DOI: 10.1007/s11099-012-0078-7 PHOTOSYNTHETICA 51 (1): 45-54, 2013

Phenotypes of Alb3p and carotenoid synthesis mutants show similarities regarding light sensitivity, thylakoid structure and protein stability

M. KUGELMANN, A. FAUSSER, F. OSSENBÜHL, and A. BRENNICKE+

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Abstract

Chloroplast proteins of the Alb3/YidC/Oxa1p family are necessary for assembly of photosynthetic complexes in the thylakoid membranes. Alb3p in *Arabidopsis thaliana* is essential for posttranslational LHCII-integration into thylakoid membranes and participates in cotranslational assembly of D1. However, the pleiotropic defects of an Alb3p mutant, *albino3*, suggest additional functions for Alb3p. To obtain an impression of such potential further Alb3p activities from phenotypic manifestations, properties of mutants disturbed in thylakoid membrane protein transport or carotenoid biosynthesis were compared with the *albino3* mutant. Specific defects observed in *albino3* were similar to those in a carotenoid synthesis mutant. While this correlation did not provide tangible evidence for Alb3p being involved in the integration of carotenoids in photosynthetic complexes, it suggests a possible avenue for future investigations.

Additional key words: *albino3*, *Arabidopsis thaliana*; carotenoids, hcf106, protein transport, thylakoid membrane.

Introduction

The thylakoid membrane protein Alb3p belongs to the conserved Alb3/Oxa1/YidC protein family. Members of this family are found in all membranes of prokaryotic origin, in archaea, bacteria, chloroplasts and mitochondria. Many organisms contain two or more homologs with different functions, *e.g*. YidC1 and YidC2 in many bacteria, Alb3p and Alb4p in chloroplasts and Oxa1p or Cox18/Oxa2p in mitochondria of *Arabidopsis thaliana* (Luirink *et al.* 2001). All the known family members are involved in a protein translocation and folding as well as co- or posttranslational integration of proteins into membranes and protein complex assembly.

In plastids, Alb4p takes part in ATPase assembly in chloroplasts (cp), its knock out has only a mild effect on the phenotype (Gerdes *et al.* 2006, Benz *et al.* 2009). The other plastid protein, Alb3p, has been shown to play a role in the cpSRP pathway through which light harvesting complex proteins (LHCPs) are integrated posttranslationally into the thylakoid membrane (Klostermann *et al.* 2002, Moore *et al.* 2000). After entering the stroma, the LHCP is bound by the GTPase cpSRP54 and the protein cpSRP43 which together build up the plastid SRP. The resulting transit complex is directed to the thylakoid membrane by the cpSRP receptor cpFtsY where Alb3p operates as insertase. This transport still functions even when both cpFtsY and cpSRP54 are missing, while Alb3p seems to be essential (Tzvetkova-Chevolleau *et al.* 2007, Falk *et al.* 2010). Parallel to the posttranslational cpSRP pathway, cp encoded proteins can also integrate into the thylakoid membrane by a cotranslational insertion mechanism analogous to bacterial plasma membranes. This was first documented for the chloroplast encoded photosystem II (PSII) reaction center protein D1 in *Chlamydomonas reinhardtii*. The translating ribosome is bound by cpSRP54, the ensuing complex is directed to the membrane and the cpSec translocase inserts the newly made protein into the thylakoid membrane. Alb3.1, the *C. reinhardtii* homolog of Alb3p, is necessary for correct cotranslational assembly of D1 into PSII (Ossenbühl *et al.* 2004). Since Alb3p interacts with many of the plastid encoded proteins, this appears to be the general protein import pathway for these proteins into the thylakoid

Received 5 April 2012, *accepted* 6 November 2012.
+Corresponding author; tel: + 49 731 502 2610, fax: + 49 731 502 2626, e-mail: mo.bo@uni-ulm.de
Abbreviations: *alb3* – *albino3* mutant; *hcf106* – high chlorophyll fluorescence mutant 106; Car(s) – carotenoid(s); Chl – chlorophyll; Cox – cytochrome-c-oxidase; cpFtsY – chloroplast receptor of the signal recognition particle; cptat – protein transport pathway across the thylakoid membrane; LHCPs – light-harvesting complex proteins; cpSRP – chloroplast signal recognition particle; Oxa1 – mitochondrial protein of the *alb3* family for insertion of proteins into the inner membrane; wt – wild type; YidC – bacterial membrane insertase of the *alb3* family for SEC-independent substrates.
Acknowledgements: We thank Bianca Wolf for excellent experimental help. We are very grateful to Kenneth Cline and Ralph Henry for their gifts of antibodies. This work was supported by grants from the Deutsche Forschungsgemeinschaft to FO and AB.

membrane. In the cyanobacterium *Synechocystis sp.*, the Alb3 homologue Slr1471p seems to play a role in carotenoid assembly into photosynthetic complexes (Ossenbühl *et al.* 2006).

These functions of Alb3p in the cotranslational assembly of D1 and the posttranslational integration of LHCPs into the thylakoid membrane alone do not explain the dramatic *albino3* phenotype in an Alb3p knock out mutant. The *albino3* mutant cannot grow in soil, while it grows with a yellowish dwarf phenotype with sucrose as an external carbon source (Sundberg *et al.* 1997). To investigate further potential functions of Alb3p in chloroplasts, here we compared mutants deficient in various protein assembly and biochemical pathways with the original *albino3* mutant.

Materials and methods

Plant growth conditions: *Arabidopsis thaliana* null mutants lacking Alb3p (*albino3*) (Sundberg *et al.* 1997) or CpSRP54 (*ffc*$^-$) (Amin *et al.* 1999) were those described previously. For knock out mutants of Hcf106 (SALK_020680), CpFtsY (SAIL_816_EM) and PSY (SALK_054288), respective lines were selected from the T-DNA insertion library. Plants with homozygous insertions were verified by PCR and causal connections to the phenotypes were confirmed by complementation with the respective WT cDNAs. The knock outs of Hcf106 and CpFtsY were additionally confirmed by Western-blot analysis. Plants were grown for 2–4 weeks on soil or –if necessary– as *in vitro* culture on sucrose containing agar media under an illumination with light intensities of 120, 50, or 12 µmol(photon) m^{-2} s^{-1}, respectively. The day/night regime was 16 h light and 8 h dark at temperatures of 21–23°C and a humidity of 50–80% in *Arabidopsis* growth chambers. All biochemical assays were repeated at least three times starting each time from fresh plants as full biological replicates.

