A second thylakoid membrane localized Alb3/Oxa1/YidC homologue is involved in proper chloroplast biogenesis in *Arabidopsis thaliana*

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Running title: Characterization of the thylakoid membrane protein Alb4

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The integral membrane proteins Alb3, Oxa1 and YidC belong to an evolutionary conserved protein family mediating protein insertion into the thylakoid membrane of chloroplasts, the inner membrane of mitochondria and bacteria, respectively. While Oxa1 and YidC are involved in the insertion of a wide range of membrane proteins, the function of Alb3 seems to be limited to the insertion of a subset of the light-harvesting chlorophyll-binding proteins. In this study, we identified a second chloroplast homologue of the Alb3/Oxa1/YidC family, named Alb4. Alb4 is almost identical to the Alb3/Oxa1/YidC domain of the previously described 110 kDa inner envelope protein Artemis. We show that Alb4 is expressed as a separate 55 kDa protein and that Artemis was identified mistakenly. Alb4 is located in the thylakoid membrane of *Arabidopsis thaliana* chloroplasts. Analysis of an *Arabidopsis* mutant (Salk_136199) and RNAi lines with a reduced level of Alb4 revealed chloroplasts with an altered ultrastructure. Mutant plastids are larger, more spherical in appearance and the grana stacks within the mutant lines are less appressed than in the wild-type chloroplasts. These data indicate that Alb4 is required for proper chloroplast biogenesis.

The insertion, folding and assembly of integral membrane proteins into high molecular weight structures is a complex process requiring numerous regulatory factors. Recently, proteins belonging to the Alb3/Oxa1/YidC family were described to have important roles for the biogenesis of membrane proteins in chloroplasts, mitochondria and bacteria (recent reviews: (1,2)).

In *Escherichia coli* most inner membrane proteins are targeted to the cytoplasmic membrane using the SRP pathway. The cotranslational insertion into the membrane is mediated via the SecY-translocase and the associated plasma membrane protein YidC. The exact function of YidC in the biogenesis of Sec-dependent membrane proteins is not clarified yet, but recent studies indicate that YidC can assist the lipid partitioning of single transmembrane domains or folding of the Sec-dependent proteins (3-6). In addition, YidC can also act independently from the SecY-translocase mediating the insertion of a subset of *E. coli* inner membrane proteins (7-9).

Mitochondria contain two homologues of YidC, Oxa1 and Cox18/Oxa2 (10). Since mitochondria do not contain a SRP and a SecY-translocase (11), Oxa1 and Cox18/Oxa2 function in a Sec-independent manner. Oxa1 is required for the insertion of a number of both mitochondrially and nuclear encoded inner membrane proteins (12,13). Oxa1 differs from Cox18/Oxa2 by a C-terminal extension that functions as a ribosome-binding domain during the cotranslational insertion of mitochondrial translation products (14). Cox18/Oxa2 is specifically required for the biogenesis of the cytochrome oxidase complex and recent studies indicate that its function is restricted to a posttranslational activity (15-17).

In chloroplasts of higher plants and algae such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* the thylakoid membrane proteins Alb3 (*A. th.* ) and Alb3.1 (*Chl. r.* ) respectively, were identified as
homologues of YidC and Oxa1 (18,19). The albino3 (alb3) null mutant of Arabidopsis shows a drastic albinotic phenotype with pigment deficiency and arrested chloroplast development (18). In vitro studies demonstrated that Alb3 is required for the membrane insertion of members of the light-harvesting chlorophyll-binding protein (LHCP) family, which are first imported into chloroplasts and then inserted using the chloroplast SRP-pathway (20,21). Chlamydomonas mutants lacking Alb3.1 are mainly characterized by a specific depletion of the light-harvesting systems (19). Interestingly, in Chlamydomonas a second Alb3-homologue, Alb3.2, was identified. The molecular function of this protein is, however, still unknown (19). In Arabidopsis, Artemis was identified as a second chloroplast member of the Alb3/Oxa1/YidC family. Artemis was described as a 110 kDa inner envelope protein consisting of three distinct modules: an N-terminal receptor kinase-like domain, a centrally positioned ATP/GTP-binding domain and a C-terminal Alb3/Oxa1/YidC domain. Arabidopsis plants containing an En-1 transposon in the N-terminal part of Artemis exhibited chloroplasts arrested in the late stages of chloroplast division (22). Notably, the Arabidopsis genome annotation of BAC clone F21J9 predicts two open reading frames in its current version for the genomic region described to encode Artemis (At1g24490/At1g24500, MIPS A. thaliana Genome Database). The upstream located open reading frame is predicted to encode a hypothetical 65 kDa protein with unknown function and the second open reading frame is predicted to encode the C-terminal Alb3/Oxa1/YidC domain of Artemis. This caused us to reinvestigate the presence of Artemis in the model plant Arabidopsis thaliana. Here, we describe that the predicted C-terminal Alb3/Oxa1/YidC domain of Artemis is expressed as a 55 kDa chloroplast protein (Alb4), that is located exclusively in the thylakoid membrane. Analysis of the ultrastructure of chloroplasts of Arabidopsis mutants showing reduced levels of Alb4 indicates that Alb4 is involved in proper chloroplast biogenesis. The existence of Artemis can no longer be maintained.

**EXPERIMENTAL PROCEDURES**

**Plant material and growth conditions**

All experiments were performed on Arabidopsis thaliana (L.) Heynh. Columbia plants (cv. Col-0; Lehle Seeds, Round Rock, USA). The T-DNA insertion line Salk_136199 (23) was obtained from Nottingham Arabidopsis Stock Centre, University of Nottingham, Loughborough, UK. Prior to sowing, seeds were surface-sterilised with 5% hypochlorite. To synchronise germination all seeds were kept at 4°C for 1 day. Plants were grown on soil or on medium containing 1% D-sucrose and 0.5x MS salts (Murashige and Skoog) at pH 5.7. Plant growth occurred in growth chambers with a 16-h light (21°C, photon-flux density of 100 µmol m⁻² sec⁻¹) and 8-h dark (16°C) cycle.

