

## RESEARCH PAPER

# *In vivo* interaction between *atToc33* and *atToc159* GTP-binding domains demonstrated in a plant split-ubiquitin system

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## Abstract

The GTPases *atToc33* and *atToc159* are pre-protein receptor components of the translocon complex at the outer chloroplast membrane in *Arabidopsis*. Despite their participation in the same complex *in vivo*, evidence for their interaction is still lacking. Here, a split-ubiquitin system is engineered for use in plants, and the *in vivo* interaction of the Toc GTPases in *Arabidopsis* and tobacco protoplasts is shown. Using the same method, the self-interaction of the peroxisomal membrane protein *atPex11e* is demonstrated. The finding suggests a more general suitability of the split-ubiquitin system as a plant *in vivo* interaction assay.

**Key words:** Heterodimerization, *in vivo*, protein–protein interaction, protoplast, split-ubiquitin, Toc GTPases.

## Introduction

More than 90% of chloroplast proteins are encoded in the nucleus and imported post-translationally. Most of these proteins are synthesized as pre-proteins with a cleavable N-terminal transit peptide. They are recognized and translocated via the action of protein complexes at the outer and inner membrane of the organelle, designated Toc (translocon at the outer envelope membrane) and Tic (translocon at the inner envelope membrane), respectively (Soll and Schleiff, 2004; Bedard and Jarvis, 2005; Kessler and Schnell, 2006). In *Arabidopsis*, the heteromeric Toc core complex contains a  $\beta$ -barrel protein-conducting channel (*atToc75*) and two GTPases (*atToc33* and *atToc159*). *atToc33* and *atToc159* confer import specificity by the recognition and binding of the transit peptide and therefore represent the import receptors at the Toc core complex. Two gene families of Toc receptor GTPases exist in *Arabidopsis*: the Toc33 family (*atToc33* and *atToc34*) and the Toc159 family (*atToc90*, *atToc120*, *atToc132*, and *atToc159*). There is evidence that all members of the subfamilies function as chloroplast import receptors with a similar mode of action

but with different substrate (pre-protein) specificities (Hiltbrunner *et al.*, 2004; Ivanova *et al.*, 2004; Kubis *et al.*, 2004).

All Toc GTPases share highly conserved GTP-binding motifs present in their respective GTP-binding domains (G-domains). *atToc33* is a 33 kDa protein anchored in the chloroplast outer membrane by a short C-terminal hydrophobic sequence. The N-terminal hydrophilic part consisting mostly of the G-domain is cytosolic. *atToc159* is a 159 kDa protein anchored in the membrane by its C-terminal M-domain. The cytosolic part of *atToc159* consists of an N-terminal acidic domain (A-domain) preceding the G-domain (Hiltbrunner *et al.*, 2001a).

Hydrolysis of GTP by Toc GTPases regulates pre-protein import, but the precise mechanisms of the two GTPases (*atToc159* and *atToc33*) during import are still unknown (Kessler and Schnell, 2006).

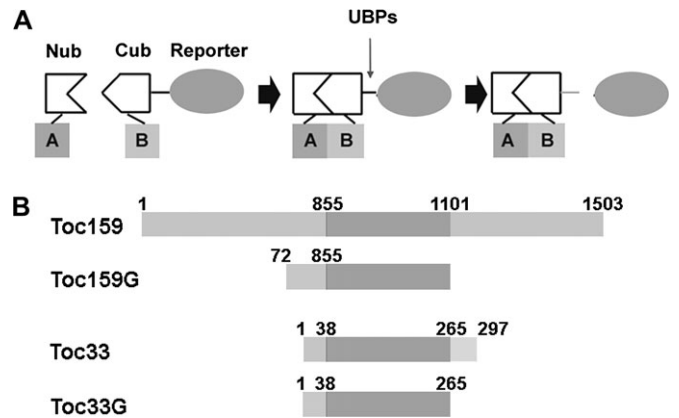
Several studies report on the *in vitro* interaction of *atToc159* and *atToc33*, suggesting that the functional mechanism of the Toc GTPases involves dimerization of

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their G-domains (Hiltbrunner *et al.*, 2001b; Bauer *et al.*, 2002; Smith *et al.*, 2002; Wallas *et al.*, 2003; Weibel *et al.*, 2003; Oreb *et al.*, 2008). When the G-domains of *Arabidopsis* or pea Toc33 (designated *psToc34*) and Toc159 are purified as soluble recombinant proteins from bacteria, they exist in a concentration-dependent equilibrium between the monomeric and dimeric state (Reddick *et al.*, 2007; Yeh *et al.*, 2007). This observation and the crystal structures available for *Arabidopsis* and pea Toc33 indicate the formation of stable homodimers of the G-domain (Sun *et al.*, 2002; Koenig *et al.*, 2008a). The positioning of an arginine residue in the pea Toc33 homodimer reminiscent of a GAP (GTPase-activating protein) arginine finger suggested reciprocal activation of one monomer by the other. However, recent studies led to the hypothesis either that additional external factors are required for catalytic activation of *atToc33/psToc34* or that activation is achieved by heterodimerization with Toc159. The Toc GTPase cycle might involve stable (non-activated) homodimers as well as more transient (self-activated) heterodimers (Koenig *et al.*, 2008a, b). Clearly, Toc GTPase homo- and/or heterodimerization are important features of the Toc GTPase cycle and are most likely crucial for the activation mechanism. While a lot of data has been gathered on homodimers, structural evidence for *atToc159-atToc33* heterodimers, however, is not available nor has the *in planta* heterodimerization been demonstrated.

To obtain more insight into the *in vivo* interaction of Toc GTPases, especially heterodimerization of *atToc159* and *atToc33*, a plant split-ubiquitin system was engineered. Originally the split-ubiquitin system was developed in yeast to monitor transient protein–protein interactions at their natural site, for example membranes in living cells (Johnsson and Varshavsky, 1994; Stagljar *et al.*, 1998). In a split-ubiquitin assay, ubiquitin is expressed in two separate parts, an N-terminal part (termed Nub, consisting of amino acids 1–37) and a C-terminal part (termed Cub, consisting of amino acids 35–76) fused to a gene coding for a reporter protein (Johnsson and Varshavsky, 1994; Stagljar *et al.*, 1998). Proteins of interest are fused either to Nub or to Cub. If the two proteins interact, the two halves of ubiquitin are brought into close proximity and a quasi ubiquitin is reconstituted and recognized by ubiquitin-specific proteases (UBPs), resulting in the cleavage of the Cub fusion and the release of the reporter protein (Fig. 1A).

