcare, Freiburg, Germany) to generate His-tagged or GST-fusion proteins. Recombinant proteins were purified by Ni-NTA (Qiagen, Hilden, Germany) or GSH affinity chromatography (GE Healthcare, Freiburg, Germany) according to protocols suggested by the manufacturer.

### Determination of the binding constant by size exclusion chromatography

For molecular weight determination, 200 µl of purified protein, typically at concentrations between 0.3 mM and 0.4 mM, was loaded onto a Superdex75 HR 10/300 gel filtration column (GE-Healthcare, Freiburg, Germany), equilibrated with 50 mM TRIS buffered at pH 8, containing 100 mM NaCl, 10 mM imidazole, 10 mM arginine, 5% glycerol, and 1 mM MgCl<sub>2</sub>. Using different starting concentrations of protein, monomeric and dimeric species were analysed by fitting two independent Gaussian distributions to the elution profile. The dissociation constant was determined according to:

$$[D] = \left( (K_D + 4 \times T) - \sqrt{(K_D + 4 \times T)^2 - 4 \times K_D \times T} \right) / 4 \quad (1)$$

where  $D$  is the dimeric fraction and  $T$  is the total amount of protein used.

### Solid phase binding assay

For all assays only freshly purified protein was used. For determination of nucleotide dependent association, the G-domain of *atToc33*-His and mutants was preloaded with nucleotides (König *et al.*, 2008) and spotted onto nitrocellulose membranes using a 96 well vacuum manifold (Bethesda Research Laboratories, Bethesda, MD, USA). Nucleotide loading was determined as described in König *et al.* (2008). Nitrocellulose membranes were saturated with 0.3% low fat milk powder with 0.03% BSA and subsequently incubated with purified GST-*atToc33* or GST-*psToc159* (35 µg ml<sup>-1</sup>) preloaded with indicated nucleotides in 20 mM Tricine-KOH at pH 7.6, containing 100 mM NaCl and 1 mM MgCl<sub>2</sub>. Background binding was controlled by incubation of GST-*atToc33* or GST-*psToc159* with saturated nitrocellulose membranes without spotted protein and spotted BSA. After two washes (10 min), the bound protein was determined by immunodetection with GST antibodies. Background staining caused by unspecific binding of GST antibodies was determined by immunodetection of membranes with spotted proteins but without an added interaction partner. Intensities were quantified with the AIDA Software (Raytest, Straubenhardt, Germany). The amount of bound protein was corrected for background binding and expressed as a comparison with the maximal binding of wild-type protein. The binding was analysed by:

$$[\text{Bound}]_{\text{norm.}} = -\sqrt{\frac{1}{4}(S + E + K)^2 - S \times E} + \frac{1}{2}(S + E + K) \quad (2)$$

where  $S$  is the concentration of the spotted protein,  $E$  the concentration of the added protein (normalized), and  $K$  reflects the dissociation constant in a non-normalized situation and an apparent dissociation constant after normalization, as described by König *et al.* (2008).

### In situ analysis of Toc complex assembly

The complementation of the *ppil* mutant with the phosphomimicking *atToc33* construct has been described previously (Aronsson *et al.*, 2006). Wild-type or mutant *A. thaliana* was grown for 2 weeks on soil in an E-36L growth chamber (Percival Sci., Perry, IA) at 16/8 h light/dark, 21/16 °C, 70% relative humidity. Approximately 5 g leaf material was ground for 2–3 s using a Polytron (Kinematica PT20) with a 13 mm rotor at 40% maximal speed in 25 ml of ice-cold homogenization buffer (0.3 M sorbitol, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 20 mM HEPES/KOH, pH 8.0, 10 mM NaHCO<sub>3</sub>, 50 mM ascorbic acid). The homogenate was filtered through Miracloth and the debris was subjected to homogenization. The procedure was iterated four times before the filtrates were combined and centrifuged at 1000 g for 5 min. Chloroplasts were resuspended in 500 µl homogenization buffer. For coimmunoprecipitation experiments, chloroplasts corresponding to 300 µg protein were used. Outer envelope vesicles (OEVs) (50 µg total protein), isolated as described by Schleiff *et al.* (2003b), were incubated with 50 µCi γ-<sup>32</sup>P-ATP/10 µM ATP-mixture in 300 µl 20 mM Tricine-KOH, pH 7.6, 1 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub> for 15 min at RT, and subsequently centrifuged at 100 000 g for 10 min at 4 °C. The pellet was solubilized with 1.5% *n*-decylmaltoside in 150 µl 20 mM Tricine-KOH, pH 7.6, 150 mM NaCl (buffer A) for 5 min at RT. The sample was centrifuged again, and the supernatant diluted 10

