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The yeast split-ubiquitin system to study chloroplast membrane protein interactions

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Abstract Each photosynthetic complex within the thylakoid membrane consists of several different subunits. During formation of these complexes, numerous regulatory factors are required for the coordinated transport and assembly of the subunits. Interactions between transport/assembly factors and their specific polypeptides occur in a membraneous environment and are usually transient and short-lived. Thus, a detailed analysis of the underlying molecular mechanisms by biochemical techniques is often difficult to perform. Here, we report on the suitability of a genetic system, i.e. the yeast split-ubiquitin system, to investigate protein–protein interactions of thylakoid membrane proteins. The data confirm the previously established binding of the cpSec-translocase subunits, cpSecY and cpSecE, and the interaction of the cpSec-translocase from *Arabidopsis thaliana* with Alb3, a factor required for the insertion of the light-harvesting chlorophyll-binding proteins into the thylakoid membrane. In addition, the proposed interaction between D1, the reaction center protein of photosystem II and the soluble periplasmic PratA factor from *Synechocystis* sp. PCC 6803 was verified. A more comprehensive analysis of Alb3-interacting proteins revealed that Alb3 is able to form dimers or oligomers. Interestingly, Alb3 was also shown to bind to the PSII proteins D1, D2 and CP43, to the PSI reaction center protein PSI-A and the ATP synthase subunit CF_0III, suggesting an important role of Alb3 in the assembly of photosynthetic thylakoid membrane complexes.

Introduction

The major photosynthetic complexes PSI, PSII, the cytochrome b_6f complex and the ATP synthase complex are located in the thylakoid membrane. Each of them consists of several subunits, which are encoded in either the chloroplast or in the nuclear genome. The biogenesis of these multi-subunit complexes requires the targeting of the single components to the thylakoid membrane, followed by the ordered assembly into high molecular weight structures. To date, four different pathways for the post-translational transport of nuclear-encoded thylakoid proteins have been described. They are called the cpTat, the cpSec, the cpSRP and the spontaneous integration pathway (for recent reviews, see Jarvis and Robinson 2004; Schünemann 2004). The cpSRP pathway that specifically mediates the transport of members of the nuclear-encoded light-harvesting chlorophyll-binding protein family (LHCPs), requires the integral membrane protein Alb3 for insertion of the LHCPs into the membrane (Sundberg et al. 1997; Moore et al. 2000). Several thylakoid membrane proteins (e.g. the reaction center proteins of PSI and PSII, PSI-A, PSI-B, D1, D2,) are encoded by the chloroplast genome and are co-translationally inserted into the thylakoid membrane. Analysis of the molecular process of D1 insertion revealed that it is mediated by the cpSec-translocase (Zhang et al. 2001). Studies with *Chlamydomonas reinhardtii* demonstrated that the loss of Alb3 led to a reduction of D1 (Bellafiore et al. 2002), and that Alb3 is involved in the assembly of D1 in PSII (Ossenbühl et al. 2004). Together, these results suggest that Alb3 can function in concert with the cpSec-translocase, supporting earlier findings that Alb3 is at least partially associated with the cpSec-complex (Klostermann et al. 2002). Moreover, in cyanobacteria, the PratA factor has been shown to be involved in PS II assembly by affecting the C-terminal processing of the D1 protein (Klinkert et al. 2004).

Despite the recent progress in identifying factors involved in protein transport, little is known about the molecular details of protein–protein interactions occurring during protein insertion and complex assembly. This is partly due to the difficulties in isolating and handling of the hydrophobic membrane proteins in biochemical experiments. The split-ubiquitin system employed in this study enables the analysis of membrane protein interac-

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tions in vivo. Initially, this genetic method was developed to analyse the interactions of membrane proteins of yeast but has since been successfully applied to study protein interactions in several different organisms (Johnsson and Varshavsky 1994; Stagljar et al. 1998; Fetchko and Stagljar 2004). The split-ubiquitin system is based on the possibility to divide ubiquitin into two parts, an N-terminal half (Nub) and a C-terminal half (Cub). When allowed to interact, the two parts spontaneously reassemble to reconstitute ubiquitin. A point mutation introduced into wild-type Nub (*Nub*I) created *Nub*G, which exhibits a considerably lower affinity to Cub (Stagljar et al. 1998). *Nub*G and Cub are only able to reconstitute ubiquitin when brought into close proximity by two interacting test proteins that are expressed as fusion proteins with *Nub*G and Cub. An ubiquitin-specific protease subsequently cleaves Cub at the C-terminus, releasing a transcriptional activator, which results in an activation of reporter genes.