Since its development, the yeast split-ubiquitin system has been successfully applied to the study of numerous protein–protein interaction pairs as well as genome-wide interaction screens (Lehming, 2002; Miller *et al.*, 2005). Proteins of higher eukaryotes were among those tested, including several, mainly plasma membrane-located, plant proteins (Reinders *et al.*, 2002a, b; Deslandes *et al.*, 2003; Ludewig *et al.*, 2003; Schulze *et al.*, 2003; Tsujimoto *et al.*, 2003; Obrdlik *et al.*, 2004; Pandey and Assmann, 2004; Park *et al.*, 2005; Pasch *et al.*, 2005; Yoo *et al.*, 2005; Orsel *et al.*, 2006; Bregante *et al.*, 2007; Ihara-Ohori *et al.*, 2007). One disadvantage of the yeast split-ubiquitin system for the study of plant protein interactions is the absence of plant-



**Fig. 1.** Schematic representation of split-ubiquitin and the Toc GTPases *atToc159* and *atToc33*. (A) In the split-ubiquitin system, ubiquitin is split into an N-terminal (Nub) and C-terminal half (Cub). Each half is fused to a protein of interest (A and B). If proteins interact, ubiquitin is reconstituted and recognized by ubiquitin-specific proteases (UBPs), resulting in the cleavage of a reporter protein. (B) *atToc159* and *atToc33* have conserved GTP binding-domains (G-domains, shown in dark grey). The boundaries of the G-domains are according to Hiltbrunner *et al.* (2001a), and numbers indicate amino acids. In addition, *atToc159* has an N-terminal acidic domain (A-domain) and a C-terminal membrane-anchoring domain (M-domain). *atToc33* has a short C-terminal hydrophobic transmembrane sequence. In this study, the coding sequence for the G-domain alone of *atToc159* (Toc159G, Toc159<sub>728–1093</sub>) was introduced into the different constructs. The *atToc33* constructs used contain the coding sequence for the G-domain (Toc33G, Toc33<sub>1–265</sub>) or for the full-length protein (Toc33).

specific factors which might influence the interaction and, for example in the case of chloroplast outer membrane proteins, the absence of the target organelle.

In the present study, the application of the split-ubiquitin protein–protein interaction assay in plants is shown for the first time. This approach demonstrates *atToc33* and *atToc159* heterodimerization *in vivo*. Furthermore, *atPex11e* (Lingard and Trelease, 2006; Orth *et al.*, 2007) was analysed as a model membrane protein of another organelle. Self-interaction of plant *atPex11e* was demonstrated, which was predicted based on knowledge of the yeast homologue (Marshall *et al.*, 1996).

## Materials and methods

### DNA constructs

To obtain the two-hybrid construct pGBKT7-Toc159G, the coding sequence of *atToc159G* (amino acids 728–1093) was amplified with primers 5'-CAT GCC ATG GGC AAG TCA GGA TGG TAC GAA A-3' and 5'-TTA TGC TAG TTA TTG CTC AG-3' from pET21d-Toc159G and cloned using *NcoI/NotI* into pGBKT7. For pGADT7-Toc33G, *atToc33G* was amplified with primers 5'-GAA ATT AAT ACG ACT CAC TAT AGG GG-3' and 5'-ACG CGT CGA CTT ACT

TTC CTT TAT CAT CAG AG-3' from pET21d-Toc33H6-sol (amino acids 1–265), subcloned using *NcoI/SalI* into pGBKT7, and cloned using *NdeI/SalI* into *NdeI/XhoI*-digested pGADT7.

The yeast split-ubiquitin constructs were derived from the *STE14-Cub-RURA3* (Wittke *et al.*, 1999), *PEX11-Cub-RURA3*, and *Nub-PEX11* constructs (Eckert and Johnsson, 2003). These constructs contain parts of the yeast *UBI4* coding sequence. All Nub (amino acids 1–37 of ubiquitin) fusions are expressed from a pRS314 plasmid under control of the *P<sub>CUP1</sub>* promoter, and all Cub–RUra3p (amino acids 35–76 of ubiquitin) fusions are expressed from a pRS313 vector under control of the *P<sub>MET17</sub>* promoter (Eckert and Johnsson, 2003). Two haemagglutinin (HA) epitopes were added to the Cub constructs by annealing the primers 5'-TCG ACC TAC CCA TAC GAC GTA CCA GAT TAC GCT GCT TAC CCA TAC GAC GTA CCA GAT TAC GCT-3' and 5'-TCG AAG CGT AAT CTG GTA CGT CGT ATG GGT AAG CAG CGT AAT CTG GTA CGT CGT ATG GGT AGG-3' and ligation into the unique *SalI* restriction site in front of the Cub coding sequence. The coding sequence of the G-domain of *atToc33* (amino acids 1–265) was amplified using a forward primer containing a *Clal* restriction site 5'-CCA TCG ATC CAT GGG GTC TCT CG-3' and a reverse primer including a *SalI* site 5'-CAT ATG GTC GAC CCT ATC TTT CCT TTA TCA TC-3', and cloned into the *Clal/SalI*-digested *STE14-Cub-RURA3* construct (Wittke *et al.*, 1999). The coding sequence of the G-domain of *atToc159* (amino acids 728–1093) was amplified using the following forward primer containing the coding sequence for a single Myc epitope tag and a *BamHI* site 5'-CCC GGG ATC CCT GGG GAT GAG GAG CAG AAG CTG-3', and a reverse primer with an *EcoRI* site 5'-CCA TCG ATC CAT GGG GTC TCT CG-3'. The resulting PCR product was ligated into the *BglII* and *EcoRI* sites of the Nub-containing plasmid *Nub-PEX11* thereby replacing *PEX11* (Eckert and Johnsson, 2003).

The plant split-ubiquitin constructs were designed with the coding sequence of plant ubiquitin *atUBQ11* (At4g05050.1) (Callis *et al.*, 1995). The sequence corresponding to the first 37 amino acids (Nub) was amplified using as a forward primer 5'-CGG GAT CCT CTA GAG TCG ACC ATG CAG ATC TTC G-3' including a *BamHI* site, and a reverse primer containing an *NcoI* site 5'-TCA TGT CAT GAC ACC ACC GCG GAG ACG G-3'. A plasmid (BUGUS) containing the *atUBQ11* coding sequence, provided by Professor Richard Vierstra (University of Wisconsin-Madison), served as template. The resulting PCR fragment was ligated into the vector pCL60 cut by *BamHI* and *NcoI*, yielding pCL60-Nub. pCL60 is a pBluescriptSK- (Stratagene) derivative containing a cauliflower mosaic virus (CaMV) 35S promoter, a nopaline synthase (NOS) terminator cassette, and the coding sequence for enhanced green fluorescent protein (EGFP; Bauer *et al.*, 2000). The I13G mutation of Nub (Nub<sup>G</sup>) was introduced into pCL60-Nub by QuikChange<sup>®</sup> Site-Directed Mutagenesis (Stratagene) using the forward primer 5'-CC GGA AAG ACC GGC

ACT CTT GAA GTT GAG AGT TCC GAC ACC-3', and the reverse primer 5'-GGT GTC GGA ACT CTC AAC TTC AAG AGT GGG GGT CTT TCC GG-3'.