times in buffer A. 750  $\mu$ l samples were incubated for 1 h at RT under constant agitation with 15  $\mu$ l of indicated antiserum, before addition of 50  $\mu$ l Protein A-Sepharose (GE-Healthcare) saturated with 0.1% (w/v) BSA. After incubation for 1 h at RT the matrix was washed three times with 500  $\mu$ l buffer A+0.2% *n*-decylmaltoside. Bound protein was eluted by boiling in SDS sample buffer for 2 min and subjected to SDS/PAGE followed by western blotting or autoradiography. OEV from pea (Schleiff *et al.*, 2003a) or envelopes from *A. thaliana* (Gerdes *et al.*, 2006) were solubilized by addition of 2% decylmaltoside. Phosphorylation of outer envelopes and complex isolation by sucrose density centrifugation was performed as described previously (Sveshnikova *et al.*, 2000; Schleiff *et al.*, 2003a).

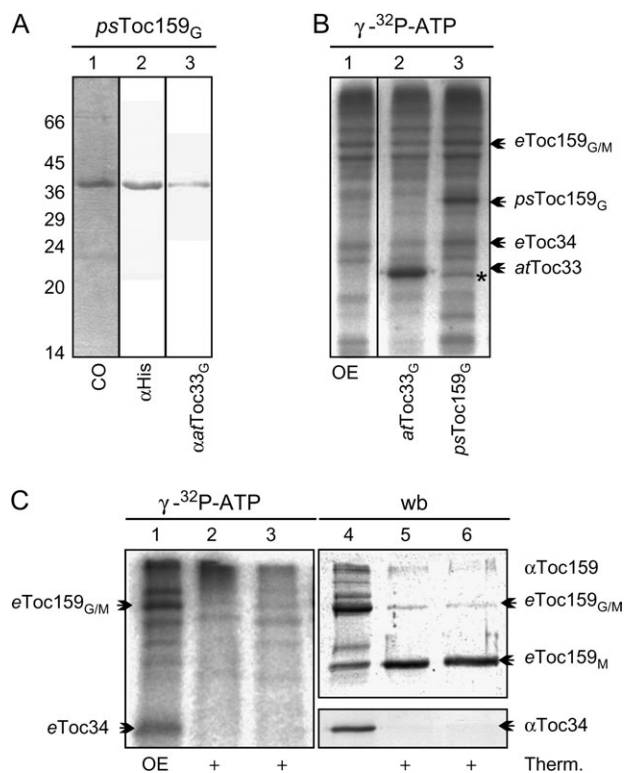
### Homology modelling

Superimposing the crystal structure of *at*Toc33 onto the monomers of the homeodimer of *ps*Toc34 (Sun *et al.*, 2002) with Yasara's SHEBA plugin (Jung and Lee, 2000) yielded the template for further modelling. The heterodimer of the G-domains of *at*Toc159 and *at*Toc33 was built with Modeller (Sali and Blundell, 1993) v8.1. The homology model of the heterodimer was further refined by loop modelling with Modeller (Fiser *et al.*, 2000). An energy minimization of the resulting heterodimer model was performed in Yasara (www.yasara.org) with the Yamber3 force field (Krieger *et al.*, 2004). Afterwards, a short MD simulation was performed in Yasara to further refine the interface of the complex.