It has recently been reported that the split-ubiquitin system can be used to investigate the intra- and intermolecular interactions of plant plasma membrane proteins such as sucrose transporters (Reinders et al. 2002a,b), ammonium transporters (Ludewig et al. 2003), K^+-channels (Obrdlik et al. 2004) and membrane proteins like TOM2 (Tsujimoto et al. 2003). Here, we report on the feasibility of this genetic system for the study of thylakoid membrane protein interactions. Our data show that the split-ubiquitin system provides a useful tool for the elucidation of processes like protein insertion and protein complex assembly within chloroplast membranes.

Materials and methods

Construction of plasmids for the split-ubiquitin system

To construct the bait plasmids, the vectors pTMBV4 and pAMBV4 encoding the Cub fragment were used. The reporter in these plasmids consists of the LexA-DNA-binding domain and the VP16-activation domain (TF) (Dualsystems Biotech AG). From the vector pADSL-Nx, which encodes the Nub fragment, the prey plasmids were constructed (Dualsystems Biotech AG). The vector pADSL-Nx also encodes a hemagglutinin–epitope tag. The mature full-length genes of cpSecY, cpSecE, Alb3 and Lhcb1 and the deletion constructs $Alb3_{57–350}$, $cpSecE_{41–133}$ and $cpSecE_{122–177}$ were obtained by polymerase chain reaction (PCR) amplification from plasmids carrying the desired genes. The complete gene sequences of D1, D2, CP43, PSI-A and CF_0III were amplified by PCR using total DNA, isolated from *Arabidopsis thaliana* (Columbia 0) by Nucleon Phytopure Kit (Tepnel), as the template. Similarly, *psbA* and *PratA* genes from *Synechocystis* were amplified from total cyanobacterial DNA. The primer combinations for all PCR are shown in Table 1. The proof-reading polymerase DyNAzyme EXT (Finnzymes) was used in all PCR set-ups. The PCR products were restricted as described in Table 1 and then cloned into the corresponding restriction sites of the bait or prey vector. In each case, successful insertion of the DNA fragments into the plasmids was verified by sequencing.

Yeast two-hybrid assay

Yeast strain DSY-1 (Dualsystems Biotech AG) was co-transformated with the indicated combinations of bait and the prey plasmids as described previously (Jonas-Straube et al. 2001). Half of the cells were plated onto synthetic medium lacking leucine and tryptophan (−LT). The other half was plated onto synthetic medium lacking leucine, tryptophan and histidine (−LTH). All culture media contained 0.004% adenine. Yeast cells were incubated for 3

Table 1 Primers used for PCR amplification

Forward (f) and reverse (r) primer pTBMV4/pAMBV4 constructs *A. thaliana*	
fcpSecY (*Xba*I)	5′-ctatctagacaaaaatggctattgaggacagttcc-3′
fAlb3 (*Xba*I)	5′-ctatctagacaaaaatgagcttaaacgagattcctc-3′
rcpSecY (*Nco*I)	5′-gtaccatggagtggatcatacttgtcaag-3′
rAlb3 (*Stu*I)	5′-atgaggccttttacagtgcgtttccgctt-3′
Forward (f) and reverse (r) primer pADSL-Nx constructs *A. thaliana*	
fcpSecE (*Bam*HI)	5′-gctggatccagtaatctg-3′
$fcpSecE_{41–133}$ (*Bam*HI)	5′-gctggatccagtaatctg-3′
$fcpSecE_{122–177}$ (*Bam*HI)	5′-gctggatccgaagtgaaggagattgag-3′
fcpSecY(*Bam*HI)	5′-gctggatccgctattgaggacagttcc-3′
fAlb3 (*Eco*RI)	5′-gagaattcgagcttaaacgagattcct-3′
fD1(*Eco*RI)	5′-gagaattcgatgactgcaattttagag-3′
fD2(*Eco*RI)	5′-gagaattcgatgactatagcccttggt-3′
fCP43 (*Eco*RI)	5′-gagaattcgatgaaaaccttatattcc-3′
fPSI-A (*Eco*RI)	5′-gagaattcgatgattattcgttcgccg-3′
fCF0III (*Eco*RI)	5′-gagaattcgatgaatccactggtttct-3′
fLhcb1 (*Bam*HI)	5′-gctggatccatgaggaagtctgctacc-3′
rcpSecE (*Sal*I)	5′-gtagtcgactcagctgaagaagtcttgaac-3′
$rcpSecE_{41–133}$ (*Sal*I)	5′-gtagtcgactcatttctgaaacgccggcc-3′
$rcpSecE_{122–177}$ (*Sal*I)	5′-gtagtcgactcagctgaagaagtcttgaac-3′
rcpSecY (*Sal*I)	5′-gtagtcgactcatggatcatacttgtcaag-3′
rAlb3 (*Sal*I)	5′-gtagtcgacttatacagtgcgtttccgctt-3′
rD1 (*Sal*I)	5′-gtagtcgacttaccatttgtagatgg-3′
rD2 (*Sal*I)	5′-gtagtcgacttaaagagcgtttccacg-3′
rCP43 (*Sal*I)	5′-gtagtcgacttagttaagaggagtcat-3′
rPSI-A (*Sal*I)	5′-gtagtcgacttatcctactgcaataat-3′
rCF0III (*Sal*I)	5′-gtagtcgacttaaacaaaaggattcgc-3′
rLhcb1 (*Sal*I)	5′-gtagtcgacttattttccgggaacaaagttg-3′
Forward (f) and reverse (r) primer pTBMV4 constructs *Synechocystis*	
fD1 (*Xba*I)	5′-atttctagaaaaaatgacaacgactctccaaca-3′
rD1 (*Stu*I)	5′-attaggcctaaaccgttgacagcaggag-3′
Forward (f) and reverse (r) primer pADSL-Nx constructs *Synechocystis*	
fD1 (*Bam*HI)	5′-attggatccatgacaacgactctccaaca-3′
fPratA (*Bam*HI)	5′-attggatccatgaatttacgctacctctg-3′
rD1 (*Eco*RI)	5′-attgaattcttaaccgttgacagcaggag-3′
rPratA (*Eco*RI)	5′-attgaattcctagagattatccagctttt-3′