The sequence corresponding to the amino acids 35–76 of UBQ11 (Cub) was amplified using the forward primer 5'-CAT GCC ATG GGA TAC CCA TAC GAC GTA CCA GAT TAC GCT GGC ATT CCT CCG GAC C-3' including a *NcoI* site and the coding sequence for a single HA tag, and the reverse primer 5'-TCA TGT CAT GAC ACC ACC GCG GAG ACG G-3' containing a *BspHI* site. The PCR product was ligated into pCL60 vector cut by *NcoI*, yielding pCL60-Cub. The primers 5'-GTA CTC ATG AAG GAG CAG AAG CTG ATC-3' (forward) and 5'-CTC AAG ACC CGT TTA GAGG-3' were used to amplify *Toc159<sub>728–1093</sub>* (*atToc159G*) with the two-hybrid construct pGBKT7-*Toc159G* as DNA template. The amplified DNA was then cloned using *NcoI* and *NotI* into pCL60-Nub. The complete sequences of *atToc33* or *atToc33G* (*Toc33<sub>1–265</sub>*) were amplified with the forward primer 5'-TGG GCC ATG GGG TCT CTC GTT CGT-3' and the reverse primers 5'-TGA ACT CAT GAG AAG TGG CTT TCC AC-3' or 5'-TGA ACT CAT GAG CTT TCC TTT ATC ATC-3', respectively. Ligation was done in the pCL60-Cub vector cut by *NcoI*. The coding sequence of *atPEX11e* (At3g61070) was amplified by the forward primer 5'-CAT GCC ATG GCA ACT ACA CTA GAT TTG ACC-3' containing an *NcoI* site, and the reverse primer 5'-CTA TAG CGG CCG CTC ATG ATT TCT TCA AC-3' including a *NotI* site. The product was ligated into pCL60-Nub cut by *NcoI* and *NotI*. To clone into pCL60-Cub cut by *NcoI*, *atPEX11e* was amplified with the same forward primer as above and the reverse primer 5'-TGA ACT CAT GAG TGA TTT CTT CAA C-3' including a *BspHI* site. The template plasmid DNA pGEM-Teasy-PEX11.2 containing the cDNA of *atPEX11e* was kindly provided by the group of Alison Baker (University of Leeds, UK).

#### Preparation of polyclonal antibodies against *Toc159G*

The coding sequence for *atToc159G* (amino acids 727–1093) was amplified with primers 5'-GG GAT CCA TGA CTA GTC AGG ATG GTA CGA A-3' and 5'-ATA AGA ATG CGG CCG CTT AAA CTC GGA AA-3', and cloned using *BamHI/NotI* into pGEX-4T-1 to generate pGEX-4T-1-*Toc159G* [encoding glutathione *S*-transferase (GST)–*Toc159G*]. After bacterial overexpression, GST–*Toc159G* was purified using Glutathione–Sepharose™ chromatography according to the specifications of the supplier (GE Healthcare). Purified GST–*Toc159G* was submitted to Eurogentec for antibody production in rabbits using a fast immunization protocol. Antibodies were affinity-purified against the antigen immobilized on Affigel-10 (Bio-Rad Laboratories).

#### Yeast two-hybrid and $\beta$ -galactosidase assay

Two-hybrid experiments were performed according to the Yeast Protocols Handbook (Clontech, a Takara Bio

Company) using the yeast strain Y190 (*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4A*, *gal80A*, *URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ*, *cyh<sup>r</sup>2*, *LYS2::GAL<sub>UAS</sub>-HIS3<sub>TATA</sub>-HIS3*, *MEL1*).

#### Yeast split-ubiquitin assay

Yeast growth was performed as described (Johnsson and Varshavsky, 1994) using yeast strain JD53 (*MAT $\alpha$* , *his3- $\Delta$ 200*, *leu2-3,112*, *lys2-801*, *trp1- $\Delta$ 63*, *ura3-5*) (Dohmen et al., 1995). Total protein extracts were prepared according to Kiel et al. (2005).

#### Plant growth

Seeds were surface-sterilized by liquid or vapour phase methods as described (Clough and Bent, 1998). *Arabidopsis thaliana* Col-2 (columbia) seedlings were plated on 0.5 $\times$  Murashige and Skoog medium (Duchefa) containing 0.8% Phyto Agar (Duchefa) and left for 2 d at 4 °C in the dark. They were then grown under short-day conditions (8 h light 120  $\mu$ mol m<sup>-2</sup> s<sup>-2</sup>, 16 h dark, 20 °C, 70% relative humidity). *Nicotiana tabacum* cv Petit Havana SR1 were grown on 1 $\times$  Murashige and Skoog medium containing 0.8% Phyto Agar under long-day conditions (16 h light, 120  $\mu$ mol m<sup>-2</sup> s<sup>-2</sup>, 8 h dark, 23 °C, 60% relative humidity).

#### Protoplast transformation

Protoplasts were transiently transformed using the polyethylene glycol method according to Jin et al. (2001) with 4-week-old *A. thaliana* or 6-week-old *N. tabacum* leaves. Fluorescence in transformed protoplasts was monitored 24–48 h after transformation using a Leica TCS 4D microscope. Green fluorescent protein (GFP) was detected with the fluorescein isothiocyanate (FITC; 488 nm) laser line, and tetramethylrhodamine isothiocyanate (TRITC; 568 nm) was used for chlorophyll autofluorescence.

#### Plant protein extraction and western blot analysis

Transiently transformed protoplasts were centrifuged for 1 min at 100 *g*. Total proteins were extracted according to Rensink et al. (1998) and 1% (v/v) protease inhibitor cocktail for plant cell extracts (Sigma P9599) was added to the extraction buffer. Proteins were concentrated by chloroform–methanol precipitation (Wessel and Flugge, 1984) and dissolved in SDS–PAGE sample buffer (50 mM TRIS pH 6.8, 10% glycerol, 2%  $\beta$ -mercaptoethanol, 0.025% bromophenol blue, 2% SDS). Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as standard.

SDS–PAGE and western blotting were carried out using standard procedures. Equal amounts of proteins were loaded on each lane and verified by amido black (naphthol blue black) staining of total proteins after transfer to a nitrocellulose membrane. Proteins were detected with monoclonal antibodies against the HA or Myc epitopes (Eurogentec, Roche) or polyclonal antibodies against

*atToc159G* (see above), *atToc75* (Bauer et al., 2000), or phosphoribulokinase (Dr Pia Stieger, Université de Neuchâtel). Blots were developed using enhanced chemiluminescence (ECL) and high performance films (GE Healthcare). Chemiluminescence signals were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>). The values obtained for cleaved and uncleaved Cub fusion proteins, respectively, were calculated using the Gel Analysing tool of the program. The sum of the two signals was defined as total Cub fusion protein (100%). The cleavage percentage was then obtained by dividing the value of cleaved Cub fusion protein by the sum of cleaved and uncleaved Cub fusion proteins. Each average was calculated from three independent experiments.