## Results

### The Toc receptors are phosphorylated within their G-domains

It was observed that both Toc receptors, Toc34 and Toc159, can be phosphorylated (Fulgosi and Soll, 2002) and the phosphorylation site of Toc34 was mapped within its G-domain (Jelic *et al.*, 2002, 2003). However, it had not been clarified whether the tripartite protein Toc159 is phosphorylated in its G-domain as well. Therefore, the heterologously expressed G-domain of *ps*Toc159 (Fig. 1A, lanes 1–3) was incubated with isolated OEVs in the presence of  $\gamma$ -<sup>32</sup>P-ATP as the donor for phosphorylation, because kinase activity has been reported within this membrane (Fulgosi and Soll, 2002). As a positive control, the G-domain of recombinant *at*Toc33 was used. Both proteins are efficiently phosphorylated (Fig. 1B, lanes 2, 3) suggesting that Toc159 might be phosphorylated within its G-domain. To confirm this observation with native proteins, the phosphoprotein pattern of the outer envelope membrane was analysed before and after thermolysin treatment, which is known to degrade the cytosolic A- and G-domains of *ps*Toc159 but leaving the membrane-anchored M-domain intact (Becker *et al.*, 2004). Indeed, after proteolysis, as confirmed by the degradation of Toc34 (Fig. 1C, lanes 4–6, western blot) the M-domain of Toc159 remains detectable (Fig. 1C, lanes 5, 6), but is not phosphorylated (Fig. 1C, lanes 2, 3, autoradiogram). Therefore it can be concluded that *ps*Toc159, like *ps*Toc34, is phosphorylated within the G-domain at the cytosol-facing side of the chloroplast outer membrane.



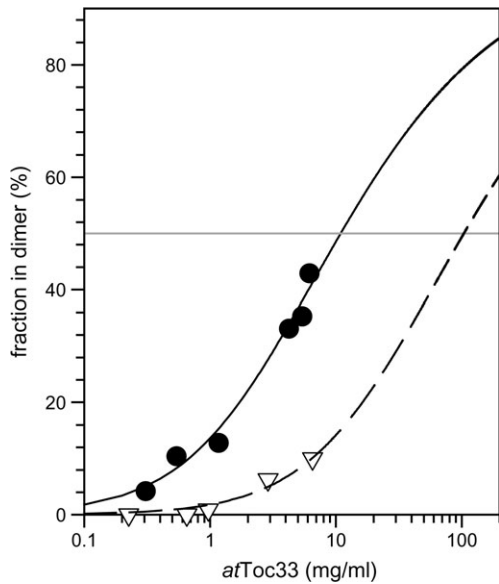
**Fig. 1.** The phosphorylation of Toc159. (A) The G-domain of *ps*Toc159 with a C-terminal His-tag was produced in *E. coli*, purified, subjected to SDS-PAGE, and stained with Coomassie Blue (lane 1) or immunodetected with antibodies against the C-terminal His-tag (lane 2) or against the G-domain of *at*Toc159 (lane 3) because the antibodies against Toc159 present in our laboratory are raised against the M-domain. The migration of the molecular weight marker is indicated on the left. (B) Pea OEVs (lane 1) were incubated with  $\gamma$ -<sup>32</sup>P-ATP in the presence of *at*Toc33<sub>G</sub> (lane 2) or *ps*Toc159<sub>G</sub> (lane 3). Proteins were subjected to SDS-PAGE and an autoradiogram is shown. The migration of the endogenous or the added receptor proteins is indicated. (C) Pea OEVs (lanes 1, 4) were phosphorylated as described (lanes 1–6) prior to proteolysis by thermolysin (lanes 2, 5) or thereafter (lanes 3, 6). Proteins were subjected to SDS-PAGE and visualized by immunodetection with the indicated antibodies (lanes 4–6) or autoradiography (lanes 1–3).

### Modification of the phosphorylation site influences the dimerization of Toc GTPases

In order to investigate the effects of phosphorylation, constructs were generated with a mutated phosphorylation site, whereby the serine 181 was substituted either with alanine or glutamate (Aronsson *et al.*, 2006; Oreb *et al.*, 2007). In a previous study, it was demonstrated that the S/E mutant exhibits a reduced capacity to bind and hydrolyse GTP (Oreb *et al.*, 2007), thus resembling the properties of the phosphorylated protein.

To investigate the effect of the S/E mutation on the dimerization, the concentration dependence of homodimerization was determined by size exclusion chromatography. It was possible to separate the monomeric and dimeric species and determined the dimeric fraction as described in the Materials and methods (Fig. 2). The apparent dissociation constants were calculated according to





**Fig. 2.** Homodimerization of *atToc33<sub>S/E</sub>*. Indicated concentrations of GDP loaded *atToc33* (circles, solid line) or *atToc33<sub>S/E</sub>* (triangles, dashed line) were subjected to gel filtration as described in the Materials and methods. The distribution of monomeric and dimeric species was analysed by fitting two Gaussian distributions. The dimer proportion is represented as a function of protein concentration. The apparent  $K_D$  was calculated according to E1 and lines represent the least square fit analysis to this equation.

equation 1. For *atToc33*, an apparent dissociation constant of 0.4 mM was determined, while the dissociation constant for *atToc33<sub>S/E</sub>* is about one order of magnitude higher (Fig. 3). This result suggests that the phosphomimicking mutant has a lower affinity for self association than the wild type.