**Plasmid construction**

Alb3, Alb4 and OEP7 (24,25) were amplified from cDNA using gene specific primers with unique restriction sites or att sites for homologous recombination (Gateway, Invitrogen). For transient expression of fluorescence tagged proteins Alb3 and Alb4 were cloned into pOL-GFP (26) and OEP7 into pK7FWG2 (27). For in vitro transcription Alb4 was cloned into pSP65 (Promega, Mannheim, Germany). Constructs were confirmed by sequencing. For RNAi, a part of Alb4 (corresponding to nucleotides 523–1257) that showed very low homologies on nucleotide level to other transcripts from Arabidopsis was amplified from cDNA using gene specific primers with att sites for homologous recombination. The PCR product was cloned into pH7GWIWG2(I) (27).

Agrobacterium-mediated transformation

Agrobacterium strain GV3101::pMK90RK carrying the RNAi construct for Alb4 in pH7GW1WG2(I) was used to transform Arabidopsis Col-0 plants (T₀) by floral dip (28). Transformed Arabidopsis lines (T₁ and T₂) were selected on MS plates containing hygromycin (50 µg/ml or 35 µg/ml, Roche, Mannheim, Germany). Hygromycin-resistant seedlings were then transferred to soil.

**Protoplast transfection**

Arabidopsis mesophyll protoplasts were isolated from leaves of 4-week-old plants and transiently transfected according to the protocol of Jen Sheen (Sheen, J. 2002, A transient expression assay using Arabidopsis mesophyll protoplasts. http://opus.mgh.harvard.edu/sheen_lab/). GFP fluorescence was observed with a TCS-SP 1 confocal-laser scanning microscope (Leica, Wetzlar, Germany). Image stacks were processed with ImageJ (Rasband, W.S., ImageJ, U. S.).

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Import

Import reactions were performed as described previously (29). 20 µg chlorophyll (chl.) were used per import reaction. Import mix contained 0.2% BSA. Import was done for 20 min at 25 °C. Import reactions were treated with 200 µg/mg chl thermolysin for 20 min on ice. Protease inhibitors were present at all subsequent steps. After protease treatment chloroplasts were lysed in hypotonic buffer (10 mM Hepes-KOH, pH 7.6) and separated into a soluble stromal and a total membrane fraction by centrifugation for 10 min at 165,000 g. Soluble proteins were precipitated with TCA. Import products were analyzed by SDS-PAGE followed by fluorography.

Protein expression and antibody production

\(\alpha\)-Alb4-1:

The C-termini of Alb3 (residues 361 – 462) and Alb4 (residues 347 – 494) were amplified by PCR using corresponding primers with unique restriction sites. The PCR products were digested with *EcoRV* and *XhoI* and cloned into the *EcoRV/XhoI* site of the overexpression vector pET29b(+) (Novagen). The recombinant proteins were expressed using the *E. coli* strain BL21(DE3) or BL21(DE3)pLysS (Invitrogen). Overexpressed protein was purified on Ni\(^{2+}\)-NTA agarose (Qiagen) under denaturing conditions as suggested by the manufacturer. The recombinant C-terminus of Alb4 was dialysed against 1x phosphate buffered saline buffer (pH 7.4) and used as antigen to raise antibodies in rabbit (SeqLab, Göttingen, Germany). The antibody was purified by affinity chromatography. 1 mg of the native purified protein was dialysed against 50 mM Hepes, pH 8.0 and coupled to 0.1 ml Affi-Gel 15 (Biorad) following the instructions of the manufacturer. For neutralization and stabilization of the antibody 20 µl 1 M Tris and 25 µl 5 mg/ml BSA were added.

\(\alpha\)-Alb4-2:

The C-terminus of Alb4 (residues 369 – 499) was amplified by PCR using corresponding primers with unique restriction sites. The PCR products were cloned into the Restriction site of the overexpression vector pET21d (Novagen). The recombinant protein was expressed using the *E. coli* strain BL21(DE3) (Invitrogen). Overexpressed protein was purified on Ni\(^{2+}\)-NTA agarose (Qiagen) under denaturing conditions as suggested by the manufacturer. The protein was concentrated using Biomax 10K Ultrafree centrifuge filters (Millipore, Billerica, USA) in 6 M urea, 250 mM imidazol, 500 mM NaCl, 1% (w/v) SDS and used as antigen to raise antibodies in rabbit (Pineda, Berlin, Germany).

\(\alpha\)-Alb4-3 and \(\alpha\)-Alb4-4:

Two peptides corresponding to amino acid residues 418-437 (QKAEAALSNQNTDKAEQDE) and 485-499 (GHDTEQQHSHETEKR) respectively, were chemically synthesized, mixed and used as antigen to raise antibodies in two rabbits (Pineda).

Isolation and fractionation of chloroplasts

*Arabidopsis* plants (150 g fresh weight, 5 week old) were homogenized in 900 ml 0.45 M sorbitol, 10 mM EDTA, 10 mM NaHCO\(_3\), 20 mM Tricine-KOH (pH 8.4) using a Warring Blender. The homogenate was filtered through two layers of miracloth and centrifuged for 5 min at 1400 g. The pellets were resuspended in 20 ml 0.3 M sorbitol, 5 mM MgCl\(_2\), 2.5 mM EDTA, 20 mM Tricine-KOH, pH 7.6 (RB) and loaded on four preformed 30 ml 50% Percoll gradients (50% Percoll in RB). After centrifugation of the Percoll gradients for 6 min at 12000 g the lower green band was removed and washed one time with RB. Chloroplasts were lysed at 1-2 mg chl./ml in 0.1 mM EDTA, 10 mM Tris-HCL, pH 7.4. Separation in stroma, thylakoids and envelopes was done according to (30).

Characterisation of the T-DNA mutant *Salk_136199*

Genomic DNA of the T3 and T4 progeny of the T-DNA insertion line *Salk_136199* was PCR-screened using the Alb4 gene-specific primer ArtTMNotI.for (5’-AATGCGGCCGCGGTACATGTTCCTTATTCCTATG-3’) in combination with the T-DNA specific left border primer LBA1 (5’-TGGTTCACGTAGTGGGCCATCG-3’). A PCR product of about 1065 bp was expected. Nevertheless, both primers generated a 1338 bp-DNA fragment on hetero- and homozygous plants. The PCR product was sequenced and the
correct insertion position determined. To identify Salk_136199 plants with the T-DNA insertion in both alleles of the Alb4 gene, we used the gene-specific sense primer ArtTMNotI.for in combination with Ex13.rev (5'-GGGGTACCCTGCGAGGACTGCATAATT-3'). DNA from homozygous Salk_136199 plants gave no amplification product, whereas the amplified region on wild-type and heterozygous DNA was 1218 bp long.