Restriction sites are underlined

days at 30°C. Activation of the −his/*lacZ* reporter genes was judged by measuring the cell growth and assessing the β-galactosidase activity by using the filter-lift assay (Breeden and Nasmyth 1985). Growth was classified as (+++), (++), (+), and (−). Growth labelled (+++) meant that the plates were virtually completely covered with colonies, and (−) meant no growth of yeast cells was observed. The filter lifts were incubated for 2 h to develop a blue colour (+) or no blue colour (−). As a positive control for pTMBV4/pAMBV4-constructs, we co-transformed yeast cells with the plasmid pAlg5-*Nub*I, which encodes the Alg5 protein fused to the *Nub*I fragment. Alg5 is an unrelated resident ER protein encoding a yeast dolichyl–phosphate glucosyltransferase. As negative controls for the pTMBV4/pAMBV4 constructs, the plasmid pAlg5-*Nub*G and pADSL-Nx expressing soluble *Nub*G were used for the co-transformations. To test the specificity of the pADSL-Nx constructs, they were co-transformed with the control-plasmid pMBV-Alg5 (Dualsystems Biotech AG), which encodes the Alg5 protein fused to the Cub fragment and the reporter and with the empty pTMBV4/pAMBV4 plasmids expressing soluble Cub.

Preparation of total protein extracts

To prepare total yeast extracts, three yeast colonies expressing either Cub-TF- or *Nub*G-fusion proteins were inoculated into 10 ml of synthetic medium, lacking leucine or tryptophane, respectively, and incubated overnight at 30°C to an OD_{600} of 1. The cells were spun down at 700 g for 5 min in a 14-ml Falcon tube. The pellet was resuspended in SD medium, and the solution transferred into 1.5-ml reaction tubes. After a further centrifugation step, 200 μl 2× SDS sample buffer and 100 μl glass beads were added to lyse the cells. These suspensions was vortexed vigorously for 1 min and was then incubated for 3 min at 98°C. After centrifugation, the supernatant was transferred into new tubes, and a sample volume of 10 μl was used for gelelectrophoresis.