Pigment measurements: To quantify the pigment contents of mutant plants, leaves were frozen in liquid nitrogen, ground in a mortar, and pigments were isolated with 80% acetone. Chlorophyll (Chl) *a* was detected photometrically at 663 nm, Chl *b* at 645 nm, total Chls at 652 nm and carotenoids (Cars) at 470 nm. Respective contents were calculated with equations taken from Lichtenthaler and Buschmann (2001) and applied to the deployed fresh mass.

***In vivo* labeling**: For *in vivo* labeling of chloroplast encoded proteins, equal amounts of leaves were incubated for 45 min under 50 µmol photons m^{-2} s^{-1} in 0.4% cycloheximide followed by a 30 or 90 min incubation in pulse solution (0.2% cycloheximide, 125 mM L-[^{35}S]-methionine). To follow the stability of the newly synthesized proteins, pulse/chase experiments were performed. Here the 30 min pulse incubation was followed by a 60 min chase incubation with chase solution (0.2% cyclohexamid, 125 mM L-[^{32}S]-methionine).

Protein isolation: Approximately 100 mg of fresh leaf material were added to 100 µl TMK buffer (10 mM Tris/HCl pH 6.8, 10 mM $MgCl_2$, 20 mM KCl) and ground with a Potter machine (*Janke&Kunkel*, Staufen, Germany) three times for 10 s. This mixture was filtered through cotton gauze and the membranes were pelleted by centrifugation. The membrane pellet was washed twice with 300 µl TMK buffer. The pellet was then resuspended in 60 to 80 µl ACA buffer [750 mM ε-aminocapric acid, 50 mM Bis/Tris pH 7.0 (HCl), 0.5 mM EDTA pH 7.0], and β-dodecylmaltoside was added to a final concentration of 1%. After incubation on ice for 10 min, the membranes were pelleted and the protein complexes containing supernatant was used for further analysis.

PAGE, blue native (BN) PAGE, 2D and Western analysis: Acrylamide gels and Western-blotting were done following standard procedures, BN-PAGE and 2D for protein complex analysis were set up and run according to Schaegger and von Jagow (1997). Denaturing analytical SDS PAGE gels were run with 12.5% polyacrylamide and the BN gels were 4–16% polyacrylamide gradient gels.

Radioactive signal detection: Gels with radioactive samples were incubated for 1 h in 40% ethanol and 10% acetic acid and for 30 min in 10% acetic acid at room temperature and afterwards vacuum dried at 80°C. Radioactive signals were detected by phosphoimaging.

Results

***albino3* showed a light sensitive phenotype**: The *albino3* mutation was first identified in 1993 as a transposon insertion plant line (Long *et al.* 1993). Our analysis of this mutant confirmed and extended the characteristics of the phenotype originally described (Sundberg *et al.* 1997). Briefly, the knock out of the *albino3* gene lead to a loss of the Alb3 protein and *albino3* plants did not develop beyond the seedling stage when grown on soil (Fig. 1). On sugar containing medium, the mutant showed yellowish leaves (Fig. 1*A*) and it was able to grow until the flowering stage, sometimes even setting seeds, which were, however, all sterile. The Chl content in leaves was