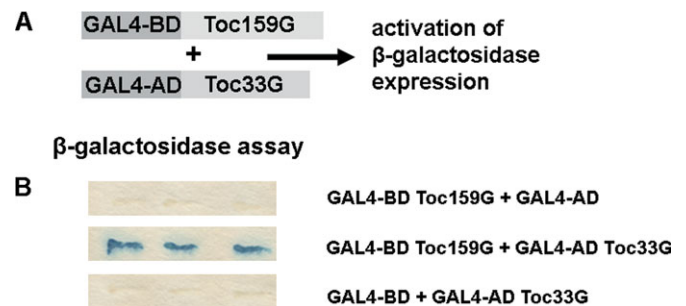
#### Separation of soluble and insoluble proteins

Transformed protoplasts were collected by centrifugation at 100 *g* for 1 min and resuspended in lysis buffer [20 mM TRIS-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 2 mM MgCl<sub>2</sub>, and 0.5% (w/v) inhibitor cocktail for plant cell extracts] followed by freezing and thawing. The lysate was centrifuged at 100 000 *g* for 1 h at 4 °C. The resulting supernatant was considered total soluble protein. Soluble protein was concentrated by chloroform–methanol precipitation. The pellet was resuspended in 50 mM TRIS-HCl, pH 7.5.

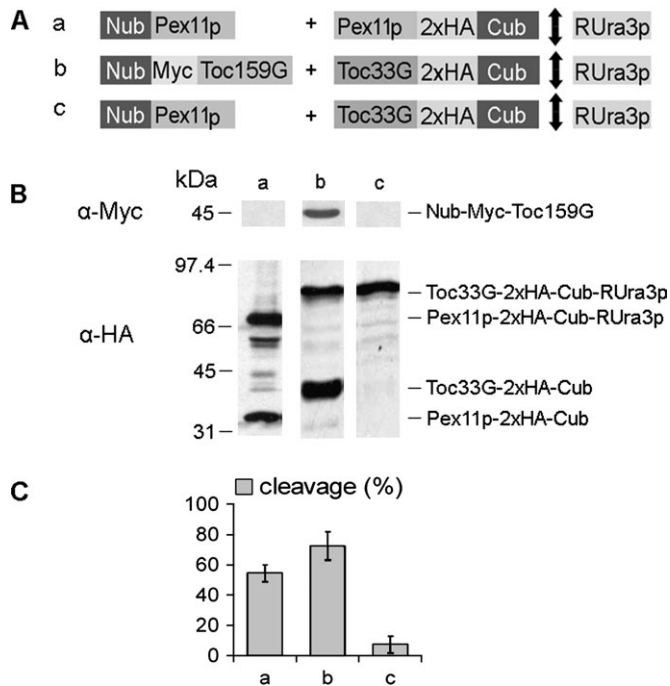
## Results

### Interaction between the G-domains of *atToc33* and *atToc159* in yeast protein–protein interaction assay systems

Before attempting *in vivo* interaction studies in plants, it was necessary to determine whether the interaction between the G-domains of Toc GTPases is detectable in the yeast two-hybrid (Fig. 2) and split-ubiquitin (Fig. 3) systems. Like split-ubiquitin, the yeast two-hybrid system is an assay system



**Fig. 2.** Yeast two-hybrid interaction of *atToc159G* and *atToc33G*. (A) *Toc159G* was fused to the GAL4-binding domain (BD) and *Toc33G* to the GAL4-activating domain (AD). (B)  $\beta$ -Galactosidase filter assays of Y190 cells transformed with constructs as indicated. The interaction of *Toc159G* with *Toc33G* leads to the expression of the  $\beta$ -galactosidase reporter gene and a blue coloration of yeast cells in the presence of a X-gal substrate solution (middle panel).



**Fig. 3.** Yeast split-ubiquitin interaction of *atToc159G* and *atToc33G*. (A) Yeast cells were co-transformed with different combinations of Nub and Cub constructs (a–c). The vertical double-headed arrows indicate the cleavage site of UBPs. (B) Western blot analysis of total cellular protein extracts using antibodies against the Myc or the HA epitope tag to detect Nub-Myc-Toc159G or the Cub fusion proteins, respectively. Co-expression of Nub-Pex11p and Pex11p-2HA-RURA3p (a) or Nub-Myc-Toc159G and Toc33G-2HA-Cub-RURA3p (b) led to partial cleavage of the RURA3p reporter, indicating interaction of these protein pairs. No cleavage was observed upon co-expression of Nub-Pex11p and Toc33G-2HA-Cub-RURA3p (c). (C) Reporter gene cleavage was quantified using ImageJ. The signal of cleaved and uncleaved proteins of one lane was estimated using the Gel Analysing tool of the program. The sum of these two signals was set to correspond to 100%. Each calculated average derives from three independent experiments. The percentage cleavage was calculated by dividing the cleaved Cub fusion protein by the total of uncleaved and cleaved.