Characterisation of the En-1 transposon footprint mutant

Progeny of plants carrying an En-1 transposon in the N-terminal part of Artemis (22) were screened for the presence of a stable footprint. A line could be identified in that En-1 was imprecisely cut out leaving a 128 bp footprint behind. PCR on genomic DNA with the primers Ex2.for (5'-ACATAAGCATCGACTGTGG-3') and Ex2.rev (5'-CTTACTAATTCCTGCGCACC-3') yielded a 527 bp product in WT and heterozygous plants. In plants heterozygous and homozygous for the footprint in Artemis a band of 655 bp was observed. In all tested plants (n=103) the footprint segregated in a Mendelian fashion (24% homozygous, 50% heterozygous, 26% WT).

RACE and RT-PCR experiments

Total RNA from Arabidopsis tissues was isolated using the Plant RNeasy Extraction kit (Qiagen, Hilden, Germany). 5'-RACE-ready cDNA was amplified from 1 µg total leaf RNA with the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, USA). 5'-RACE was performed with the gene specific primer 5'RACE-1633.rev (5'-GACGACGACGGTTGTGCTGAAACG-3') using Advantage 2 Polymerase Mix (Clontech). The RACE products were TA-cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany).

Full-length cDNA of Alb4 was synthesized using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) and the gene specific primers 5'-CAAGTTATGTCTTCAATAATCC and 5'-GAAACAGAGAAGAGGTAATTC. The full-length PCR products were TA-cloned into the vector pGemT-easy (Promega).

To analyze splicing variants in the Alb4 mutant (Salk_136199), cDNA from 7-day-old cotyledons and 4-week-old leaves was prepared as depicted before (31). The primers E7.fw (5'-TCCACACGTGTTGTGC-3') and E7.rv (5'-CAGGTTCGGGGGCATC-3') were used to amplify specific products for wild-type Alb4 (497bp) and mutant transcripts (389bp, see Fig. 7). The RT-PCR products were TA-cloned into pCR2.1-TOPO (Invitrogen).

All RACE and RT-PCR products were fully sequenced in the respective plasmid vectors.

Quantitative Real-time RT-PCR

To quantify the transcript abundance in alb4 mutants, total RNA was extracted from cotyledons of 7-day-old A. thaliana seedlings or from leaves of 5-week-old plants, DNase digested and reverse transcribed into cDNA as described (31). Quantification of transcripts by real-time RT-PCR was performed as depicted before (32) using a LightCycler (Roche). For Alb4 we constructed the following gene-specific primers: LCfw (5'-ACCAAGAGAGAGGTGTGCT-3'), LCrv (5'-GCAATGGCCGAACTGT-3'). The primer pair was designed to amplify a product specific for the C-terminal end of Alb4 (316 bp). To prevent amplification of contaminating genomic DNA, the primers were selected to flank intron 9. cDNA quantities were calculated by using LIGHTCYCLER 3.1 software (Roche). All quantifications were normalized to actin 2 cDNA fragments, amplified by ACTfw (5'-GAGTGATGGTGTGTCT-3') and ACTrev (5'-ACTGAGCAATGTTAC-3').

Electron microscopy

Samples of A. thaliana were prepared as follows: cotyledons of 7-day-old plants were fixed over night in 4% glutaraldehyde in 50 mM sodium cacodylate buffer pH 7.0. The samples were washed thoroughly in the same buffer. Post-fixation was over night in 1% osmium tetroxide in sodium cacodylate buffer. After washing in distilled water, samples were dehydrated in a graded water/acetone series (10%, 20%, 40%, 60%, 80%, 2×100%) and embedded afterwards in Spurr’s resin. Ultra thin sections were cut with a Reichert-Jung Ultracut E ultramicrotome from the middle part of the cotyledons and collected on copper grids. Samples were stained by flotation on droplets of 2% uranyl acetate and Reynolds lead citrate solution. All sections were examined with a EM10 A transmission electron microscope (Zeiss, Oberkochen, Germany).

Protein isolation

Arabidopsis leaves were grinded in liquid nitrogen, mixed with buffer (50 mM Tris pH 6.8, 50 mM EDTA, 0.1% β-Mercaptoethanol, 300 µM PMSF) and centrifuged for 10 min with
256,000 g. Soluble proteins in the supernatant and membrane proteins from the pellet were subject to SDS-PAGE followed by immunoblot analysis using antibodies against Alb4, LHCP (as a marker protein for thylakoid membranes), Tic32 (as a marker protein for the inner envelope membrane) and the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (LSU, as a marker protein for the stroma).

**RESULTS**

**The genomic region predicted to encode the C-terminal Alb3/Oxa1/YidC domain of Artemis contains an open reading frame for Alb4**

Difficulties to amplify full-length cDNA encoding Artemis by RT-PCR and the in silico prediction of two open reading frames within the genomic sequence led to experiments reexamining the existence of Artemis. Fig.1 A shows a partial alignment of the amino acid sequence of Artemis and the N-terminus of the predicted ORF encoding an Alb3 homologue, referred to as Alb4. Except for the first 20 residues Alb4 is identical to the C-terminal Alb3/Oxa1/YidC domain of Artemis. To prove the expression of Alb4, the corresponding full-length cDNA was amplified by RT-PCR using RNA extracted from Arabidopsis leaves. The observation that the Alb4 cDNA could be amplified using a 5’-primer whose sequence is specific for Alb4 and not present in the Artemis cDNA, demonstrated the existence of an ORF encoding Alb4 (submitted to GenBank, accession number AM177312). In addition, sequence analysis of the 130 bp long 5’-untranslated region obtained by 5’-RACE experiments identified a stop codon 30 basepairs upstream of the start codon of Alb4 excluding the possibility of a longer ORF (Fig. 1 B). The exon/intron structure for Alb4 was confirmed by Northern Blot experiments. Total RNA was extracted from 6-week-old plants, transferred to filters, and hybridized with probes encoding the C-termini of Alb3 and Alb4 (Fig. 2 A). The specificity of the probes was verified by control experiments using in vitro generated transcripts of Alb3 and Alb4 (Fig. 2 B). Fig. 2 A shows that the Alb3 probe detected the expected 1.7 kb Alb3 transcript. The Alb4 specific probe generated a single signal of ~1.9 kb, a size that corresponds to the predicted length of the Alb4 mRNA. Even after prolonged exposure of the blots treated with the Alb4 probe no signal could be found at ~ 3.5 kb, the predicted size of the Artemis transcript.