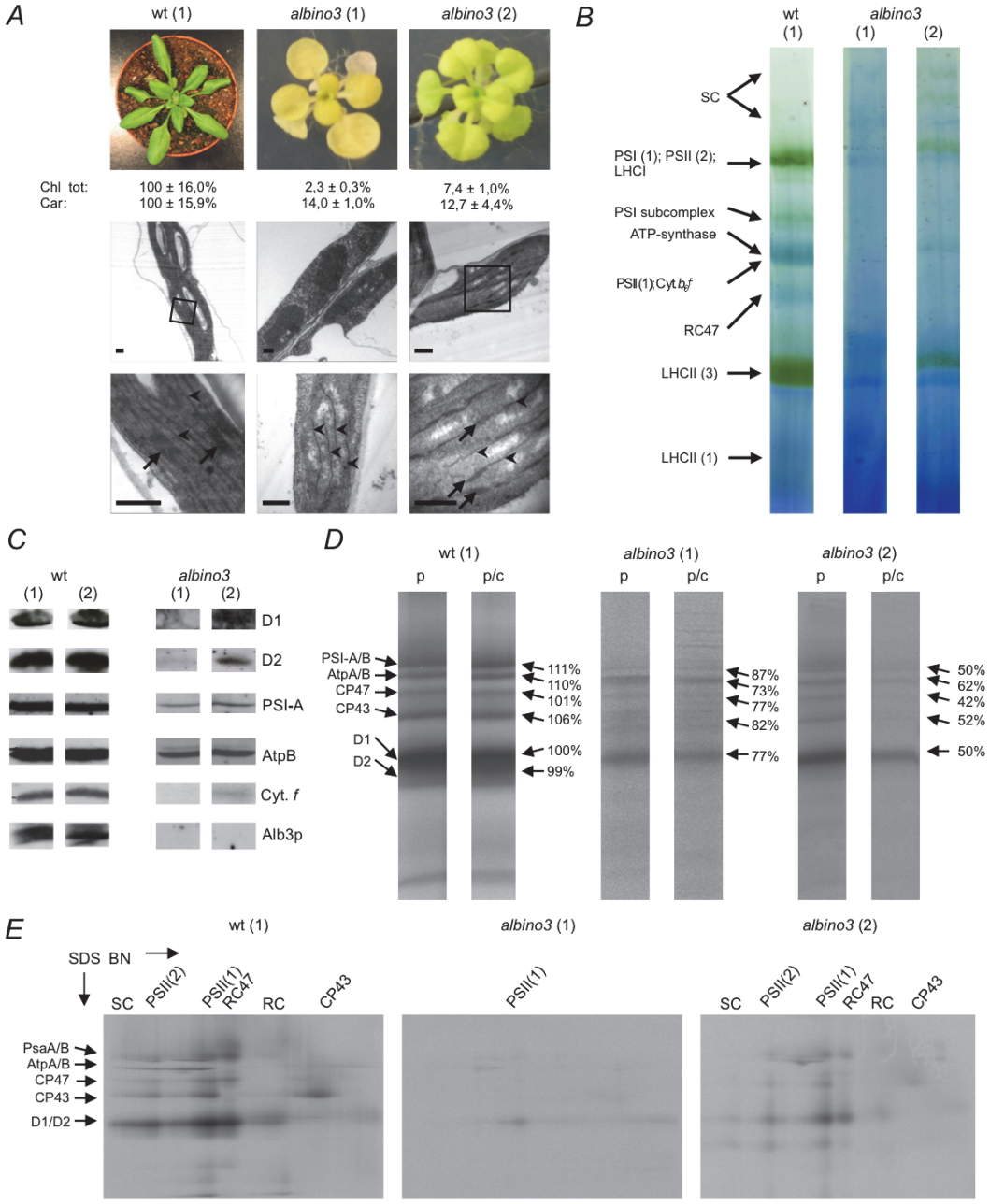

Fig. 1. Analysis of chloroplast protein complexes in the *albino3* mutant. (*A*) The mutant plants grown at (*1*) 120 µmol m^{-2} s^{-1} or at (*2*) 12 µmol m^{-2} s^{-1}. The macroscopic and electron microscopic phenotype in comparison to wild type (wt) plants. Chlorophyll (Chl) and carotenoid (Car) contents are given as % of those in wt plants. Black bar – 500 µm. *Arrows* – thylakoid stacks; *arrow heads* – stromal thylakoids. (*B*) Total cellular protein complexes from rosette leaves separated by BN-PAGE. The most prominent complexes are those from chloroplasts as indicated at the lane of the wt proteins, which were mostly absent in the mutant. (*C*) Western-blot analysis of several plastid proteins from the *albino3* mutant and wt plants. (*D*) Newly made proteins analysed by SDS-PAGE of *in vivo* labeled proteins of rosette leaves of *albino3* and wt. p = 30 min pulse of ^{35}S methionine, p/c = 30 min pulse followed by 60 min chase with cold ^{32}S methionine. Numbers show the intensities of the p/c-signals relative to the p-signals taken as 100%. (*E*) Integration of the newly synthesized proteins into the chloroplast protein complexes investigated by BN/SDS 2D analysis of *in vivo* labeled total proteins from rosette leaves of the *albino3* mutant and wt plants. SC – super complexes; PSI (1) – photosystem I monomer; PSII (2) – photosystem II dimer; LHCI – light-harvesting complex I; PSII (1) – photosystem II monomer; Cyt. b_6f – cytochrome b_6f complex; RC47 – CP43-free PSII monomer; RC – reaction center of PSII; LHCII(3) – light harvesting complex II trimer; LHCII (1) – LHCII monomer. D1 and D2 – reaction center proteins of PSII; CP47 – chlorophyll binding protein (47 kDa); CP43 – chlorophyll binding protein (43 kDa); PSI-A/B – reaction center proteins of PSI; AtpA/B – subunit A/B of ATP-synthase; Cyt *f* – cytochrome *f* subunit of cytochrome b_6f complex.

reduced to about 2% of that in wild type (wt) plants, the total carotenoid content to about 14%. This resulted in a ratio of Chl to Car of 1.17 (6.89 for wt), indicating photoxidative stress (Lichtenthaler and Buschmann 2001). TEM pictures revealed plastids of variable sizes, most of which contained none or only a few stromal thylakoid membranes (Fig. 1*A*).

The light sensitivity and resulting photoxidative stress were confirmed by the observation that *albino3* plants, when grown at very low light intensities, became slightly greener and concomitantly developed the Chl content almost threefold increased (Fig. 1*A*). Since Cars remained at almost the same level as under the normal light, the resulting altered ratio of Chl/Cars of 3.67 indicated that photooxidative stress was reduced, although still apparent. The enhanced greening under a very low light was accompanied by changes in the internal membranes towards more normal structures, and the plastids contained thylakoid structures with small membrane stacks.

The light-sensitive phenotype was reflected in BN-PAGE analyses of protein complexes in rosette leaves (Fig. 1*B*). *Albino3* plants grown at 120 µmol(photon) m^{-2} s^{-1} showed almost no photosynthetic complexes, while under a low light almost all complexes, detectable in the wt plants, also appeared in *albino3*. Especially LHCII trimers and PSII monomers and dimers were enriched; the increase of PSII was confirmed by Western-blot (Fig. 1*C*). The PSII core complex proteins, D1 and D2, were not detectable in *albino3* plants grown at the normal growth light, but they appeared under the low light. Proteins of PSI, the cytochrome b_6f complex and the ATP synthase also became slightly enriched, as proteins PSI-A showed for PSI, AtpB for the ATP synthase and cyt. *f* for the cytochrome b_6f complex. These observations were remarkable since Alb3p is supposed to play a central role in co- and posttranslational cpSRP transport of LCHPs and of plastid encoded subunits of the photosynthetic complexes (Klostermann *et al.* 2002, Moore *et al.* 2000, Schünemann 2007).

Since all proteins transported by Alb3p were found in thylakoid membranes even when Alb3p was absent, their reduced amounts in the *albino3* plants might be caused not by import problems but possibly by an altered stability or assembly into the respective complexes. The stability of plastid encoded proteins was evaluated by pulse and pulse/chase experiments, which showed that signal intensities of all plastid encoded proteins declined during the chase independent of the light conditions (Fig. 1*D*). This observation suggests a generally decreased protein stability for the chloroplast encoded subunits of photosynthetic complexes in the mutant.

This result raised the question if the protein stability was reduced by a disturbed complex assembly. While in wt leaves the typical signal pattern with the assembly intermediates of PSII, free CP43, RC, RC47, PSII monomer and dimer, and the supercomplexes could be seen (Fig. 1*E*), the signals in *albino3* grown at 120 µmol (photon) m^{-2} s^{-1} were so strongly reduced that only D1 and D2 were detected in the PSII monomer. In low light grown *albino3* plants, the signal pattern was similar to wt plants although reduced. This confirmed the BN-PAGE results and showed that all photosynthetic complexes could assemble correctly despite the *albino3* mutation. This finding reinforced an influence of additional Alb3p functions beyond the cpSRP protein transport pathway.

***ffc1-2* and *Δcpftsy*, two mutants of the cpSRP transport pathway**: To evaluate directly the influence of the cpSRP pathway, we investigated two specific mutants, the *ffc1-2* mutant (Amin *et al.* 1999) and a new T-DNA insertion line, *Δcpftsy*. Both these genes encode essential factors of the cpSRP pathway. CpSRP54, which is disturbed in the *ffc1-2* mutant, is a part of the signal recognition particle stabilizing LHCPs in the transit complex; cpFtsY is supposed to be the SRP receptor directing the transit complex to the thylakoid membrane.