Preparation of cytosole and membrane extracts

Yeast colonies expressing Cub-TF- or *Nub*G-fusion proteins were inoculated into 200-ml SD medium and incubated at 30°C overnight to an OD_{600} of 1. The cells were harvested at 700 g for 5 min. The pellets were re-suspended in 3 ml of cold lysis buffer [50 mM Tris–HCl pH 7.5 containing a protease inhibitor cocktail (Roche)]. The suspension was transferred into 2-ml tubes, and 200 μl of glass beads were added. Next, the cells were vortexed vigorously for 30 s, cooled on ice for 1 min and vortexed again. This step was repeated seven times. The samples were centrifuged at 700×*g* for 20 min at 4°C. The supernatant was carefully transferred into new 1.5-ml reaction tubes and spun down again for 10 min. The supernatant was transferred into polyallomer tubes and spun down for 2 h at 150,000 g at 4°C. The supernatant (cytosolic fraction) was taken off, and 1 ml of the suspension was precipitated using trichloracetic acid. The resulting pellet was washed with 200 μl cold lysis buffer containing protease inhibitors. Then 200 μl lysis buffer containing 1% Triton X-100 was added, and the pellet was resuspended. The suspension was centrifuged again for 2 h at 150,000×*g*, and only the supernatant was kept. The protein extracts were concentrated by methanol–chloroform precipitation (Wessel and Flügge 1984). The precipitated proteins were resuspended in SDS sample buffer and analysed using gelelectrophoresis.

Western blot analysis

Standard protocols were used for SDS-Page and Western Blot analysis of the protein extracts. To detect bait proteins (encoded by the pAMBV4 or pTMBV4 constructs) the rabbit anti-LexA polyclonal antibody was used (1:5,000 dilution, Invitrogen). To locate the prey proteins (encoded by the pADSL-Nx constructs) the mouse anti-HA monoclonal antibody was used (1:2,500 dilution, Roche).

Results

The cpSec-translocase consists of the known integral thylakoid membrane proteins cpSecE and cpSecY. CpSecE spans the thylakoid membrane once, whereas cpSecY contains ten transmembrane helices. The subunits are known to form a stable complex that is involved in the translocation of proteins across the thylakoid membrane (Schuenemann et al. 1999). To test whether the split-ubiquitin system is suitable for investigating interactions between thylakoid membrane proteins, cpSecY was cloned into the vector pTMBV4 to generate the fusion protein cpSecY-Cub-TF, and cpSecE was cloned into the vector pADSL-Nx to generate the fusion protein *Nub*G-cpSecE. The *S. cerevisiae* strain *DSY-1* carrying the *lacZ* and *HIS3* reporter genes was co-transformed with both plasmids and plated onto medium lacking histidine (−His). After 3 days of incubation, a strong growth of yeast cells together with high β-galactosidase levels was observed, indicating a stable interaction of cpSecY and cpSecE in the yeast cells (Fig. 1a, Table 2). Activation of *HIS3* and *lacZ* was also present in cells co-expressing cpSecY-Cub-TF and the Alg5-*Nub*I construct. The latter is a fusion of the resident yeast ER protein Alg5 with wild-type Nub and served as a positive control. This result confirms that cpSecY-Cub-TF is expressed in these cells, and that its reporter moiety Cub-TF is properly positioned at the cytosolic side of the membrane. In contrast, expression of the negative control combinations cpSecY-Cub-TF together with Alg5-*Nub*G, or *Nub*G-cpSecE together with Alg5-Cub-TF, did not lead to an activation of the reporter genes (Fig. 1a, Table 2). To gather further evidence that cpSecY-Cub-TF is suitable to be used as a bait protein in the split-ubiquitin system, Western blot experiments using anti-LexA antibodies and