**Alb4 is a thylakoid membrane protein**

Alb4 is predicted to have a chloroplast transit peptide with a processing site after amino acid residue 45 based on the ChloroP prediction program (33). To test this prediction, Arabidopsis mesophyll protoplasts were transiently transformed with constructs for Alb3-GFP or Alb4-GFP. Merging of the GFP and autofluorescence images indicated a thylakoid localisation of Alb4. The GFP distribution for Alb4 is similar to that of Alb3 and not to that of outer envelope protein AtOEP7 (Fig. 3). To test this assumption in vitro translated radiolabelled Alb4 was imported into isolated pea chloroplasts. Non-imported protein was degraded by protease treatment, and chloroplasts were fractionated into a soluble and a membrane fraction. Fig. 4 A shows that Alb4 is imported and processed to a smaller protease-resistant mature protein of 50 kDa that was present in the membrane fraction after extraction with 4 M urea. The import behaviour was under all conditions similar to that of the precursor protein of the oxygen evolving complex subunit of 33 kDa OE33, which is localized in the thylakoid lumen. Since pOE33 has a bipartite transit peptide an intermediate form is detected in the stroma fraction (Fig. 4 B).

To confirm that Alb4 is localized in the thylakoid membrane and not in the envelope membranes an antibody was raised against a recombinant C-terminal fragment of Alb4. Alb4 is highly similar to Alb3 (55% identity, 72% similarity). To exclude a possible cross-reaction of the anti-Alb4-1 antibody with Alb3 and to demonstrate the specificity for Alb4 we tested anti-Alb4-1 thoroughly using overexpressed protein and in vitro translation products for both Alb4 and Alb3. The anti-Alb4-1 antibody recognized the antigen and detected the in vitro translation product of the full-length Alb4 precursor (Fig. 5 A/B). No cross-reaction was detected between the anti-Alb4-1 antibody and the recombinant C-terminus of Alb3 and the in vitro translation product of full-length Alb3 (Fig. 5 A/B).

Chloroplasts were isolated from 6-week-old Arabidopsis plants and fractionated into stroma, envelope and thylakoid membranes. Immunoblot analysis of these fractions using the anti-Alb4-1 antibody and control antibodies against marker proteins of the different chloroplast subfractions were conducted. The
results demonstrated that Alb4 is exclusively located in the thylakoid membrane (Fig. 6 A). This result was further substantiated by the use of additional antibodies raised against different parts of Alb4 (α-Alb4-2, α-Alb4-3 and α-Alb4-4) (Fig. 6 B).

Overexpressed protein corresponding to exon 7 of Artemis (and thus exon 1 of Alb4) had been used to raise the α-ArtA antibody (22). α-ArtA recognized a 110 kDa protein in the inner envelope (Artemis) and failed to recognize Alb4 in the thylakoid membranes in Arabidopsis (Fig. 6 B) confirming results with chloroplast fractions from pea (22). Notably, none of the anti-Alb4 antibodies, that were raised against different parts of Alb4 and should also detect the putative protein Artemis, recognized a protein of 110 kDa in the envelope membranes (Fig. 6 A and B). These results demonstrate clearly that Artemis does not exist as a 110 kDa envelope protein and we conclude that the α-ArtA antibody reacts nonspecifically with an inner envelope protein but not with an Alb3/Oxa1 isofrom.

Arabidopsis Alb4 mutants exhibit a defect in chloroplast development

The Arabidopsis T-DNA insertion lines of the Salk collection were screened for lines containing a T-DNA insertion within Alb4. A single Arabidopsis line, Salk_136199, was described to have a T-DNA insertion in the fifth intron of Alb4 (http://signal.salk.edu/cgi-bin/tdnaexpress). Plants were raised and the location of the T-DNA insertion was analysed by PCR using gene specific and T-DNA specific primers followed by sequencing of the PCR product. The insertion position was found to be further downstream than annotated (Fig. 7 A). Plants homozygous for the T-DNA insertion in intron 6 were used for all further studies.

When we analyzed the expression of Alb4, two products were obtained with RT-PCR using the primers E7.fw and E7.rv (Fig. 7 A) on cDNA made from 7-day-old cotyledons, indicating the presence of two mRNA populations in homozygous Salk_136199 plants (Fig. 7 B). Sequencing of these products revealed that the larger one is identical to the product obtained from wild-type mRNA. In the smaller variant, exon 7 is completely spliced out and exon 6 is directly fused in frame to exon 8 (Fig. 7 C). When we probed for mRNA in 4-week-old leaves of single mutant plants, both transcript populations were present as well, showing that the mutant splice variant does not segregate with individual mutant lines (data not shown). In all mutant plants tested, both transcripts were present in approximately equal amounts.

Since the message for this smaller Alb4 isoform was abundant and stable we tested if we could also detect a second smaller Alb4 isof orm by immunoblot. However, a 4 kDa smaller protein corresponding to the transcript without exon 7 was not detected in thylakoid membranes from mutant plants (Fig. 7 D), indicating that the protein is either not translated or not stably integrated into thylakoids. Alb4 is predicted to possess five transmembrane regions and the loss of exon 7, that encodes the putative fourth transmembrane domain, probably leads to an altered topology of Alb4.

Real time PCR experiments using RNA extracted from cotyledons of 7-day-old seedlings and from leaves of 5-week-old homozygous plants revealed that the transcript level was reduced by 55% and 50% when compared to wild-type (Fig. 8 A and 9 G). Please note that the measured transcripts include Alb4 full-length as well as the incorrectly spliced RNA missing exon 7 (primers LC.fw and LC.rv, compare Fig. 7 A and B). The alb4 mutants showed also a significant reduction of Alb4 on protein level as demonstrated by immunoblot analysis of thylakoid membrane extracts using the anti-Alb4-1 antibody or an antibody against cpSecY as a loading control (Fig. 8 B). A smaller Alb4 protein was also not detected in these individual mutant plants.