The *ffc1-2* phenotype showed only a slight reduction of Chl (84%), but no differences in the growth or leaf size were found (Fig. 2*A*). The likewise slightly reduced Car content resulted in a stable Chl to Car ratio which gave no evidence for photooxidative stress in the *ffc1-2* plants. All other investigated parameters, the ultrastructure of the chloroplast membranes, chloroplast membrane protein complexes and respective subunits showed no difference in comparison with wt plants (Fig. 2*B* and 2*C*). However, the protein stability assays suggested that the larger chloroplast encoded subunits of photosynthetic complexes were slightly destabilized (Fig. 2*D*) and a slight reduction of the larger complexes (*e.g.* supercomplexes, PSII dimer) was seen (Fig. 2*E*). But since all supercomplexes were detected, the slightly reduced protein stability did not seem to be a major problem for the plants.

The homozygous T-DNA insertion in *Δcpftsy* was confimed by PCR and complementation with the wt allele. *Δcpftsy* was viable on soil, developed flowers and it was fertile, but it had a bleached dwarf phenotype (Fig. 3*A*). In a green alga, an orthologous mutant (*tla2*) is viable and actually grows better under a bright light (Kirst *et al.* 2012). As in the *albino3* mutant, the Chl to Cars ratio (3.4) was reduced, which was an indication of photooxidative stress. Like the *albino3* mutant, *Δcpftsy* was also light sensitive; at low light the phenotype was weaker and the ratio of Chl to Cars of 6.07 reached almost wt level. Under both light conditions all photosynthetic complexes were present (Fig. 3*B*), but they were more reduced under the normal than under the low light. Particularly, membrane-internal photosystem proteins were reduced, as visualized for D1 and D2 for PSII and PSI-A for PSI (Fig. 3*C*). The Alb3p, the ATP synthase and the cytochrome *f* proteins showed almost no difference between the two light conditions and the wt, respectively. Protein stability and assembly patterns showed no differences to the wt except for a slight reduction of all complexes (Fig. 3*D,E*).

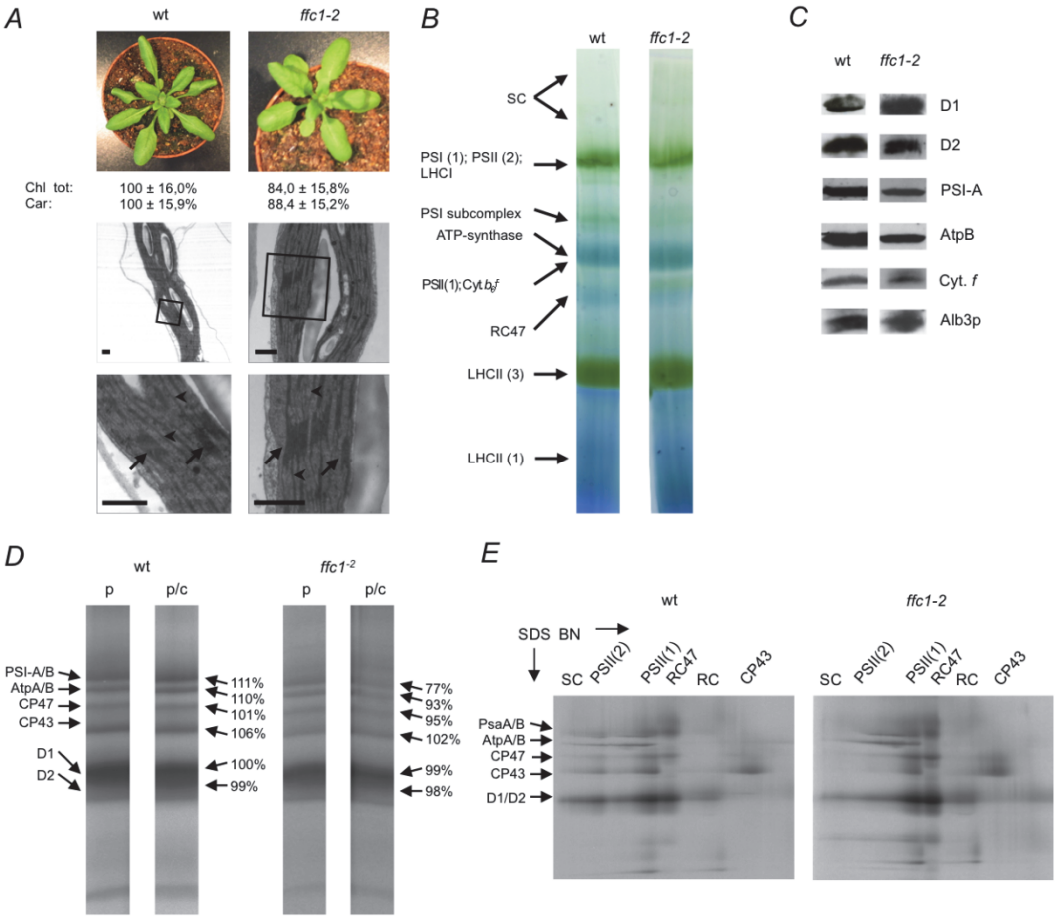

Fig. 2. Analysis of the *ffc1-2* mutant deficient in the cpSRP pathway. (*A*) Plants grown at normal growth light intensity of 120 µmol m^{-2} s^{-1}. Comparison of the macroscopic and electron microscopic phenotype of *ffc1-2* with the wt. Chlorophyll (Chl) and carotenoid (Car) contents are given as percentage of the content in wt plants. Black bars in the TEM pictures represent 500 µm. *Arrows* – thylakoid stacks; *arrow heads* – stromal thylakoids. (*B*) Total cellular protein complex analysis by BN-PAGE from rosette leaves from *ffc1-2* plants and from the wt. (*C*) Western-blot analysis of several plastid proteins from the *ffc1-2* mutant and wt plants. Abbreviations are as in Fig. 1. (*D*) SDS-PAGE of *in vivo* labeled proteins of rosette leaves of *ffc1-2* and wt shows the sets of proteins synthesized in both plant lines. p = 30 min pulse of ^{35}S methionine, p/c = 30 min pulse followed by 60 min chase with ^{32}S methionine. Numbers show the intensities of the p/c-signals relative to the p-signals taken as 100%. (*E*) BN/SDS 2D analysis of *in vivo* labeled total proteins from rosette leaves of the *ffc1-2* mutant and wt plants confirms the competence of the mutant.

The Alb3p protein was absent in the *Δhcf106* mutant: The Hcf106 protein is part of the receptor component in the cptat protein transport pathway across the thylakoid membrane (Cline and Dabney-Smith 2008, Voelker and Barkan 1995). A homozygous T-DNA insertion line was verified by PCR and the knock out by Western-blot (Fig. 4*C*) and complementation.