Given that both homo- and heterodimerization of Toc GTPases is thought to be functionally relevant and a similar interaction interface for both types of associations is possible (Fig. 3A–E; see Discussion), this property was further investigated by a recently described membrane binding assay (König *et al.*, 2008). Here, His-tagged variants were immobilized on a nitrocellulose membrane and incubated with soluble GST-fused Toc proteins. The binding efficiency was subsequently analysed by immunodetection of the GST-moiety and densitometric quantification of the signals. This approach was chosen because it allows the effects of different nucleotide loading states and mutations in a homo- and heterodimeric context to be assessed in parallel.

The affinity of the S/A mutant for dimerization with wild type *atToc33* is even slightly enhanced in comparison to the homodimerization of the wild-type receptor when both molecules are charged with GMPPNP (Fig. 3F, green bar, left), but only moderately, if at all, decreased when at least one dimerization partner is in the GDP loaded state (Fig. 3F, green bar, middle). This is also the case for the formation of the heterodimeric complex of the

G-domains of Toc159 and *atToc33<sub>S/A</sub>* (Fig. 3F, black bar). A far more pronounced effect is observed with the S/E mutant, especially for heterodimerization when *atToc33<sub>S/E</sub>* is charged with GMPPNP (Fig. 3F, red bar, left and right). This finding documents that the phosphomimic has a more drastic effect on heterodimerization than on homodimerization. Furthermore, heterodimerization is mostly affected when *atToc33* is in the GMP-PNP state and homodimerization when one of the complex components is in the GDP state.

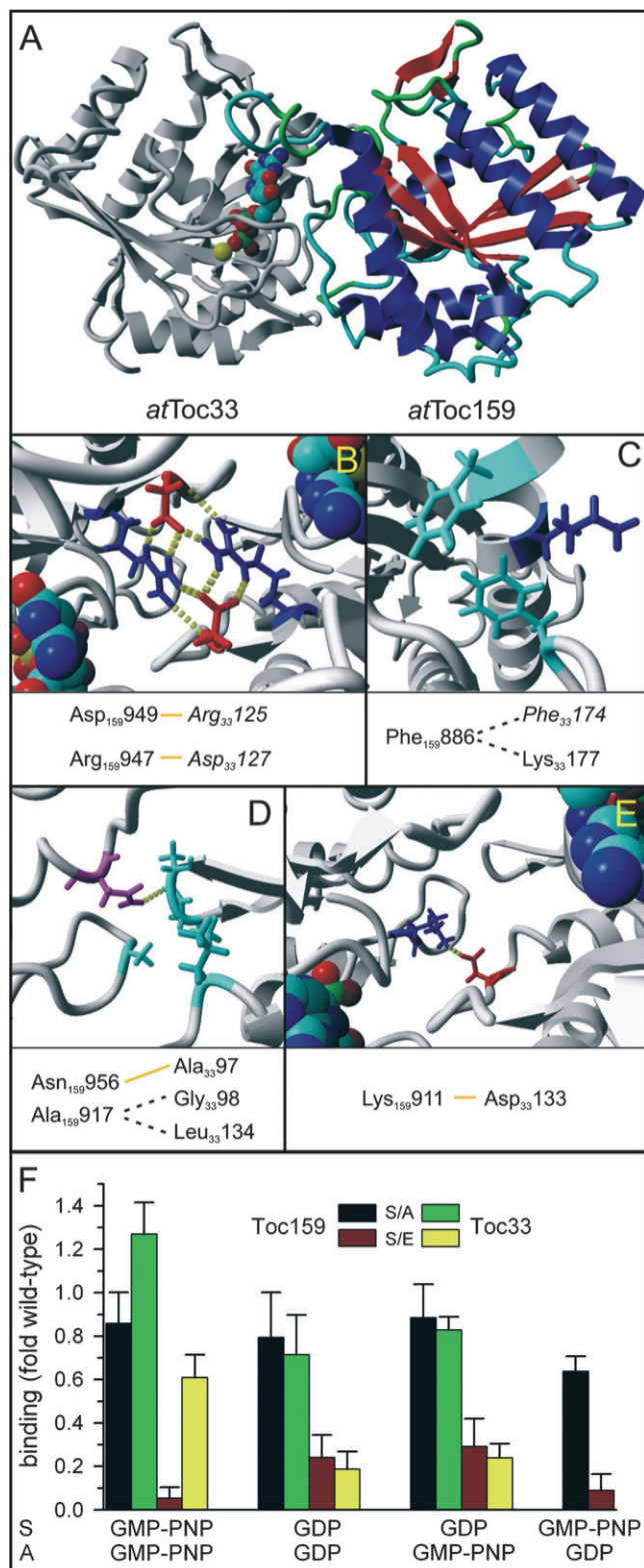
#### *Phosphorylation modulates the assembly of atToc33 with the Toc core complex*