Electron microscopy of 7-day-old plants from the homozygous T-DNA line revealed that the reduction of Alb4 is affecting the shape of chloroplasts (Fig. 9 C, D, E and F). Plastids of the Salk line 136199 are somewhat larger (~20%) and more spherical in appearance compared to that of wild-type plants of the same stage and in the same tissue. The grana stacks within the T-DNA line are less appressed than in the wild-type chloroplasts whereas the structure of the stacks is not affected.

Further T-DNA lines for Alb4 were identified (see Supplemental Material) but no complete loss of function mutant could be found. Therefore, we used an RNAi approach to reduce the abundance of Alb4. Several independent mutant lines were obtained. The chosen T2 plants contained only about 12% residual message and from leaves of 5-week-old homozygous plants revealed that the transcript level was reduced by 55% and 50% when compared to wild-type (Fig. 10 A). Immunoblot analysis indicated that also the protein level of Alb4 is drastically reduced in comparison to wild-type plants (below 10 % of wild-type level). At the same time the amount of LHC protein did not decrease correspondingly.
(Fig. 10 B). Though the RNAi lines grew without any visible phenotype on soil, the ultrastructure of the chloroplasts was changed (Fig. 10 E-H). Chloroplasts were larger, their shape was pronounced ball-shaped, the stroma region was enlarged and thylakoids were less well organized and not so appressed.

**Alb4 is independent of Artemis N-terminus**

The original results for Artemis were obtained using mutant *Arabidopsis* plants that carried a transposable *En-1* element in the second exon of *Artemis* (22). To simplify further studies we screened for plants with a stable footprint instead of the often somatically excised complete transposon. In the total membrane fraction of plants that were homozygous for a 128 bp footprint we could still detect Alb4 at normal levels, demonstrating that the expression of Alb4 is not affected in these plants (Fig. 11).

**DISCUSSION**

Analysis of the *Arabidopsis* genome led to the identification of six genes encoding putative members of the Alb3/Oxa1/YidC family (34,35). One of these loci was described to encode Artemis, an inner envelope 110 kDa protein of chloroplasts involved in chloroplast division (22). In this report we reinvestigated the existence of Artemis because a revised annotation of the *Arabidopsis* genome indicated a gene model which predicts two coding sequences for the locus (At1g24490/At1g24500, MIPS *A. thaliana* Genome Database). We show that indeed the At1g24500 gene product, that corresponds to the C-terminal Alb3/Oxa1/YidC domain of Artemis, is expressed as a separate 55 kDa protein (Alb4). The exact localisation and function of the At1g24490 gene product is not yet resolved. However, chloroplasts from *Arabidopsis* plants containing an *En-1* transposon element in this region were described to appear duplicated or triplicated and developing cotyledons contained tripolar plastids (22). Current work is in progress to confirm this phenotype. In this work we demonstrate that this phenotype is independent of Alb4. First, the protein level of Alb4 is not reduced in the *En-1* footprint transposon plants (Fig. 11). Second, the chloroplast ultrastructure of *Arabidopsis* mutants (Salk_136199, RNAi) containing reduced amounts of Alb4 is altered but does not point to a specific defect in chloroplast division.

The amino acid sequence of Alb3 and its homologue Alb4 show high conservation with 72% similarity and 55% identity. Both proteins are expressed in the green tissue of *Arabidopsis* and are located in the same compartment, the thylakoid membrane. This leads to the question whether these homologues have redundant functions. The strong phenotype of the alb3 null mutant indicates that Alb4 can not compensate efficiently the loss of Alb3 and therefore must have a specialized function (18). *Arabidopsis* mutants with a strong reduction (~ 90 %) of the Alb4 level are vital and do not have an apparent visual phenotype under normal growth conditions. However, an effect on chloroplasts as well as thylakoid membrane structure could be observed. Thylakoid membranes are less appressed than in wild-type, which is probably due to the enlarged chloroplasts in the mutant. The chloroplasts in the mutant lines appear more circular in shape than in wild-type. This is especially pronounced in the RNAi line where chlorolasts are much more spherical and not lense shaped. These results could be interpreted in a way that Alb4 is not essential for chloroplast development like Alb3, but is still necessary for the formation of proper chloroplast ultrastructure. However, it is possible that the residual 10 % of Alb4 are sufficient to support the biogenesis of photosynthetic active chloroplasts and that an alb4 null mutants would exhibit a stronger phenotype. It is also possible that the mild phenotype of the Alb4 mutants analysed in this study is caused by a functional replacement of Alb4 by Alb3.

We observed reduced amounts of wild-type-like Alb4 protein in the mutant line Salk_136199 (Fig. 8 B) but no smaller protein that would be translated from the transcript lacking exon 7 (Fig. 7 D and 8 B). One possible explanation for this would be that the truncated protein is unstable and therefore prone to immediate proteolysis. A proper integration into the thylakoid membranes might also be hindered by the lack of the putative fourth transmembrane span that is encoded on exon 7.

In previous studies it was shown that preincubation of thylakoid membranes with anti-Alb3 antibodies led to a drastic reduction of the insertion of a group of light-harvesting chlorophyll-binding proteins into the thylakoid membrane (20,21). Using the same type of antibody it was demonstrated that Alb3 docks to the cpSRP-cpFtsY complex, two components required for the targeting of the light-harvesting chlorophyll-binding proteins (36) and that Alb3...
is also at least partially associated with the cpSecY complex (37). It should be noted that the antibody used in these studies was generated against the first stromal loop of Alb3, a region that is highly conserved in Alb4. Experiments analysing the specificity of this type of anti-Alb3 antibody revealed that it also detects Alb4 (data not shown). Therefore, it will be very important for future studies to use antibodies that recognize exclusively either Alb3 or its homologue Alb4.