The *Δhcf106* plants were not viable on soil or on sucrose containing medium under normal light conditions (like wt). Plants grown under the 'intermediate' low light of 50 µmol(photon) m^{-2} s^{-1} showed a phenotype similar to *albino3* in their first leaves (Figs. 4*A,* 1*A*). The leaves were bleached and contained about 3% Chl and 11% Cars of the wt contents (Chl/Cars = 1.90). The plastids in these leaves showed no significant internal membrane structure and they appeared to be smaller than normal chloroplasts. In later developmental stages, the leaves showed a light green colour, their plastids became larger and showed single huge membrane stacks combined with some stroma thylakoids. When grown under 'very low' light conditions, the plants showed the green leaves earlier and Chl increased threefold, the Car content about twofold compared with plants grown under the 'intermediate' low light. Plastids of such *Δhcf106* plants always showed an internal membrane structure composed of enlarged single membrane stacks with some stromal thylakoids.

These big thylakoids were accompanied by enriched LHCII trimers in low light grown plants (Fig. 4*B*). While no other photosynthetic complexes were detected in the BN gel, signals for ATP synthase were detectable in silver stained second dimension gels, explaining the Western-blot results (Fig. 4*C*), where only ATP synthase subunit B and a very low signal for cytochrome *f* were found. Surprisingly, no Alb3p was detectable. This might

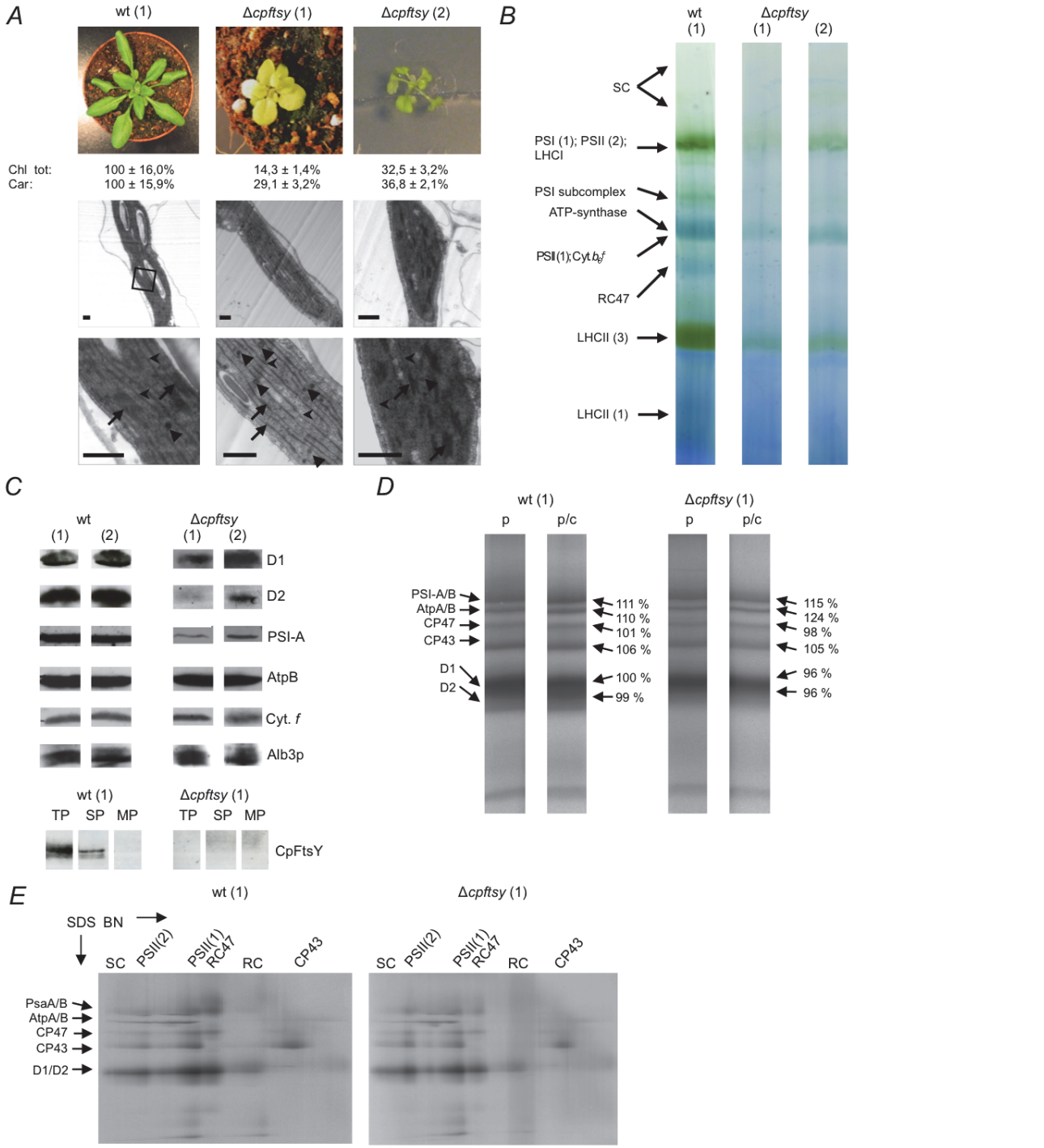

Fig. 3. Analysis of the *Δcpftsy* mutant deficient in the cpSRP. (*A*) Plants grown at (*1*) 120 µmol m^{-2} s^{-1} or (*2*) 12 µmol m^{-2} s^{-1} light intensity. The macroscopic examination: the growth sizes and chlorophyll (Chl) and carotenoids (Cars) contents of the mutant plants *vs*. the wt. Electron microscopy: the numbers and sizes of the thylakoid stacks. Chl and Car contents are given as % of the wt plants. Black bars in the TEM pictures represent 500 µm. *Arrows* – thylakoid stacks; *arrow heads* – stromal thylakoids. (*B*) BN-PAGE of protein complexes from *Δcpftsy* rosette leaves. (*C*) Western-blot analysis of selected plastid proteins to determine the presence of specific photosystem complexes as seen with the BN-PAGE analysis in *Δcpftsy* mutant and in wt plants. At the bottom a Western analysis verification of the *Δcpftsy* knock out is shown. TP – total proteins, SP – soluble proteins, MP – membrane proteins. Other abbrev. as in Fig. 1. (*D*) SDS-PAGE of *in vivo* labeled proteins of rosette leaves of *Δcpftsy* and wt. p = 30 min pulse of ^{35}S methionine, p/c = 30 min pulse followed by 60 min chase with cold methionine. Numbers show the intensities of the p/c-signals relative to the p-signals taken as 100%. (*E*) The capacity of complex assembly was investigated by BN/SDS 2D of *in vivo* labeled total proteins from rosette leaves of the *Δcpftsy* mutant and of wt plants.