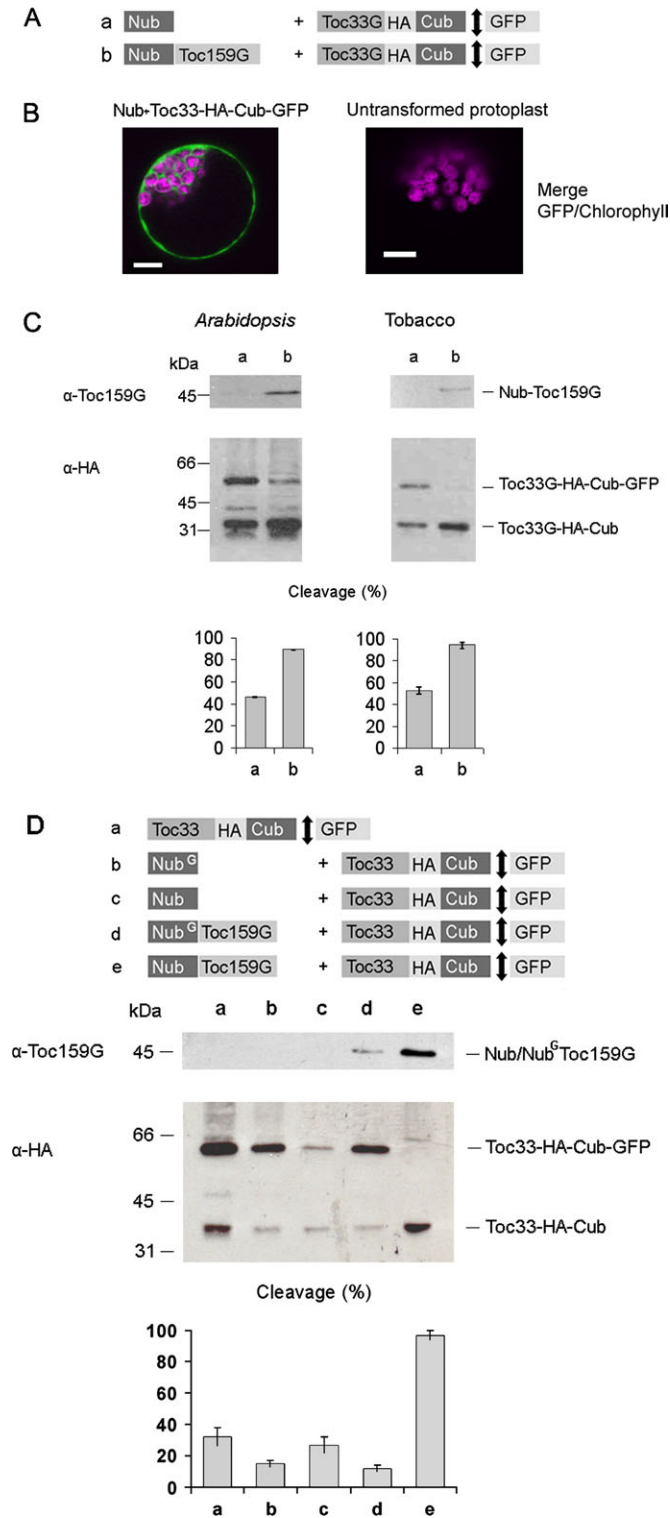
based on protein complementation. Proteins of interest are fused to two separate parts of a transcription factor (e.g. GAL4). A positive interaction leads to the reconstitution of a functional GAL4 transcription factor and transcriptional activation of a reporter gene (e.g.  $\beta$ -galactosidase). Constructs encoding the G-domains of *atToc33* (Toc33<sub>1–265</sub>) and *atToc159* (Toc159<sub>728–1093</sub>) were engineered (Fig. 2). For the yeast two-hybrid studies, *atToc159G* was fused to the GAL4 DNA-binding domain (BD) and *atToc33* to the GAL4-activating domain (AD) (Fig. 2A) in the vectors pGBKT7 and pGADT7, respectively. Yeast cells (strain Y190) were transformed with these two constructs, and the  $\beta$ -galactosidase reporter gene activity of transformants was determined. The co-transformation of pGBKT7-Toc159G and pGADT7-Toc33G resulted in blue colonies in the

presence of the X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside) substrate, and neither of these constructs activated  $\beta$ -galactosidase expression in combination with the empty AD or BD vectors by themselves (Fig. 2B), indicating that the two proteins interact in yeast cells. For yeast split-ubiquitin studies, split-ubiquitin fusion constructs were generated by replacing *STE14* or *PEX11* in the constructs *STE14-Cub-RURA3* (Wittke *et al.*, 1999) or *Nub-PEX11* (Eckert and Johnsson, 2003) by *atToc33G* or *atToc159G*, respectively. To allow for subsequent western blot analyses, two HA epitope tags were introduced upstream of Cub, and a Myc epitope downstream of Nub. Constructs encoding Nub-Pex11p and Pex11p-2HA-Cub-RURA3p were used as a positive control in experiments as these two fusion proteins were shown to homodimerize using this system (Eckert and Johnsson, 2003). Originally, the arginine-URA3 (RURA3) element was designed to serve as metabolic marker for the interaction between the Nub and Cub fusion proteins in growth assays, but here the interaction was monitored using immunoblotting.

Yeast cells (strain JD53) were co-transformed with the different constructs as shown in Fig. 3A. Equal amounts of cellular protein of the transformants were subjected to western blot analysis with anti-Myc and anti-HA antibodies to test for the presence of Nub-Myc-Toc159G and for cleavage of the Cub fusion proteins as an indicator of interaction (Fig. 3B). Cleavage of the Toc33G-Cub fusion protein was observed when it was expressed in the presence of Nub-Myc-Toc159G (Fig. 3A–C, b) whereas no cleavage was observed upon co-expression with a Nub fusion of the peroxisomal protein Pex11p (Fig. 3A–C, c). In this negative control experiment, only a single band corresponding to the Toc33G-Cub-HA-RURA3 (73 kDa) fusion protein was detected. In a positive control experiment, the same Nub-Pex11p fusion protein induced cleavage of Pex11p-2HA-Cub-RURA3p (Fig. 3A–C, a), consistent with Pex11p homodimerization (Eckert and Johnsson, 2003).

#### *Toc GTPase interaction in Arabidopsis protoplasts*

For the plant split-ubiquitin system, plant ubiquitin *AtUBQ11* (At4g05050.1) was used instead of *ScUBI4*. The EGFP was used as reporter protein. *AtUBQ11* is 97% identical to yeast ubiquitin, differing from *Saccharomyces cerevisiae* Ubi4p by only two amino acid substitutions (S28A and S57A). The N- and C-terminal ubiquitin parts were defined as in yeast, Nub consisting of amino acids 1–37 and Cub of amino acids 35–76. Constructs were engineered in the pCL60 vector (Bauer *et al.*, 2000), containing a CaMV 35S promoter and a NOS terminator. A HA epitope tag was included in the Cub constructs for subsequent western blot analysis. Isolated *Arabidopsis* protoplasts were transformed with constructs encoding *atToc33G* fused to HA-Cub-GFP (Toc33G-HA-Cub-GFP) in combination with constructs encoding Nub alone or for an Nub-*atToc159G* fusion protein (Fig. 4A). The GFP reporter protein of the Cub construct allowed assessment of the protoplast transformation efficiency (estimated at 30%



**Fig. 4.** Plant split-ubiquitin interaction between *atToc159G* and *atToc33*. (A) Protoplasts were co-transformed with Nub and Cub constructs as indicated (a and b). (B) Use of the GFP reporter to assess protoplast transformation visually via confocal microscopy. Due to partial background cleavage, all Cub–GFP fusions gave the same green cytosolic fluorescence pattern as exemplified here for Toc33-HA-Cub-GFP (bar = 5 μm). Green, GFP fluorescence; purple, chlorophyll autofluorescence. (C) Interaction of Toc159G and Toc33G in *Arabidopsis* or tobacco protoplasts. Total proteins

in most of the experiments, data not shown) by confocal microscopy (Fig. 4B).