To transfer the *in vitro* findings into a physiological context, wild-type *Arabidopsis* plants and *pil* plants complemented with *atToc33<sub>S/E</sub>* (line 3; Aronsson *et al.*, 2006, Oreb *et al.*, 2007) were analysed with respect to the association of *atToc33* with the Toc complex. The chloroplasts envelope membranes were isolated, solubilized, subjected to sucrose density centrifugation, and the protein distribution determined by western-blotting using  $\alpha$ Toc75 and  $\alpha$ Toc33 antibodies (Fig. 4A). The Toc complex containing both Toc75 and Toc33 is present in high density fractions (Fig. 4A, lanes 8, 9). Fractionating envelopes from *pil-S181E-3* plants reveals that a significant portion of *atToc33<sub>S/E</sub>* is not assembled with the complex and migrates at a lower density (Fig. 4F, lanes 1–3). This sedimentation profile is similar to the phosphorylated form in the wild-type background (compare wt+<sup>32</sup>P-ATP with western blots) and represents a receptor population not associated with the Toc complex. The data suggest that phosphorylated *atToc33* is no longer assembled in the Toc complex in endogenous membranes.

To confirm that *atToc33<sub>S/E</sub>* indeed has a lower affinity for the core complex, the Toc complex was coimmunoprecipitated by  $\alpha$ Toc75 antibodies after solubilization of isolated chloroplasts. *atToc33*, but not *atToc33<sub>S/E</sub>*, can efficiently be co-immunoprecipitated with *atToc75* as determined by immunodecoration with  $\alpha$ Toc33 antibodies (Fig. 4B, lanes 2, 4). The reduction becomes most obvious when the precipitation efficiency between Toc159 and Toc33 is compared (Fig. 4B, upper panel versus lower panel). This confirms a specific effect of the S/E exchange and invalidates effects due to up-regulation of *atToc33<sub>S/E</sub>* in the transgenic plants (Oreb *et al.*, 2007). While the *in vitro* data have shown a change in dimerization behaviour of *atToc33<sub>S/E</sub>*, these experiments now show that assembly of the native Toc core translocon is also impaired.

#### *The effect of phosphorylation on complex assembly is independent of the positioning of the phosphorylation site of Toc34 in different species*

Phosphorylation not only regulates *atToc33* (Jelic *et al.*, 2003), but *psToc34* is similarly regulated by this



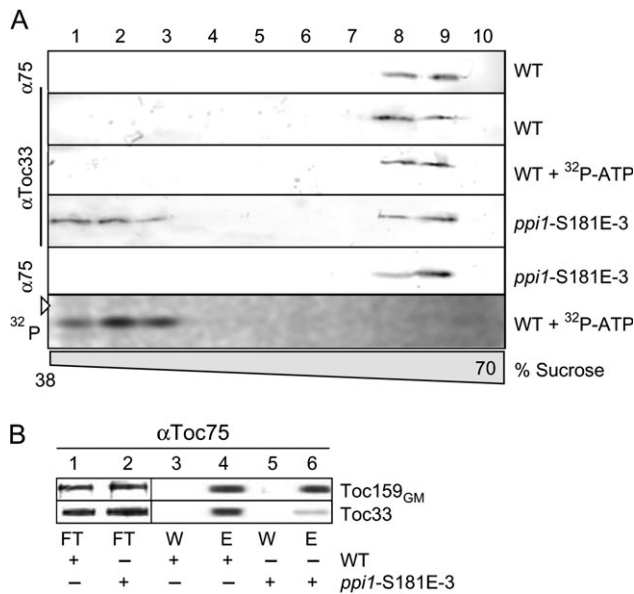
**Fig. 3.** Phosphorylation influences heterodimerization of the G-domains of the Toc receptors *in vitro*. (A) The homology model of the heterodimer generated as described in the Materials and methods section is shown. The model of the G-domain of Toc159 is shown in colour, where structural elements are coloured differently. (B–E) The positions of amino acids previously identified to be involved in