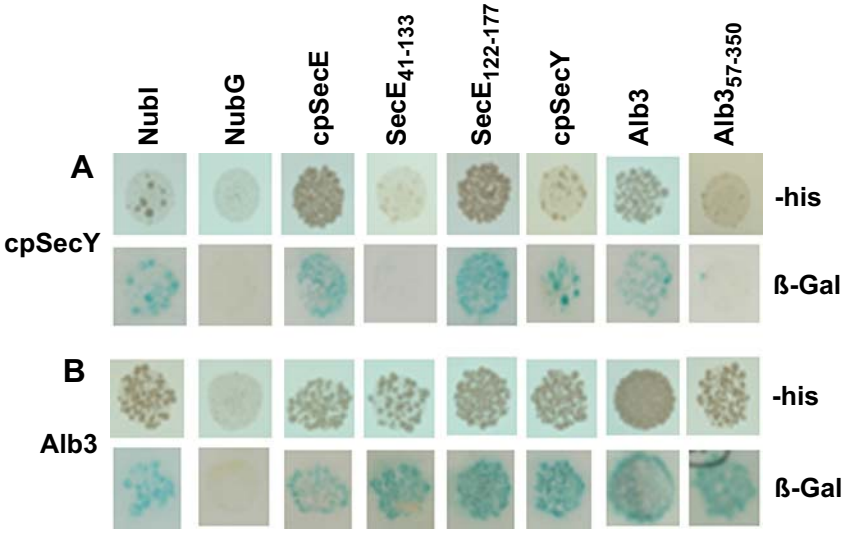

Fig. 1 Growth and β-galactosidase activity of yeast cells expressing cpSecY-Cub-TF (**a**) or Alb3-Cub-TF (**b**) with different *Nub*G-fusion proteins. Yeast cells containing the indicated combinations of bait and prey proteins were spotted onto minimal medium lacking leucine, tryptophane and histidine and grown for 3 days at 30°C (−*his*). The activity of β-galactosidase (*β-gal*) was visualised by filter assays (*blue colour* development is positive). The construct Alg5-*Nub*I was used as positive control, and the construct Alg5-*Nub*G as negative control. The constructs $cpSecE_{41–133}$ and $cpSecE_{122–177}$ correspond to the stromal N-terminal part and the transmembrane region including the C-terminus of mature full-length cpSecE (amino acids 41–177), respectively. The construct $Alb3_{57–350}$ was generated by the deletion of the 112 C-terminal amino acids of mature full-length Alb3 (amino acids 57–462)

protein extracts of yeast cells expressing cpSecY-Cub-TF were performed. A single protein with an apparent molecular mass of 82 kD was detected in the membrane fraction of these yeast cells (Fig. 2a). No cross reaction occurred with the cytosolic extract (Fig. 2a) nor with protein samples from non-transformed yeast cells (data not shown). These results prove that full-length cpSecY-Cub-TF is exclusively situated in the membrane fraction of these yeast cells. Likewise, the presence of full-length *Nub*G-cpSecE (20 kD) in the membrane fraction of transformed yeast cells was confirmed using anti-HA antibodies (Fig. 2b).

To determine the part of cpSecE, which mediates the interaction with cpSecY, constructs encoding the N-terminal stromal part of mature cpSecE ($cpSecE_{41–133}$) and constructs encoding the transmembrane helix together with the short C-terminal part of the protein ($cpSecE_{122–177}$) were generated, and the interaction with cpSecY was tested. The construct $cpSecE_{122–177}$ interacted as strongly with cpSecY as full-length cpSecE, whereas no interaction occurred with $cpSecE_{41–133}$ (Fig. 1a, Table 2). To exclude a false-negative result, effective expression of $cpSecE_{41–133}$ was confirmed by Western blot analysis of total-cell extracts using anti-HA antibodies (Fig. 2b).

It has previously been demonstrated that Alb3 is at least partially associated with the cpSec-translocase (Klostermann et al. 2002). To test whether the split-ubiquitin system can be used to characterise the nature of this interaction in more detail, the interaction of cpSecY-Cub-TF with *Nub*G-Alb3 was analysed. As shown in Table 2 and Fig. 1a, strong binding between these proteins was observed. In-

Table 2 Protein–protein interactions between subunits of the cpSecY/E translocase and Alb3

pADSL-Nx	pTMBV4-cpSecY			pAMBV4-Alb3			pMBV4-Alg5	
	-his	β-gal	*n*	-his	β-gal	*n*	-his	*n*
Alg5-*Nub*I	+	+	2	++	+	5	+	1
Alg5-*Nub*G	−	−	2	−	−	7	−	1
cpSecE	+++	+	7	++	+	4	−	4
$cpSecE_{41–133}$	−	−	3	+	+	2	−	1
$cpSecE_{122–177}$	+++	+	3	++	+	2	−	1
cpSecY	+	+	3	++	+	3	−	5
Alb3	++	+	2	+++	+	5	−	4
$Alb3_{57–350}$	−	−	3	++	+	3	−	2

Yeast strain DSY-1 was co-transformed with pTMBV4-cpSecY, pAMBV4-Alb3 or the control vector pMBV4-Alg5 and the indicated *Nub*G-fusion proteins. Activation of the −his and *lacZ*-reporter genes was determined as described in Materials and methods. No activation of reporter genes was seen in control experiments using empty pTMBV4/pAMBV4 and pADSL-Nx plasmids expressing soluble Cub and *Nub*G, respectively (data not shown)