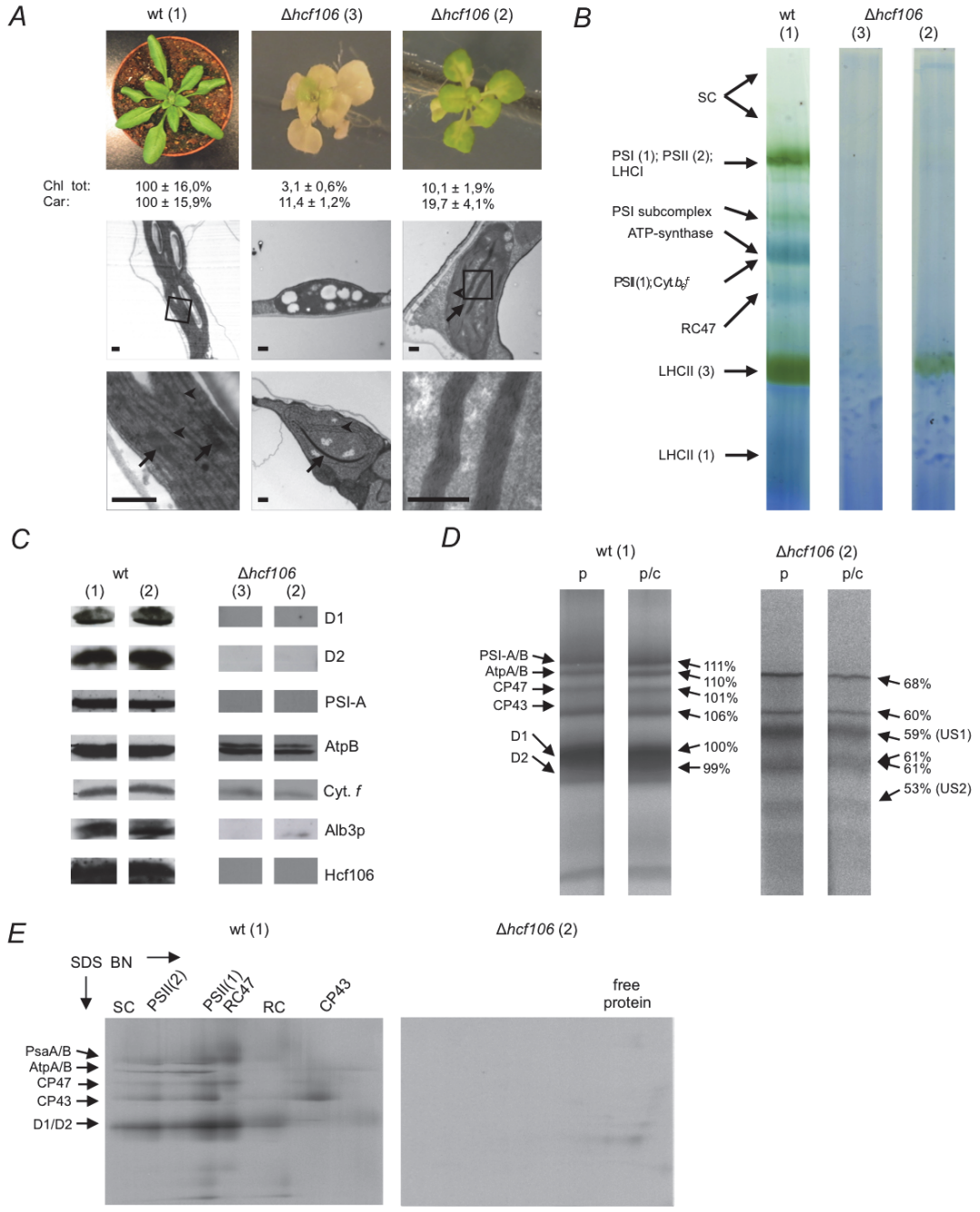

Fig. 4. Analysis of the *Δhcf106* mutant deficient in the cptat protein transport pathway. (*A*) The light-sensitive phenotype becomes apparent when plants were grown at light intensities of (*1*) 120 µmol m^{-2} s^{-1}, (*2*) 12 µmol m^{-2} s^{-1}, or (*3*) 50 µmol m^{-2} s^{-1}. The *Δhcf106* mutant plants could not survive on soil under normal light,they were therefore grown on sugar supplemented agar under reduced light (2,3). The TEM pictures show thylakoid structures in the mutant. Chlorophyll (Chl) and carotenoid (Car) contents are given relative to wt plants. Black bars in TEM pictures indicate 500 µm. *Arrows* –thylakoid stacks; *arrow heads* – stromal thylakoids. (*B*) BN-PAGE analysis in *Δhcf106* rosette leaves in comparison with the wt. (*C*) Western-blot anaysis of selected chloroplast proteins to analyze their abundance in the *Δhcf106* mutant. The bottom blot shows the verification of the *Δhcf106* mutant as a knock out mutant. (*D*) Synthesis and stability of some plastid proteins was determined by SDS-PAGE analysis of *in vivo* labeled proteins of rosette leaves of *Δhcf106*. Lanes are labelled p = 30 min pulse, p/c = 30 min pulse followed by 60 min chase. The percentages show the p/c signals relative to the p signals. US1 and US2 = unknown signals 1 and 2. (*E*) Comparison of the BN/SDS 2D analysis of *in vivo* labeled proteins from rosette leaves of *Δhcf106* and wt plants to investigate the assembly of proteins into thylakoid complexes in the mutant relative to the wt.