post-translational modification (Sveshnikova *et al.*, 2000) even though the position of the phosphorylation sites differs (Jelic *et al.*, 2002, 2003). Thus, it was important to generalize our observations regarding Toc complex assembly using *Pisum* as an additional system. Hence, complex assembly in purified OEVs from pea chloroplasts, preincubated with radioactive ATP, was analysed by sucrose density centrifugation (Fig. 5A). Again, the phosphorylated, radioactively labelled form of *psToc159* and *psToc34* is not detectable in high density fractions, containing the assembled Toc complex (Fig. 5A, bottom, silver-stained gel, fractions 9–11). In more detail, the phosphorylated form of *psToc34* remains at the top of the gradient (Fig. 5A, fractions 1–4; inset grey squares), whereas phospho-*psToc159* migrates further into the gradient (Fig. 5A, fractions 1–6; inset black circles). This could be explained by a remaining association with *psToc75*, and thus association of Toc complex components was examined by coimmunoprecipitation. Using *psToc75*-antibodies, both *psToc159* and *psToc34* can be efficiently coimmunoprecipitated (Fig. 5B, wb, lane 4). While *psToc159*, when phosphorylated, remains present in the complex, *psToc34* is not associated with *psToc75* (Fig. 5B, P<sup>32</sup>, lane 4). The specificity of this assay was controlled by immunoprecipitation using antibodies against the OE protein of 37 kDa, Oep37 (Fig. 5B, lanes 5–8; Schleiff *et al.*, 2003b). This result shows that phosphorylation of the Toc components results in a soluble pool of the small GTPase Toc34.

## Discussion

The significance of the phosphorylation of Toc receptors, even though it has been documented *in vitro* and *in vivo* (Sveshnikova *et al.*, 2000; Jelic *et al.*, 2002), is controversial (Aronsson *et al.*, 2006; Oreb *et al.*, 2007). This controversy has three reasons: physiological effects of a phospho-mimicking mutant can only be observed under specific conditions (Aronsson *et al.*, 2006; Oreb *et al.*,

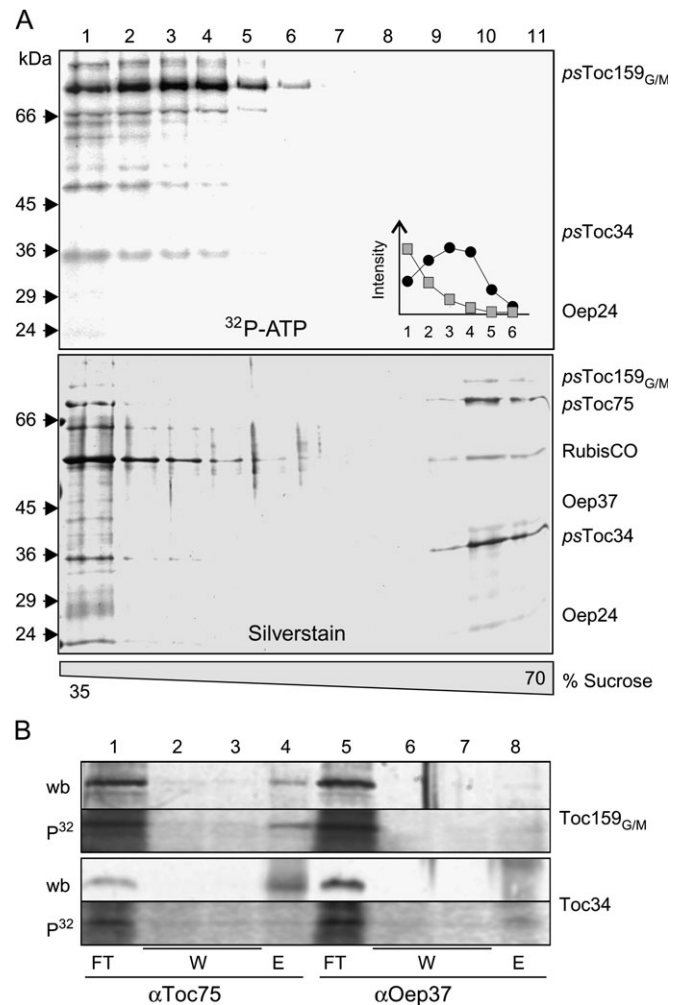
homodimerization (in italics) and additional amino acids of *atToc33* putatively involved in interactions within the heterodimeric conformation are shown. Yellow lines indicate putative hydrogen bonds and dashed lines putative hydrophobic interactions. Molecular graphics were created with Yasara ([www.yasara.org](http://www.yasara.org)) and Povray ([www.povray.org](http://www.povray.org)). (F) *atToc33*-His (for comparison), *atToc33*<sub>S/A</sub>-His (black and green bars) or *atToc33*<sub>S/E</sub>-His (red and yellow bars) loaded with GMPPNP or GDP were immobilized on nitrocellulose. The membrane was subsequently incubated with GST-*atToc33* (green and yellow bars) or GST-*psToc159*<sub>G</sub> (black and red bars) loaded with GDP or GMPPNP (The combinations of nucleotides are indicated at the bottom, whereby S denotes the loading state of the spotted protein and A the loading state of the added soluble protein). The amount of bound protein was quantified as described in the Materials and methods and normalized to the ratio of wild-type receptor binding (*atToc33*/*atToc33* or *atToc33*/*psToc159*). The average of at least six independent experiments including the three different concentrations is shown.