Western blots were performed on protein extracts of transformed protoplasts using anti-HA antibodies to determine whether cleavage had occurred (Fig. 4C, lower panel). Antibodies raised against *atToc159G* were used to monitor Nub–Toc159G expression (Fig. 4C, upper panel). When Nub–Toc159G and Toc33G-HA-Cub-GFP were co-expressed, >80% cleavage of the GFP reporter was observed (Fig. 4C, b). In the control experiment in which Nub alone was co-expressed together with Toc33G-HA-Cub-GFP, non-specific cleavage in the range of 40% of the GFP reporter gene was observed (Fig. 4C, a). Similar results were observed when the same experiment was performed in isolated *Arabidopsis* or tobacco protoplasts (Fig. 4C). Although the rate of non-specific cleavage in the plant split-ubiquitin system is higher than the rate of background cleavage observed in the yeast split-ubiquitin assays, the clear increase in cleavage by co-expressing *atToc159G* and *atToc33G* indicates the interaction of the two GTPases.

One of the objectives of the present work is to study Toc GTPase interactions and mechanisms at their target membrane. Therefore, an experiment was performed using Nub–Toc159G and a Cub construct containing the full-length cDNA coding for *atToc33* including its C-terminal hydrophobic transmembrane sequence (Toc33-HA-Cub-GFP) (Fig. 4 D). Co-expression of Nub–Toc159 together with this construct yielded the same high level of cleavage (Fig. 4D, e) as observed with the Toc33 G-domain Cub fusion, pointing towards interaction between Toc159G and full-length Toc33.

To address the issue of background cleavage, additional controls were carried out (Fig. 4D). First, to test if the high level of background cleavage is due to spontaneous association of the Nub and Cub moieties, protoplasts were transformed with the Toc33-HA-Cub-GFP fusion only (Fig. 4D, a). In addition, a Nub moiety bearing a I13G (Nub<sup>G</sup>) mutation was used (Fig. 4D, b and d). The I13G mutation decreases the conformational stability of Nub. As the efficiency of ubiquitin reconstitution depends on the conformational stability of Nub, this mutation has been exploited to reduce background cleavage in yeast split-ubiquitin approaches (Johnsson and Varshavsky, 1994). Expression of the Toc33-HA-Cub-GFP fusion protein alone (Fig. 4D, a) yielded about the same level of background cleavage as observed when co expressing Toc33-HA-Cub-GFP with Nub (Fig. 4D, c). Thus, background cleavage is most probably not due to spontaneous association of Nub

were extracted and analysed by western blotting using antibodies raised against Toc159G and anti-HA to check for the presence of Nub–Toc159G and the HA-tagged Toc33G Cub fusion protein, respectively. (D) Plant split-ubiquitin interaction among Toc159G and full-length Toc33. *Arabidopsis* protoplasts were co-transformed with Nub and Cub constructs as indicated (a–e). Note that experiments b and d were carried out with the I13G mutant of Nub. The graph below shows the results of chemiluminescence quantification of three independent experiments.

and Cub but due to an unspecific proteolytic action on the Cub fusion protein itself. In line with this observation, use of Nub<sup>G</sup> resulted in only a slight reduction of background cleavage compared with Nub (compare Fig. 4E, b and c). The increase in cleavage by co-expressing Nub-Toc159G together with Toc33-HA-Cub-GFP could no longer be observed when the Nub moiety fused to Toc159G contained the I13G mutation (Fig. 4E, d). Considering the other control experiments, it is not thought that this loss of cleavage hints at an unspecific interaction between *atToc159G* and *atToc33* but rather at the weak or transient nature of the interaction. The Nub I13G mutation could further weaken or retard the interaction-induced reconstitution of ubiquitin and therefore inhibit detection of the interaction by split-ubiquitin.

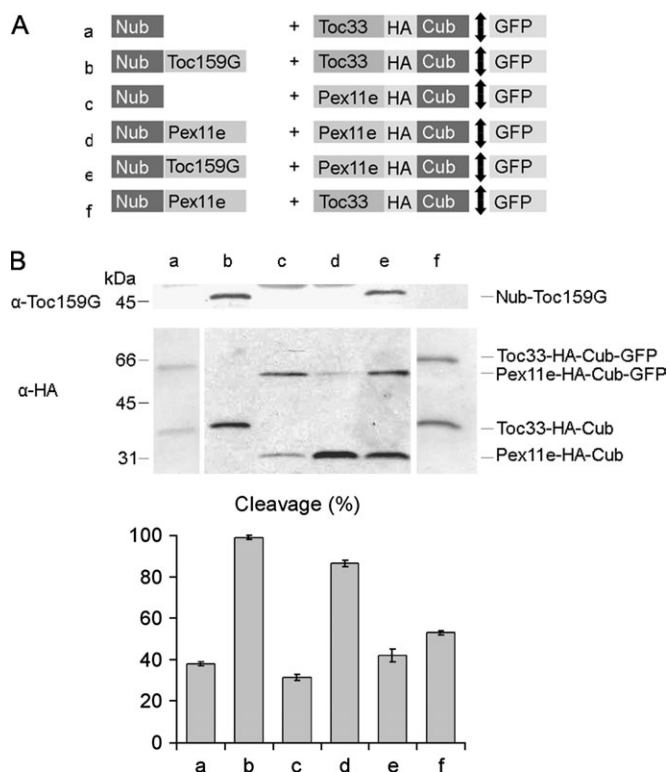
#### *AtPex11e* self-interaction

To substantiate further the specificity of Toc GTPase interaction in the plant split-ubiquitin system, constructs encoding Nub and Cub fusions to an *Arabidopsis* homologue of yeast Pex11 were engineered. Five Pex11 homologues were identified in *Arabidopsis* (*atPex11a–e*), all representing peroxisomal membrane proteins involved in peroxisome proliferation (Lingard and Trelease, 2006; Orth *et al.*, 2007). Two out of these five homologues, *atPex11c* and *atPex11e*, have been demonstrated partially to complement the *S. cerevisiae pex11* null mutant (Erdmann and Blobel, 1995), indicating a conserved function in peroxisome biogenesis and similar interaction patterns (Orth *et al.*, 2007). *atPex11e* was chosen as a model protein for the following reasons. First, *atPex11e* was expected to homodimerize like *Saccharomyces* Pex11p and therefore to give a positive interaction in the plant split-ubiquitin system. In the yeast split-ubiquitin system, *ScPex11p* homodimerization was demonstrated with the full-length protein (Eckert and Johnsson, 2003, and Fig. 3) and therefore it was likely that plant split-ubiquitin could work with full-length, membrane-inserted *atPex11e* as well. Finally, *atPex11e* localization in a different cellular compartment (peroxisome) and its function in peroxisome multiplication made it unlikely to interact with a component of the chloroplast protein import machinery.

Constructs encoding Nub-Pex11e and Pex11e-HA-Cub-GFP (Fig. 5A) were engineered in order to test for *atPex11e* self-interaction. Co-expression of Nub-Pex11e and Pex11e-HA-Cub-GFP in isolated tobacco protoplasts gave ~85% reporter GFP cleavage (Fig. 5d). In contrast, control experiments with Pex11e-HA-Cub-GFP and either Nub alone (Fig. 5c) or Nub-Toc159G (Fig. 5e) resulted in only 30–40% cleavage. Similarly, co-expression of Nub-Pex11e with Toc33-HA-Cub-GFP resulted in ~45% cleavage of the GFP reporter (Fig. 5f). Thus, the cleavage observed when co-expressing Toc GTPases with Pex11e is at the level of unspecific background cleavage.