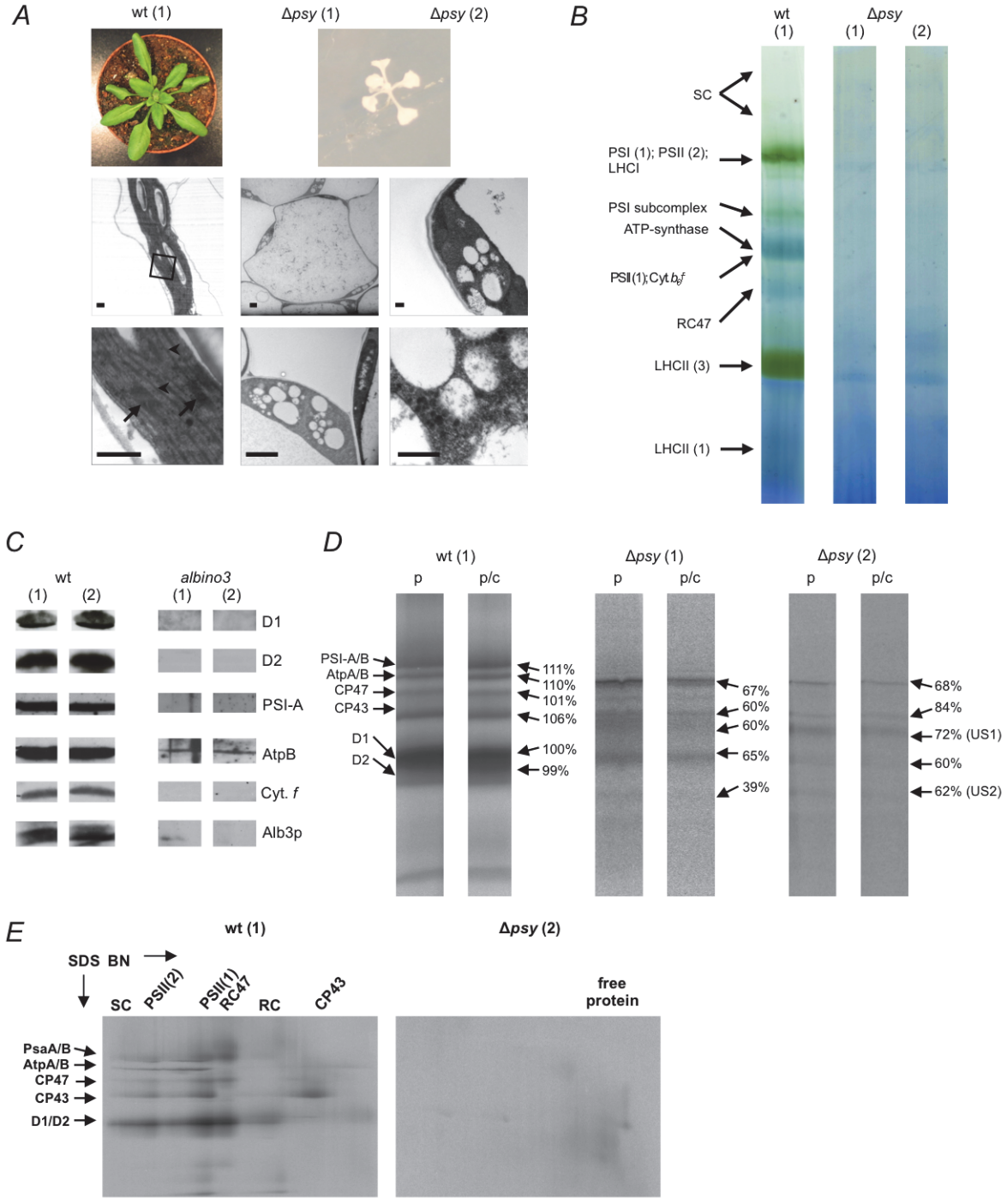

Fig. 5. Analysis of a *Δpsy* mutant deficient in carotenoid biosynthesis. (*A*) Phenotypes of mutant plants grown at light intensities of (*1*) 120 µmol m^{-2} s^{-1} or (*2*) 12 µmol m^{-2} s^{-1} or 50 µmol m^{-2} s^{-1}; only one plant is shown. Macroscopic and electron microscopic examination compare the *Δpsy* mutant phenotype with the *albino3* mutant. The bar in the TEM pictures indicates 500 µm. *Arrows* – thylakoid stacks in the wt. (*B*) Protein complex sorting by BN-PAGE of *Δpsy* rosette leaves in comparison with wt protein preparations. (*C*) Analysis of proteins involved in photosynthesis complexes in a Western-blot of proteins from *Δpsy* mutant and wt leaves. (*D*) Analysis of the synthesis of proteins involved in photosynthesis such as PSI-A/B in an SDS-PAGE spread of *in vivo* labeled proteins from rosette leaves of *Δpsy* and wt. The proteins in lanes marked p were labelled by a 30-min pulse, those marked p/c were labelled in a 30 min pulse which was followed by a 60 min chase. Relative intensities of p/c to p signals are indicated. US1 and US2 – unknown signals 1 and 2. (*E*) Analysis of the thylakoid proteins being synthesized and their integration into complexes by BN/SDS 2D gels of protein complexes from the *in vivo* labeled proteins from *Δpsy* rosette leaves.

be explained if the HCF106 protein was directly or indirectly required for import of Alb3p. The observed phenotype could include secondary effects caused by the absence of the Alb3p protein. To investigate if the photosynthetic proteins were not found because they were not expressed or because they were degraded, pulse/chase experiments were carried out. PSI-A/B and CP47 were not labeled *in vivo*, while two new signals (US1 and US2 for unknown signal) appeared in the mutant (Fig. 4*D*). All expressed proteins showed lowered stability in the pulse/chase experiment. No photosynthetic complexes and no PSII assembly intermediates could be seen in the assembly assay (Fig. 4*E*). Therefore, it seemed that the *Δhcf106* mutant could only assemble LHCII trimers and very low amounts of ATP synthase.

Δpsy showed a similar but more severe phenotype compared to *albino3*: In the cyanobacterium *Synechocystis*, the Alb3p homolog Slr1471p is supposed to play a role in Car assembly into the thylakoid membrane complexes. To investigate the analogous possibility in *Arabidopsis thaliana*, we analysed a T-DNA insertion line of the *PSY* gene coding for the phytoene synthase, the initial enzyme of Car biosynthesis as confirmed by complementation with the wt gene (Scolnik and Bartley 1994). The mutant plants were completely white and neither Cars nor Chls could be extracted from leaves of this *Δpsy* mutant (Fig. 5*A*). The cells contained a few small plastids with electron bright inclusions (probably filled with starch) and occasional accumulations of plastoglobuli but no internal membranes. This phenotype did not change under different light conditions.

No photosynthetic complexes were seen in the BN gels (Fig. 5*B*). Very small amounts of ATP synthase and LHCII trimers could be detected in the second-dimension gels and in Western-blots of ATP synthase subunit B (Fig. 5*C*) and LHCII proteins. The protein synthesis assay showed a very low expression of most plastid encoded proteins, but some did not seem to be made at all (*e.g.* PSI-A/B; Fig. 5*D*). Proteins that were synthesized appeared to be rapidly degraded since all signal intensities in the chase were lower than in the respective pulse lane. No complex assembly was seen in *Δpsy* in 2D analysis of labelled chloroplast proteins (Fig. 5*E*).