n Number of independent experiments

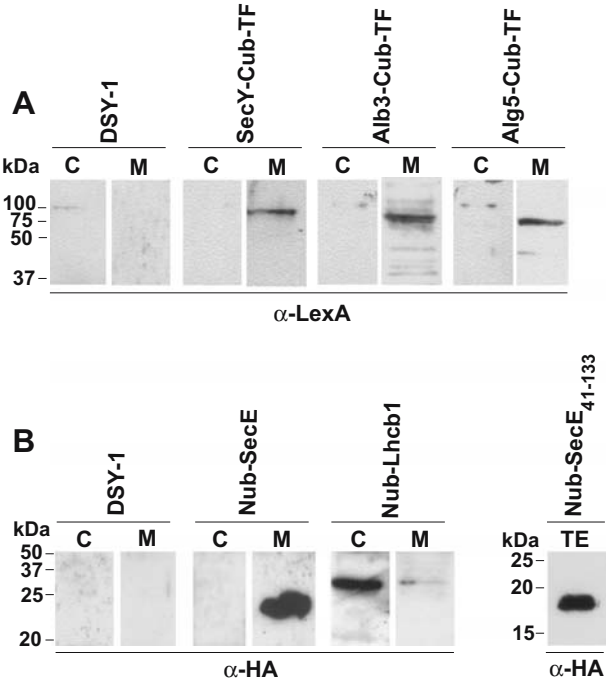

Fig. 2 Western analysis of yeast cell extracts containing Cub-TF or *Nub*G-fusion proteins. Yeast cells were transformed with the indicated pAMBV4, pTMBV4 or pADSL-Nx constructs and grown in minimal medium lacking either leucine or tryptophane. The transformed yeast cells were either used for the preparation of total protein extracts (*TE*) or for the preparation of a cytosolic (*C*) and a membrane fraction (*M*) as described in Materials and methods. **a** Western blots of yeast cell extracts expressing Cub-TF-fusion proteins using α-LexA antibodies. **b** Western blots of yeast cell extracts expressing *Nub*G-fusion proteins using α-HA antibodies. Lanes labelled with DSY-1 contained cell extracts from the untransformed yeast strain DSY-1 as negative controls

terestingly, removal of the C-terminus of Alb3 (construct $Alb3_{57–350}$) led to a complete loss of binding to cpSecY, suggesting that this region of Alb3 is crucial for its interaction with cpSecY. To confirm the binding between Alb3 and cpSecY, Alb3 was cloned as bait in the vector pAMBV4 to generate Alb3-Cub-TF. The induction of the reporter genes in cells expressing Alb3-Cub-TF and Alg5-*Nub*I and the lack of reporter gene activation in cells expressing Alb3-Cub-TF together with Alg5-*Nub*G proved that the Alb3-Cub-TF construct was able to serve as bait (Fig. 1b, Table 2). The expression of full-length Alb3-Cub-TF in the membrane fraction of yeast cells transformed with pAMBV4-Alb3 was confirmed by Western blot (Fig. 2a). As expected, co-expression of Alb3-Cub-TF and *Nub*G-cpSecY resulted in strong induction of the reporter genes. However, binding of Alb3 to the cpSec-translocase seems not to be mediated exclusively by the interaction with cpSecY, because an interaction was also observed between Alb3-Cub-TF and *Nub*G-cpSecE (Fig. 1b, Table 2). As shown in Fig. 1b and Table 2, Alb3-Cub-TF is also able to interact with *Nub*G-Alb3, suggesting that Alb3 is able to form dimers or oligomers. In contrast to the complex formation with cpSecY, the C-terminus of Alb3 is not essential for oligomerization, because binding of Alb3-Cub-TF to *Nub*G- $Alb3_{57–350}$ was observed in the split-ubiquitin system (Fig. 1b, Table 2).

Recently, it has been shown that Alb3 is involved in assembly of D1 in PSII from *C. reinhardtii* and co-immunoprecipitation experiments indicated that there is direct contact between Alb3 and D1 (Ossenbühl et al. 2004). Therefore, we wanted to test whether a direct interaction between Alb3 and D1 from *A. thaliana* can be observed in the split-ubiquitin system. Combining Alb3-Cub-TF and *Nub*G-D1 resulted in a strong activation of *HIS3* and *lacZ* as shown in Table 3. We were also able to demonstrate that Alb3 interacts with other chloroplast-encoded subunits of PSII like D2 and CP43 (Table 3). In addition, binding of Alb3 to the PSI reaction center protein PSI-A and the ATP synthase subunit CF_0III was observed (Table 3). Taken together, these results confirm the important function of Alb3 in PSII assembly, and suggest that Alb3 might play a general role in the biogenesis of chloroplast-encoded thylakoid membrane proteins.