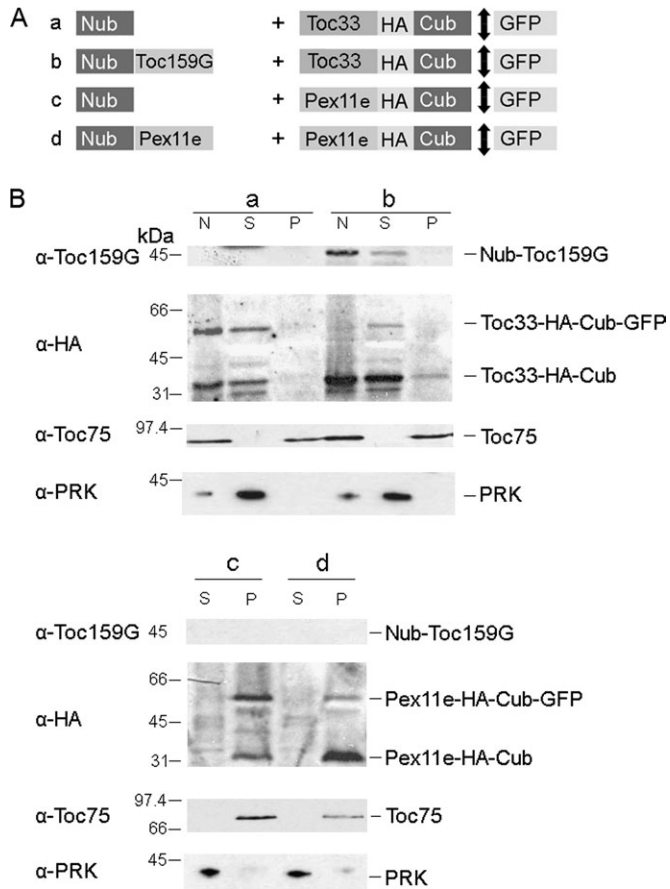
#### *Toc* protein–protein interactions in the protoplast cytosol

To test whether the fusions to the membrane proteins *atToc33* and *atPex11e* insert into membranes, the split-



**Fig. 5.** Toc and Pex protein interactions in the plant split-ubiquitin system. (A) Tobacco protoplasts were co-transformed with Nub and Cub constructs as indicated (a–f). (B) Total proteins were extracted and analysed by western blotting using antibodies raised against Toc159G to check for the presence of Nub–Toc159G or anti-HA for the Cub fusion proteins. Interacting protein pairs result in almost complete cleavage of GFP [NubToc159G and Toc33-HA-Cub-GFP (b), NubPex11e and Pex11e-HA-Cub-GFP (d)] and non-interacting protein pairs result in partial background cleavage of the reporter gene [Nub and Toc33-HA-Cub-GFP (a), Nub and Pex11e-HA-Cub-GFP (c), Nub-Toc159G and Pex11e-HA-Cub-GFP (e), Nub-Pex11e and Toc33-HA-Cub-GFP (f)]. The graphs below show the results of chemiluminescence quantification of three independent experiments.

ubiquitin experiments shown in Fig. 5 were repeated including an additional cell fractionation step. Extracts of transformed tobacco protoplasts were centrifuged at 100 000 g to separate soluble proteins (Fig. 6, S ‘soluble’) from membrane proteins (Fig. 6, P ‘pellet’). Western blot analysis with anti-HA revealed that both the uncleaved and cleaved forms of full-length Toc33-HA-Cub-GFP were predominantly located in the soluble fraction (Fig. 6a, b, S). Only upon co-expression of Nub-Toc159G was a small portion of cleaved Toc33-HA-Cub detected in the 100 000 g pellet fraction (Fig. 6b, P). These data suggest that the C-terminal HA-Cub-GFP fusion prevents insertion of *atToc33* into the membrane, and that only upon cleavage of the bulky GFP is *atToc33* membrane insertion possible. Therefore, the interaction observed between Nub-Toc159G and full-length Toc33-HA-Cub-GFP in the plant split-ubiquitin system most probably occurs in the protoplast cytosol. The uncleaved and cleaved fusions of the second membrane protein tested,



**Fig. 6.** Membrane association of full-length Toc33 and Pex11e in plant split-ubiquitin assays. (A) Tobacco protoplasts were co-transformed with Nub and Cub constructs as indicated (a–d). (B) Co-transformed protoplasts were lysed and separated into soluble and pellet fractions by centrifugation at 100 000 *g* for 1 h. Equal amounts of protein of non-fractionated protoplasts (N), soluble (S), and pellet (P) fractions were analysed by immunoblotting with antibodies against Toc159G, the HA epitope, Toc75, and phosphoribulokinase (PRK). Toc75 and PRK served as the membrane and soluble marker, respectively. Co-expression of Nub–Toc159G with full-length Toc33–HA–Cub–GFP (b) or Nub–Pex11e with Pex11e–HA–Cub–GFP (d) resulted in increased cleavage. Uncleaved and cleaved forms of the Toc33–HA–Cub fusion (a, b) are mainly present in the soluble fraction, suggesting inhibition of Toc33 membrane insertion by the C-terminal fusion part. In marked contrast, uncleaved and cleaved fusions of the integral membrane protein Pex11e (c, d) are both located in the pellet fraction.

*atPex11e*, were mainly located in the insoluble fraction (Fig. 6c, d, P). This indicates that in contrast to Toc33, membrane insertion of *atPex11e* is probably not affected by the C-terminal fusion partner. Moreover, it appears likely that the observed *atPex11e* self-interaction occurs at the target membrane.

## Discussion

In response to an increasing interest in *in vivo* protein–protein interaction data, a variety of *in vivo* protein–protein

interaction assay systems have been developed in the recent past. Many of these are based on protein fragment complementation and have been demonstrated to be applicable to plant cells as well (Subramaniam *et al.*, 2001; Bhat *et al.*, 2006; Ehlert *et al.*, 2006; Fujikawa and Kato, 2007; Kerppola, 2008). The receptor GTPases at the chloroplast outer surface are presumed to undergo short-lived and dynamic interactions with chloroplast pre-proteins and among themselves. Therefore, an *in vivo* protein–protein interaction assay system is required that allows for the analysis of transient protein–protein interactions at the cytosolic face of organelles. In the present study, the yeast split-ubiquitin system possessing the characteristics desired for plant cells was adapted, and the interaction between *atToc159* and *atToc33* as well as *atPex11e* self-interaction were demonstrated.