In *Chlamydomonas* two Alb3 homologues, Alb3.1 and Alb3.2, were identified (19). Both proteins are closely related to Alb3. Alb3.1 displays 46% identity and 6% similarity to Alb3 and sequence comparison of Alb3.2 and Alb3 revealed even higher values of 53% identity and 71% similarity. *Chlamydomonas* mutants lacking Alb3.1 are mainly characterized by a drastic reduction of the light-harvesting systems. In addition, the *alb3.1* mutant has a reduced amount of photosystem II and a defect in the assembly of D1 into photosystem II (19,38). However, the mutant is still able to grow photoautotrophically and therefore, exhibits a much milder phenotype than the *alb3* mutant of *Arabidopsis*. Interestingly, *Synechocystis* sp. PCC6803 contains just a single Alb3 gene (slr1471). It was shown that this Alb3 homologue is essential for cell viability and that depletion of this protein results in a severe damage of thylakoid biogenesis (39). These data indicate that Alb3 plays a general role in protein insertion in *Synechocystis* like in other bacteria.

Currently, little is known about the function of Alb4. Recently, it was shown that the transmembrane region of Alb4 (Artemis) could functionally replace Oxa1p in yeast to some extent (35) indicating that Alb4 is possibly involved in the insertion or assembly of membrane proteins.

Thylakoid membranes contain proteins of dual genetic origin. Some are encoded on the chloroplast genome, synthesized inside the organelle and integrated into the thylakoid membrane in a cotranslational manner most likely involving a bacterial-like Sec-system, composed of the membrane localized subunits SecY (40) and SecE (41) and the homologue of the bacterial ribosome receptor FtsY (42,43). Other are encoded in the nucleus, imported posttranslationally into the chloroplasts and then inserted into the thylakoid membrane. The major intrinsic thylakoid protein LHCP uses the soluble SRP-like protein in cooperation with Alb3. Other integral thylakoid membrane proteins like cpSecE, PsbW, PsbX and PsaK use a so called spontaneous insertion pathway (reviewed in (44)), which seems not to require proteinaceous components. However, the spontaneous insertion pathway which was proposed to exist in a similar fashion in bacteria, was recently shown to involve the essential component YidC, the bacterial relative to Alb4. It is therefore tempting to speculate that Alb4 might be involved in this pathway in chloroplasts, although the weak phenotype of the RNAi lines does not point to an essential function of Alb4.

However the conversion from a cyanobacterial endosymbiont to an eukaryotic organelle with the necessity to accommodate both cotranslational protein insertion as well as postranslational protein integration might require two YidC homologues namely Alb3 and Alb4 for proper thylakoid differentiation. Further protein-interaction studies and the analysis of a loss of function mutant are required to elucidate the precise role of Alb4 in chloroplast biogenesis.

**REFERENCES**

1. Dalbey, R. E., and Kuhn, A. (2004) *J Cell Biol* **166**, 769-774.
2. Yi, L., and Dalbey, R. E. (2005) *Mol Membr Biol* **22**, 101-111.
3. Beck, K., Eisner, G., Trescher, D., Dalbey, R. E., Brunner, J., and Müller, M. (2001) *EMBO Rep* **2**, 709-714.
4. Urbanus, M. L., Scotti, P. A., Froderberg, L., Saaf, A., de Gier, J. W., Brunner, J., Samuelson, J. C., Dalbey, R. E., Oudega, B., and Luirink, J. (2001) *EMBO Rep* **2**, 524-529.
5. Houben, E. N., ten Hagen-Jongman, C. M., Brunner, J., Oudega, B., and Luirink, J. (2004) *EMBO Rep* **5**, 970-975.
6. Nagamori, S., Smirnova, I. N., and Kaback, H. R. (2004) *J Cell Biol* **165**, 53-62.
7. Samuelson, J. C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) Nature 406, 637-641.
8. Chen, M., Samuelson, J. C., Jiang, F., Muller, M., Kuhn, A., and Dalbey, R. E. (2002) J Biol Chem 277, 7670-7675.
9. Serek, J., Bauer-Manz, G., Struhalla, G., van den Berg, L., Kiefer, D., Dalbey, R., and Kuhn, A. (2004) Embo J 23, 294-301.
10. Funes, S., Nargang, F. E., Neupert, W., and Herrmann, J. M. (2004) Mol Biol Cell 15, 1853-1861.
11. Glick, B. S., and Von Heijne, G. (1996) Protein Sci 5, 2651-2652.
12. Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1998) Proc Natl Acad Sci U S A 95, 2250-2255.
13. Hell, K., Neupert, W., and Stuart, R. A. (2001) Embo J 20, 1281-1288.
14. Herrmann, J. M., and Neupert, W. (2003) IUBMB Life 55, 219-225.
15. Jia, L., Dienhart, M., Schramp, M., McCauley, M., Hell, K., and Stuart, R. A. (2003) Embo J 22, 6438-6447.
16. Szyrach, G., Ott, M., Bonnefoy, N., Neupert, W., and Herrmann, J. M. (2003) Embo J 22, 6448-6457.
17. Preuss, M., Ott, M., Funes, S., Luirink, J., and Herrmann, J. M. (2005) J Biol Chem 280, 13004-13011.
18. Sundberg, E., Slagter, J. G., Fridborg, I., Cleary, S. P., Robinson, C., and Coupland, G. (1997) Plant Cell 9, 717-730.
19. Bellafiore, S., Ferris, P., Naver, H., Gohre, V., and Rochaix, J. D. (2002) Plant Cell 14, 2303-2314.
20. Moore, M., Harrison, M. S., Peterson, E. C., and Henry, R. (2000) J Biol Chem 275, 1529-1532.
21. Woolhead, C. A., Thompson, S. J., Moore, M., Tissier, C., Mant, A., Rodger, A., Henry, R., and Robinson, C. (2001) J Biol Chem 276, 40841-40846.
22. Fulgosi, H., Gerdes, L., Westphal, S., Glockmann, C., and Soll, J. (2002) Proc Natl Acad Sci U S A 99, 11501-11506.
23. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubak, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeke, A., Crosby, W. L., Berry, C. C., and Ecker, J. R. (2003) Science 301, 653-657.
24. Salomon, M., Fischer, K., Flügge, U. I., and Soll, J. (1990) Proc Natl Acad Sci U S A 87, 5778-5782.
25. Lee, Y. J., Kim, D. H., Kim, Y. W., and Hwang, I. (2001) Plant Cell 13, 2175-2190.
26. Peeters, N. M., Chapron, A., Giritch, A., Grandjean, O., Lancelin, D., Lhomme, T., Vivrel, A., and Small, I. (2000) J Mol Evol 50, 413-423.
27. Karimi, M., De Meyer, B., and Hilson, P. (2005) Trends Plant Sci 10, 103-105.
28. CloUGH, S. J., and Bent, A. F. (1998) Plant J 16, 735-743.
29. Waegemann, K., and Soll, J. (1995) Methods Cell Biol 50, 255-267.
30. Li, H. M., Moore, T., and Keegstra, K. (1991) Plant Cell 3, 799-717.
31. Clausen, C., Ilkavets, I., Thomson, R., Philippar, K., Vojta, A., Mohlmann, T., Neuhaus, E., Fulgosi, H., and Soll, J. (2004) Planta 220, 30-37.
32. Philippar, K., Ivashikina, N., Ache, P., Christian, M., Luthen, H., Palme, K., and Hedrich, R. (2004) Plant J 37, 815-827.
33. Emanuelsson, O., Nielsen, H., and von Heijne, G. (1999) Protein Sci 8, 978-984.
34. Yen, M. R., Harley, K. T., Tseng, Y. H., and Saier, M. H., Jr. (2001) *FEMS Microbiol Lett* **204**, 223-231
35. Funes, S., Gerdes, L., Inaba, M., Soll, J., and Herrmann, J. M. (2004) *FEBS Lett* **569**, 89-93
36. Moore, M., Goforth, R. L., Mori, H., and Henry, R. (2003) *J Cell Biol* **162**, 1245-1254
37. Klostermann, E., Droste Gen Helling, I., Carde, J. P., and Schünemann, D. (2002) *Biochem J* **368**, 777-781
38. Ossenbühl, F., Gohre, V., Meurer, J., Krieger-Liszkay, A., Rochaix, J. D., and Eichacker, L. A. (2004) *Plant Cell* **16**, 1790-1800
39. Spence, E., Bailey, S., Nenninger, A., Moller, S. G., and Robinson, C. (2004) *J Biol Chem* **279**, 17664-17667
40. Laidler, V., Chaddock, A. M., Knott, T. G., Walker, D., and Robinson, C. (1995) *J Biol Chem* **270**, 12177-12182
41. Schuenemann, D., Amin, P., Hartmann, E., and Hoffman, N. E. (1999) *J Biol Chem* **274**, 12177-12182
42. Kogata, N., Nishio, K., Hirohashi, T., Kikuchi, S., and Nakai, M. (1999) *FEBS Lett* **447**, 329-333
43. Tu, C. J., Schuenemann, D., and Hoffman, N. E. (1999) *J Biol Chem* **274**, 27219-27224
44. Di Cola, A., Klostermann, E., and Robinson, C. (2005) *Biochem Soc Trans* **33**, 1024-1027