Alb3 is also required for the post-translational cpSRP-dependent insertion of Lhcb1 into the thylakoid membrane (Moore et al. 2000). We therefore tested whether Alb3 is able to interact with Lhcb1 in the split-ubiquitin system. As shown in Table 3, no interaction between Alb3-Cub-TF and *Nub*G-Lhcb1 was detected. However, Western blot experiments showed that only a small amount of *Nub*G-Lhcb1 is located in the membrane fraction, whereas most is present in the cytosolic fraction of the transformed yeast cells (Fig. 2b). The lack of interaction between Alb3

Table 3 Interaction of cpSecY-Cub-TF or Alb3-Cub-TF with different *Nub*G-fusion proteins

pADSL-Nx	pTMBV4-cpSecY			pAMBV4-Alb3			pMBV4-Alg5	
	-his	β-gal	*n*	-his	β-gal	*n*	-his	*n*
D1	−	−	2	++	+	7	−	3
D2	−	−	2	++	+	3	−	3
CP43	/	/	/	++	+	2	−	1
PSI-A	−	−	2	++	+	4	−	1
CF_0III	/	/	/	++	+	2	−	2
Lhcb1	−	−	4	−	−	4	−	2

Yeast strain DSY-1 was co-transformed with pTMBV4-cpSecY, pAMBV4-Alb3 or the control vector pMBV4-Alg5 and the *Nub*G-fusion proteins listed below. Activation of the -his and *lacZ*-reporter genes was determined as described in Materials and methods
n Number of independent experiments, / not analysed

Table 4 Interaction of D1 and PratA from *Synechocystis* in the split-ubiquitin system

pADSL-Nx	pTMBV4-D1		pTMBV4-SecY		pMBV4-Alg5
	-his	β-gal	-his	β-gal	-his
Alg5-*Nub*I	+	+	+	+	+
Alg5-*Nub*G	−	−	−	−	−
PratA	+	+	−	−	−
D1	+	+	/	/	−

Yeast cells expressing the indicated combinations of D1-Cub-TF, cpSecY-Cub-TF or Alg5-Cub-TF, together with PratA-*Nub*G, were plated onto minimal medium lacking histidine. Activation of the −his and *lacZ*-reporter was measured as described in Materials and methods. CpSecY-Cub-TF and Alg5-Cub-TF were used as negative controls. Each experiment was repeated three times

/ Not analysed

and Lhcb1 may therefore have been due to the incorrect expression of Lhcb1 in the transformed yeast cells.

Recently, Zhang et al. published that the thylakoid membrane protein cpSecY is involved in the insertion of D1 by interacting transiently with D1 intermediates during elongation (Zhang et al. 2001). However, in contrast to the clear interaction between Alb3 and D1, we detected no interaction between cpSecY and D1 using the split-ubiquitin system. Similarly, no interaction between cpSecY and D2, or the reaction center protein of PSI, PSI-A, was observed (Table 3).

The experiments mentioned above concentrated on thylakoid membrane proteins from *A. thaliana*. As a further step, the feasibility of the split-ubiquitin system to study the interaction of proteins found in cyanobacteria was tested. Using a standard yeast two-hybrid system, binding of the soluble periplasmic protein PratA from *Synechocystis* spp. PCC 6803 to the soluble 68-amino-acids comprising C-terminus of the D1 protein had already been demonstrated (Klinkert et al. 2004). Our experiments using the split-ubiquitin system confirmed the binding of PratA to full-length D1 localized in the membrane (Table 4). Furthermore, the data suggest a dimerization of D1, which one would expect, considering the tight binding of D1 to the closely related D2 protein in PSII.

In summary, the split-ubiquitin system can be used to analyse interactions between thylakoid membrane proteins, but also to investigate binding between soluble proteins, like PratA, and thylakoid membrane proteins, like D1 for example.

Discussion

After the complete sequencing of the *Arabidopsis* genome, the functional analysis of the proteome will be a major task in plant research. One key aspect in understanding the function of a protein is the identification of its binding partners. This is particularly challenging with regards to integral membrane proteins, because their hydrophobic nature excludes the application of many biochemical techniques. In this work, we employed the split-ubiquitin system to study the interactions of thylakoid membrane proteins for the first time. First, we selected binding partners, whose physical interaction had previously been identified. Using the split-ubiquitin system, we confirmed the complex formation between cpSecY and cpSecE (Schuenemann et al. 1999), the association of Alb3 with the cpSecY/E complex (Klostermann et al. 2002), the interaction of Alb3 with D1 [previously shown for the corresponding proteins from *Chlamydomonas* (Ossenbühl et al. 2004)] and the interaction of the D1 protein from *Synechocystis* with the soluble protein Prat A (Klinkert et al. 2004). In addition, we demonstrated that Alb3 interacts strongly with itself, a result that had been anticipated because this feature is also described for YidC and Oxa1p, the bacterial and mitochondrial homologues of Alb3 (van der Laan et al. 2001; Nargang et al. 2002). We thus demonstrated that the split-ubiquitin system is suitable to discover and analyse thylakoid membrane protein interactions.