Conclusion and hypothesis: The pleiotropic phenotype of the *albino3* mutant in higher plants, *i.e. A. thaliana*, has been enigmatic since its first identification (Sundberg *et al.* 1997). Since the mutant does not grow very vigorously, biochemical and further genetic analyses have been difficult. Here we took a different approach by comparing various physiological, ultrastructural, and biochemical parameters between the *albino3* mutant and mutants deficient in selected pathways of chloroplast assembly and thylakoid function. Surprisingly, the *albino3* mutant showed a photosensitive phenotype, which has not been noted previously. The *Δhcf106* mutant, which also lacks the Alb3p protein, was similarly photosensitive. Whether this photosensitive phenotype of the *Δhcf106* mutant was caused by the absence of the Alb3p protein, which apparently could not be imported into the thylakoid membrane in this *Δhcf106* mutant, needs to be investigated.

The observed similarity of affected functions between the *albino3* mutant and the *Δpsy* Car biosynthesis mutant was an intriguing correlation. This similarity suggests that further analyses of Alb3p protein functions might be directed to investigate a potential role in Car integration and assembly into the respective photosynthetic complexes and their organisation in the thylakoid membrane. Direct investigations of the Car content in the photosynthetic complexes of the *albino3* mutant and of relative pigment concentrations may yield more detailed information about the background of the correlations between the *albino3* and the *Δpsy* Car biosynthesis mutants.

We are aware that the hypothesis of an involvement of the Alb3p protein in Car assembly in photosynthetic complexes is a potential connection which was not proven by hard data. Here this hypothesis is based on comparisons of phenotypes and thus it is a suggestion by an analogy. Nevertheless, these observations pointed out new directions, in which the next level of biochemical investigations of the Alb3p protein function and ordered Car integration in photosynthetic complexes might be directed. It will be interesting to see whether heterologous compensation for the defect in the *albino3* mutant could be achieved *via* the Car biosynthesis and/or assembly pathway. Such experiments would prove or disprove the inferences drawn here, which suggest that the Alb3p protein might be involved in Car integration into and/or at the photosynthetic complexes.

References

Amin, P., Sy, D.A.C., Pilgrim, M.L., *et al.*: Arabidopsis mutants lacking the 43- and 54-kilodalton subunits of the chloroplast signal recognition particle have distinct phenotypes. – Plant Physiol. **121**: 61-70, 1999.

Benz, M., Bals, T., Gügel, I.L., Piotrowski, M., Kuhn, A., Schünemann, D., Soll, J., Ankele, E.: Alb4 of Arabidopsis promotes assembly and stabilization of non chlorophyll-binding photosynthetic complex, the CF_1CF_O-ATP synthase. – Mol. Plant **2**: 1410-1424, 2009.

Cline, K., Dabney-Smith, C.: Plastid protein import and sorting: different paths to the same compartments. – Curr. Opin. Plant Biol. **11**: 585-592, 2008.

Falk, S., Ravaud, S., Koch, J., Sinning, I.: The C terminus of the Alb3 membrane insertase recruits cpSRP43 to the thylakoid membrane. – J. Biol. Chem. **285**: 5954-5962, 2010.

Gerdes, L., Bals, T., Klostermann, E. *et al.*: A second thylakoid

membrane-localized Alb3/OxaI/YidC homologue is involved in proper chloroplast biogenesis in *Arabidopsis thaliana*. – J. Biol. Chem. **281**: 16632-16642, 2006.

Kirst, H., García-Cerdán, J.G., Zurbriggen, A., Melis, A.: Assembly of the Light-Harvesting Chlorophyll antenna in the green alga Chlamydomonas reinhardtii requires expression of the TLA2-CpFTSY gene. – Plant Physiol. **158**: 930-945, 2012.

Klostermann, E., Droste Gen Helling, I., Carde, J.P., Schünemann, D.: The thylakoid membrane protein ALB3 associates with the cpSecY-translocase in *Arabidopsis thaliana*. – Biochem. J. **368**: 777-781, 2002.

Lichtenthaler, H.K., Buschmann, C.: Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. – Curr. Prot. Food Anal. Chem. F4.3.1 – F4.3.8, 2001.

Long, D., Martin, M., Sundberg, E. *et al*.: The maize transposable element system Ac/Ds as a mutagen in *Arabidopsis*: identification of an albino mutation induced by Ds insertion. – Proc. Natl. Acad. Sci. USA. **90**: 10370-10374, 1993.

Luirink, J., Samuelsson, T., de Gier, J.W.: YidC/Oxa1p/Alb3: evolutionarily conserved mediators of membrane protein assembly. – FEBS Lett. **501**: 1-5, 2001.

Moore, M., Harrison, M.S., Peterson, E.C., Henry, R.: Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes. – J. Biol. Chem. **275**: 1529-1532, 2000.

Ossenbühl, F., Göhre, V., Meurer, J., *et al*.: Efficient assembly of photosystem II in Chlamydomonas reinhardtii requires Alb3.1p, a homolog of *Arabidopsis* ALBINO3. – Plant Cell **16**: 1790-1800, 2004.

Ossenbühl, F., Inaba-Sulpice, M., Meurer, J., Soll, J. and Eichacker, L.A.: The *Synechocystis* sp PCC 6803 Oxa1 homolog is essential for membrane integration of reaction center precursor protein pD1. – Plant Cell **18**: 2236-2246, 2006.

Schaegger, H., von Jagow, G.: Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. – Anal. Biochem. **199**: 223-231, 1991.

Schünemann, D.: Mechanisms of protein import into thylakoids of chloroplasts. – Biol. Chem. **388**: 907-915, 2007.

Scolnik, P.A., Bartley, G.E.: Nucleotide sequence of *Arabidopsis* cDNA for phytoene synthase. – Plant Physiol. **104**: 1471-1472, 1994.

Sundberg, E., Slagter, J.G., Fridborg, I., *et al*.:ALBINO3, an *Arabidopsis* nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria. – Plant Cell **9**: 717-730, 1997.

Tzvetkova-Chevolleau, T., Hutin, C., Noël, L.D., *et al*.: Canonical signal recognition particle components can be bypassed for posttranslational protein targeting in chloroplasts. – Plant Cell **19**: 1635-1648, 2007.

Voelker, R., Barkan, A.: Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. – EMBO J. **14**: 3905-3914, 1995.