**Fig. 4.** Phosphorylation and phospho-mimicking of *atToc33* influences the Toc complex integrity. (A) Envelope membranes from wild type (WT) or mutant *A. thaliana* (*ppi1*-S181E-3) were solubilized with decylmaltoside after incubation with  $^{32}$ P-ATP (WT+ $^{32}$ P-ATP) adjusted with ATP to 1  $\mu$ M (final) and subjected to a 25–70% sucrose density gradient centrifugation. Fractions of the density region from 38% to 70% were analysed using  $\alpha$ Toc33 or  $\alpha$ Toc75 ( $\alpha$ T75) antibodies. The last panel shows the autoradiogram of the *atToc33* blot ( $\alpha$ Toc33/WT+ $^{32}$ P-ATP). The triangle indicates the migration of the 36 kDa molecular weight standard. (B) *atToc33* and *atToc159* were coimmunoprecipitated using  $\alpha$ Toc75-antibodies from equivalent amounts of chloroplasts isolated from wt or *ppi1*-S181E-3 plants. For analysis, 4% of the flow through (FT), 4% of the wash (W), and 50% of the eluate (E) fractions were subjected to SDS-PAGE and immunodetection with  $\alpha$ Toc159 and  $\alpha$ Toc33 antibodies, respectively.

2007), the envelope localized kinases (Fulgori and Soll, 2002) still await their identification, and the molecular effect of phosphorylation in endogenous membranes remains unclear. In the present study, the last question was targeted and evidence provided that phosphorylation of the G-domains of the Toc receptors (Fig. 1) regulates the association of Toc34 with the Toc core complex. Two underlying mechanisms can be proposed. The first considers the fact that the Toc complex integrity in general (Becker *et al.*, 2004), and the association between Toc34 and Toc159 in particular (Wallas *et al.*, 2003), are dependent on the ability of Toc GTPases to bind GTP. It can be expected to influence all nucleotide-dependent properties, including the integrity of the Toc complex, since phosphorylation inhibits binding of GTP. Contradicting such a conclusion is that *atToc33<sub>S/E</sub>* is still able to bind GTP (Oreb *et al.*, 2007), but shows a reduced affinity for complex assembly as well. Furthermore, the strongest effect of the phospho-mimicking mutation on heterodimerization was observed when *atToc33* is loaded with GMP-PNP, the non-hydrolysable analogue of GTP (Fig. 3).