**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1 The Alb4 transcript
(A) Scheme of the domain organization of Artemis and Alb4. A partial sequence alignment of Artemis and Alb4 shows that Alb4 is identical to the Alb3/Oxa1/YidC domain of Artemis except for the N-terminal located twenty amino acids. This work demonstrates that the genome annotation predicting the ORF encoding Alb4 is correct. The previously described existence of Artemis could not be verified. (B) 5'-UTR (*italics*) of Alb4 from Arabidopsis was determined by 5'-RACE and RT-PCR. mRNA and corresponding amino acid sequence are shown (*coding sequence underlined*). Extending the reading frame upstream leads to a stop-codon (*) excluding the possibility of a longer ORF. Numbering starts with first transcribed nucleotide.

Fig. 2 The Alb4 gene is expressed in leaves of Arabidopsis.
(A) Total RNA (15 µg/lane) was prepared from 6-week-old wild-type plants. RNA gel blots were hybridized with [*32P*]-CTP labeled DNA probes specific for Alb3 (lane 1) or Alb4 (lane 2). The DNA probes encode the C-terminal regions of Alb3 and Alb4 used for protein expression as described in “Experimental Procedures”. (B) The specificity of the used DNA probes for the detection of the Alb3- or Alb4-mRNA was verified by performing Northern Blot experiments using *in vitro* transcribed mRNA of Alb3 (lane 2 and 4) and pAlb4 (lanes 1 and 3). Samples in lane 1 and 2 were hybridized with the Alb3 specific probe and samples in lanes 3 and 4 were hybridized with the Alb4 specific probe.

Fig. 3 Subcellular localization of Alb3-GFP and Alb4-GFP fusion proteins
Arabidopsis mesophyll protoplasts were transiently transformed with constructs for Alb3- or Alb4-GFP. Maximum intensity signals from confocal images are shown for chlorophyll autofluorescence, GFP fluorescence and an overlay of both. OEP7-GFP is included as a marker for the chloroplast envelope. Bar represents 5µm.

Fig. 4 Alb4 is imported into chloroplasts
(A) *In vitro* translated [*35S*] methionine-labeled Alb4 precursor (pAlb4) was imported into isolated pea chloroplasts (equivalent to 20 µg chl) for 20 min at 25°C in the dark. After completion of the import reaction, chloroplasts were reisolated through a 40% (v/v) Percoll cushion and washed once. Chloroplasts were then either not treated (-) or treated (+) with the protease thermolysin. Organelles were lysed and separated into a soluble stroma (S) and membrane fraction (M). All samples were subjected to SDS-PAGE (only 10% of the soluble fraction was loaded) and labeled proteins were detected by fluorography. [*35S*]-labeled translation product (TP) is shown as an internal standard, 10% of which was added to a standard import reaction. p, precursor; m, mature. (B) The precursor protein of the oxygen evolving complex 33 kDa subunit (OE33) was used as a control and was imported into the thylakoids, where the processed mature form is resistant to thermolysin.

Fig. 5 Anti-Alb4 antibodies are specific for Alb4.
(A) C-terminal fragments of Alb3 and Alb4 were expressed as his-tag fusion proteins (cAlb3-his or cAlb4-his) in *E. coli*, purified and separated on SDS-PAGE. Gels were stained (left panel) or subjected to immunoblot analysis using an antibody generated against cAlb4-his (α-Alb4-1) or preimmune serum (α-PI). (B) *In vitro* translation products of Alb3 or the Alb4 precursor (pAlb4) were separated on SDS-polyacrylamidgels and subjected to immunoblot analysis using the anti-Alb4-1 antibody or an anti-Alb3 antibody (37). *: nonspecific band (is also detected in control experiments using just wheat germ extract for blotting (data not shown)).