To ensure that the interactions detected by the split-ubiquitin system were specific for each individual protein, an unrelated membrane protein (Alg5) or the soluble ubiquitin domains Cub or *Nub*G were used as bait or prey proteins in control experiments. The observation that the outermost C-terminus of Alb3 seems to be essential for the interaction with cpSecY, but is not needed for dimerization, further supports the reliability of the split-ubiquitin system. The interaction of cpSecE with cpSecY is specifically mediated by the C-terminal part of cpSecE, which contains the transmembrane region and the outermost C-terminus. This result is consistent with the previous findings that the third, but not the first and second transmembrane domain of bacterial SecE, plays an important role in binding of SecY (Schatz et al. 1991; Kaufmann et al. 1999). The third transmembrane domain of bacterial SecE is homologous to the single transmembrane region of cpSecE.

All prey proteins studied in this work were expressed with *Nub*G fused to the N-terminus. The fact that the *Nub*G-fusion proteins were functioning in the split-ubiquitin system indicates that the N-terminus of these proteins is located at the cytosolic side of the yeast cell membrane. Provided that all molecules of a protein are inserted in the same orientation, one would expect that prey proteins with an uneven number of transmembrane helices would not interact with their corresponding bait partner if *Nub*G is added at the C-terminus. Therefore, we tested the interaction of cpSecY-Cub with cpSecE-*Nub*G and Alb3-Cub with D1-*Nub*G (data not shown). The observation that in neither case an interaction occurred indicates that in these set-ups, the C-terminal *Nub*G is not accessible for the cytosolic Cub domain and, hereby, validates the functionality of the split-ubiquitin system. However, expression levels of proteins containing *Nub*G at their C-terminus were considerably lower than those of the *Nub*G-fusion proteins (data not shown). Therefore, it is possible that the lack of interaction of cpSecE-*Nub*G and D1-*Nub*G was due to inadequate protein levels and not due to the orientation

of the *Nub*G-domain. Alb3 and D1 are predicted to have five transmembrane segments. In the split-ubiquitin system, both proteins can be used as constructs with Cub-TF fused to the C-terminus or *Nub*G fused to the N-terminus. This suggests that these proteins are inserted in the yeast membranes in different orientations.

In further experiments, we extended the study to identify novel interaction partners of Alb3. Alb3 is involved in the assembly of D1 into photosystem II, and evidence is accumulating that YidC, the bacterial homologue of Alb3, is involved in the assembly of a large number of membrane protein complexes like the SecYEG translocase or the F_0 sector of the bacterial F_1F_0 ATP synthase (Yi et al. 2003; van Bloois et al. 2004; recent review, Dalbey and Kuhn 2004). This led to the assumption that Alb3 might be involved in the integration or assembly of additional chloroplast-encoded thylakoid membrane proteins. In addition to D1, Alb3 did indeed clearly interact with other tested chloroplast-encoded thylakoid membrane proteins, namely, D2, CP43, PSI-A and the ATP synthase subunit CF_0III. These results suggest that Alb3 is involved in the insertion, folding or assembly of membrane proteins belonging to the major thylakoid protein complexes and, therefore, represents an important component in thylakoid membrane biogenesis. In contrast to the clear interaction between Alb3 and the subunits of the photosynthetic complexes, no interaction was seen between these proteins and cpSecY. In vivo, the interaction of cpSecY with D1 is transient and occurs while D1 is translated at the ribosome (Zhang et al. 2001). It seems reasonable to argue that cpSecY is not able to bind to full-length D1 in a fashion that would activate reporter gene expression in the split-ubiquitin system, and that the lack of interaction with cpSecY is therefore not surprising. The ability of Alb3 to interact with D1, D2, CP43, PSI-A and CF_0III in our system supports the current model of co-translational protein insertion that Alb3 probably acts downstream of cpSecY during or after the release of the substrate proteins from the cpSec-translocase.

In conclusion, we have shown that the split-ubiquitin system represents a suitable and easy-to-use technique to analyse the molecular details of protein–protein interactions between integral thylakoid membrane proteins or between membrane proteins and their soluble interaction partners. In addition, it provides a tool to screen for novel interaction partners using cDNA libraries, which can be cloned into the prey vector of the split-ubiquitin system.

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