The second possibility would be a direct modulation of protein–protein interactions by the bulky and negatively



**Fig. 5.** Phosphorylation of *psToc34* in OEVs influences the Toc complex integrity. (A) Pea OEVs were solubilized with decylmaltoside after incubation with  $^{32}$ P-ATP adjusted with ATP to 1  $\mu$ M (final) and subjected to a 25–70% sucrose density gradient centrifugation. Fractions of the density region from 35–70% were collected and subjected to SDS-PAGE followed by silver staining (lower panel) and visualization of radioactivity (upper panel). The inset shows the distribution of phosphorylated *psToc34* (squares) or *psToc159<sub>GM</sub>* (circles). (B) Pea OEVs were phosphorylated as described in the Materials and methods followed by immunoprecipitation using  $\alpha$ Toc75 or  $\alpha$ Oep37 antibodies. Flow through (10%, FT), wash (10%, W), and precipitate fractions (50%, E) were analysed by western blots (wb) using  $\alpha$ Toc159 (Toc159<sub>GM</sub>) or  $\alpha$ Toc34 antibodies. Phosphorylation was visualized by autoradiography ( $P^{32}$ ).

charged phosphate at the interface of interacting proteins. The observation that the *atToc33* phospho-mimicry mutant has a lower affinity for heterodimerization with *atToc159*, even in the GDP-loaded state (Fig. 3), supports this notion. Furthermore, on the basis of *psToc34* and *atToc33* crystal structures (Sun *et al.*, 2002; König *et al.*, 2008) the phosphorylation site of *atToc33* can be mapped to the homodimerization interface. *In vitro*, the ability of the phospho-mimicking *atToc33<sub>S/E</sub>* mutant to form homodimers is reduced (Figs 2, 3). Given the high similarity of G-domains of *atToc33* and *atToc159*, the interface for



homodimerization can also serve as an interaction site for heterodimerization (Fig. 3). This hypothesis is supported by the observation that *in vitro*-translated *atToc33*, which can be assumed to be exclusively monomeric due to its low concentration in the translation mixture, was shown to bind to Toc159 (Smith *et al.*, 2002). Analysing the amino acids assigned as interacting in the homodimer (Sun *et al.*, 2002) in the model of the heterodimer showed that many of these interactions are conserved. For instance, the network of electrostatic interactions by Arg<sub>33</sub>125 and Asp<sub>33</sub>127 can be identified (Fig. 3B), as well as the van der Waals interaction of Phe<sub>33</sub>174 (Fig. 3C), Phe<sub>33</sub>67 (not shown, with putative interactions with Phe<sub>159</sub>989, Gln<sub>159</sub>1001, and Ile<sub>159</sub>1001) or of Gly<sub>33</sub>98 and Leu<sub>33</sub>134 (Fig. 3D). In addition, some new possible contacts could be allocated (Fig. 3C–E), possibly compensating the loss of the interaction of the network involving Arg<sub>33</sub>130 (Sun *et al.*, 2002), which is not conserved in Toc159. Hence, it can be speculated that (at least in the *Arabidopsis* system) the phosphate group sterically inhibits the contacts between the G-domains even in the heterodimeric context. This interpretation, however, can not be directly applied to the *Pisum* orthologues, because the phosphorylation site of *psToc34* is distant from the dimerization interface. Hence, in pea, another interaction, for example with the cytosolic domain of the translocation channel Toc75 (Ertel *et al.*, 2005), might be affected. In turn, it is possible that the phosphorylation of the Toc159 G-domain (Fig. 1) disturbs the contacts with Toc34 in pea. The precise determination of the phosphorylation site in Toc159 and the physical mapping of the interaction network within the Toc complex will therefore be necessary to understand the regulation of the system in pea.

In general, regulation of protein complexes by phosphorylation is a widespread regulatory principle, even in protein translocation (Wang and Johnsson, 2005; Glavy *et al.*, 2007). An extensively studied example is the regulation of the light-harvesting complex protein II (LHCPII) by phosphorylation when the activity of photosystem I becomes limiting (Allen, 2005). The phosphorylated LHCPII dissociates from photosystem II and assembles with the photosystem I. The subsequent oxidation of the plastoquinone pool inactivates the kinase and activates the phosphatase, restoring the interaction of LHCPII with photosystem II. Thereby, this cycle fine-tunes the activity of a membrane complex. A similar mechanism can be envisioned for the Toc translocon to switch between different functionalities as proposed and to enable the exchange of receptors. However, our results and the structural context with the phosphorylation site being localized on the dimerization interface suggests that phosphorylation does not serve as a signal for dissociation of the receptor, but rather as an inhibitor of association. Finally, the question about the physiological function of the regulatory circuit of Toc34 will have to be answered,

a question which will require the identification and analysis of the Toc-phosphorylating kinase(s).

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