### *Toc* GTPase heterodimerization *in vivo*

In many *in vitro* studies, homo- or heterodimerization of the G-domains of *atToc33* and *atToc159* has been observed (Hiltbrunner *et al.*, 2001b; Bauer *et al.*, 2002; Smith *et al.*, 2002; Sun *et al.*, 2002; Weibel *et al.*, 2003; Reddick *et al.*, 2007; Yeh *et al.*, 2007; Oreb *et al.*, 2008). Working with recombinant or *in vitro* translated proteins, stable homodimers of *atToc159* and *atToc33* are much more easily obtained than heterodimers, leading to the assumption that *atToc159* and *atToc33* do not form stable heterodimers or that heterodimers are formed only transiently *in vivo* (Li *et al.*, 2007). A short-lived interaction between *atToc159* and *atToc33* fits well with a model of a dynamic, nucleotide-dependent Toc GTPase cycle in chloroplast protein import. In the present work, the *in vivo* heterodimerization between the Toc GTPases *atToc159* and *atToc33* is demonstrated for the first time in three different interaction assay systems: (i) the yeast two-hybrid system; (ii) the yeast split-ubiquitin system; and (iii) the plant split-ubiquitin system. The latter was especially developed for this purpose. Surprisingly, and in contrast to *in vitro* studies mentioned above, it was not possible to observe *atToc33G–atToc33G* or *atToc159G–atToc159G* homodimerization in the yeast two-hybrid system (data not shown). For this reason, studies on homodimerization using split-ubiquitin were not pursued further. However, the present results supply evidence that heterodimerization indeed occurs *in vivo*. This supports the leading hypotheses of pre-protein translocation across the outer chloroplast membrane in which heterodimerization between the G-domains of Toc33 and Toc159 is central (Bedard and Jarvis, 2005). Both *atToc159* and *atToc33* are receptors for chloroplast pre-proteins. In the current models, the *atToc159* and *atToc33* receptor–receptor interaction has been implicated in the pre-protein transfer from one receptor GTPase to the other before pre-protein insertion into the *atToc75* channel. The mechanistic details of the Toc complex remain for the most part unresolved. For example, it is not clear which of the two GTPases acts as the initial receptor, making the first contact with the pre-protein, and whether pre-protein binding occurs to a receptor monomer or to a receptor dimer. The published



stoichiometry for the pea Toc complex (1:4–5:4 for *psToc159:psToc34:psToc75*) contradicts the existence of Toc159 dimers but favours the existence of Toc33 homodimers in the Toc complex (Schleiff *et al.*, 2003). Recent studies indicate that Toc33 homodimers are most probably not self-activated and might need the exchange of one homodimeric subunit by Toc159 for activation (switch hypothesis) (Koenig *et al.*, 2008a, b). Thus the physiological role of *atToc159–atToc33* heterodimerization in the Toc complex might be acceleration of GTP hydrolysis, and pre-protein transfer could be directly linked to this process. Currently, the sole evidence for this interaction stems from *in vitro* experimentation using recombinant proteins. The present results indicate that G-domain heterodimerization occurs in the *in vivo* setting, thereby lending support to a critical element in the prevalent models of chloroplast outer membrane translocation. To gather more information on the residues involved in *atToc159–atToc33* heterodimerization, the yeast two-hybrid interaction may be used as a tool to screen for mutations altering the binding properties of *atToc159G* for *atToc33G* and vice versa (Steffan *et al.*, 1998). The resulting mutations could subsequently be further tested *in planta* using the split-ubiquitin system. Cell fractionation using ultracentrifugation demonstrated that the interaction between full-length *atToc159G* and *atToc33* observed in the plant split-ubiquitin system occurred almost entirely in the cytosol and not at the chloroplast membrane (Fig. 6). Most probably, the bulky C-terminal GFP fusion interfered with *atToc33* membrane insertion. These data suggest that the C-terminus of *atToc33* must be freely accessible for membrane insertion. This is supported by the insertion of a small portion of Toc33-HA-Cub upon cleavage of the GFP. In general, for a split-ubiquitin experiment involving an integral membrane protein to be successful the fusion proteins have to be designed carefully as the topology as well as the presumed targeting mechanism have to be considered. The Nub and Cub fusion parts have to be located in the cytosol and may not interfere with membrane targeting. According to the results of the cell fractionation experiment conducted here, the next generation of experiments will be performed using N-terminal Nub or Cub fusions to *atToc33*.

#### *AtPex11e* self-interaction

At the start of this study homodimerization had been reported of Pex11 and Pex11-related proteins from yeast (Eckert and Johnsson, 2003; Tam *et al.*, 2003; Rottensteiner *et al.*, 2003) and mammals (Li and Gould 2003). No such data were available on physical interaction of the *Arabidopsis* Pex11 family comprising five members (a–e). By means of the plant split-ubiquitin experiments carried out in this study, it was possible to show *in vivo* homodimerization of *atPex11e*. In the case of *atPex11e* (in contrast to Toc33-HA-Cub-GFP) the C-terminal Cub–GFP fusion was almost entirely present in the membrane pellet after centrifugation at 100 000 *g* (Fig. 6). The C-terminal GFP therefore did not appear to interfere with membrane insertion. This result

(Fig. 6) demonstrates that the plant split-ubiquitin may be useful to determine and analyse interactions between integral membrane proteins and allow conclusions regarding molecular constraints of the insertion mechanism. As plant split-ubiquitin worked successfully for *atPex11e*, it is most probably a suitable assay system to test for dimerization of the remaining *Arabidopsis* isoforms as well. In a recently published study (Lingard *et al.*, 2008), homo- and hetero-oligomerization of all five Pex11p isoforms at the peroxisome membrane have been demonstrated by bimolecular fluorescence complementation (BiFC). The observation of *atPex11e* self-interaction by another *in vivo* interaction system further substantiates the usefulness of plant-split ubiquitin.

#### *Future modification and improvement of the plant split-ubiquitin system*

For the future use of the plant split-ubiquitin system, further improvement, particularly with regard to the reduction of background cleavage, is recommended. A higher level of background cleavage was observed in the plant than in the yeast split-ubiquitin assays. This is not due to a higher rate of spontaneous *in vivo* association of the Nub and Cub fragments in plants as the same level of background cleavage was observed when the Cub fusion proteins were expressed in the absence of free Nub or Nub fusion proteins (Fig. 4D, a, and data not shown). Possible explanations are that substrate recognition by plant UBPs is less dependent on a complete ubiquitin moiety or that the overall activity of UBPs in plants is higher than in yeast. The latter appears likely as about twice as many deubiquitinating enzymes (DUBs) have been identified in *A. thaliana* compared with *S. cerevisiae* (Yang *et al.*, 2007). Reduction of the background cleavage in the plant split-ubiquitin system could be achieved by performing the assays in protoplasts derived from mutant plants in which selected, non-essential UBPs are knocked out.

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