Fig. 6 Alb4 is located in the thylakoid membrane.
(A) Chloroplasts were fractionated into thylakoid membranes (T), stroma (S) and envelopes (E) as described in “Experimental Procedures”. Total chloroplast proteins and proteins of the subfractions were separated on SDS-polyacrylamidgels and subjected to immunoblot analysis using antibodies against Alb4, LHCP (as a marker protein for thylakoid membranes), OEP37 (as a marker protein for the outer envelope membrane) and Tic22 (as a marker protein for the inner envelope membrane). (B) Three further antibodies against Alb4 were used to immunodecorate chloroplast fractions. α-ArtA
recognizes a protein in the envelope described as Artemis. LHCP, Tic32 (as a marker protein for the inner envelope membrane) and GAPDH subunits A and B (as a marker protein for the stroma) serve as controls for the fractions.

Fig. 7 The Arabidopsis T-DNA insertion line Salk_136199 transcribes two versions of Alb4 mRNA. (A) Schematic representation of Alb4 gene. Exons are represented by black boxes, 5'-UTR and 3'-UTR by white boxes, introns are indicated as lines. Position of T-DNA and its left border (LB) in mutant line Salk_136199 are depicted in black. The wrongly annotated position for the T-DNA in intron 5 is shown in grey. Primers used for detailed transcript analysis (E7) or quantitative real-time PCR (LC) are shown as arrows. The grey stretch represents the part additionally spliced out in the mutant line. (B) RT-PCR was performed with cDNA transcribed from total RNA of 7-day-old plants and products separated on agarose gel. Mutants homozygous for the T-DNA insertion (Salk_136199) have two Alb4 transcripts, WT plants only one. (C) RT-PCR products were cloned and sequenced. One transcript in the T-DNA mutant is the same as in WT plants. In the other transcript exon 7 (underlined) is spliced out together with intron 7. RNA and corresponding amino acid sequence are shown. Positions of WT introns are marked with open triangles. The dashed line indicates the position of the putative fourth transmembrane domain (predicted by the programme TMPred). Numbering starts with first transcribed nucleotide or the first translated amino acid, respectively. (D) Chloroplasts of plants homozygous for the T-DNA in Alb4 were fractionated into stroma (S) and thylakoid membranes (T) as described in “Experimental Procedures”. Proteins of the subfractions were separated on SDS-polyacrylamidgels and subjected to immunoblot analysis using antibodies against Alb4, LHCP and LSU.

Fig. 8 Analysis of 5-week-old plants of the Arabidopsis T-DNA insertion line Salk_136199 (A) The expression of Alb4 in the T-DNA insertion line Salk_136199 was tested by real time PCR using four independent 5-week-old homozygous plants (white bar). Wild-type plants were used as controls (black bar). (B) Membrane proteins from the same four individual 5-week-old homozygous Salk_136199 mutants were prepared from 200 mg leaf material, separated on SDS-polyacrylamidgels and subjected to immunoblot analysis using the anti-Alb4-1 antibody (upper panel). Loading controls were done using an antibody against the integral membrane protein cpSecY (41) (lower panel).

Fig. 9 Analysis of 7-day-old seedlings of the Arabidopsis T-DNA insertion line Salk_136199 (A, B, C, D, E, F) Electron microscopic pictures of mesophyll cells from 7-day-old Arabidopsis plants. Bar represents 2µm. (A, B) Overview and chloroplast from wild-type. (C, D, E, F) Overviews and chloroplasts from the mutant line Salk_136199. (G) Quantification of Alb4 transcripts in cotyledons of 7-day-old seedlings from homozygous Salk_136199 mutants (white bar) and wild-type (black bar). Transcript density (n = 3 ± SD) was measured by quantitative real-time RT-PCR with the primer pairs LC.fw and LC.rv (see 7 A) as described in “Experimental Procedures”. The Alb4 mRNA content was calculated relative to AtActin 2/8 transcripts and normalised to the amount in wt (black bar), which was set to 100 % (arbitrary units).

Fig. 10 Analysis of Arabidopsis plants containing an RNAi construct to reduce Alb4 expression (A) The expression of Alb4 in the RNAi line was tested by quantitative real-time PCR using leaves of four independent plants in the rosette stage. Wild-type plants were used as controls. (B) Membrane proteins were prepared from leaves of the same plants, separated by SDS-PAGE and analysed by immunoblot for the presence of Alb4 and LHCP. WT protein extract was diluted 1/2, 1/5 and 1/10. (C, D, E, F, G, H) Electron microscopic pictures of mesophyll cells from the same plants. Bar represents 2µm. (C, D) Overview and chloroplast from wild-type. (E, F, G, H) Overviews and chloroplasts from the RNAi line.

Fig. 11 Alb4 is independent of Artemis N-terminus Total proteins were isolated from 6-week-old WT plants or plants homozygous for an En-1 transposon footprint (Trsp-Fp) in Artemis N-terminus. Proteins were fractionated into soluble (S) and membrane (M) proteins, subsequently separated by SDS-PAGE and subjected to immunoblot analysis using antibodies against Alb4, LHCP (as a marker protein for thylakoid membranes), Tic110 (as a marker
protein for chloroplast inner envelope) and GAPDH subunits A and B (as a marker protein for the stroma).
Fig. 1
Fig. 3
Fig. 4

Fig. 5
Fig. 6
Fig. 7
Fig. 8

**A**

Alb4 transcript content in %

|          | 136199 | WT |
|----------|--------|----|
| 1        | 50.0   |    |

**B**

|          | 136199 |
|----------|--------|
| WT       | 1 2 3 4 |

Alb4

cpSecY

Fig. 8
|       | WT   | Trsp-Fp |
|-------|------|---------|
|       | S    | M       | S    | M       |
| α-Alb4-2 | ![Image](image1.png) | ![Image](image2.png) |
| α-LHCP    | ![Image](image3.png) | ![Image](image4.png) |
| α-Tic110 | ![Image](image5.png) | ![Image](image6.png) |
| α-GAP A/B | ![Image](image7.png) | ![Image](image8.png) |

Fig. 11
A second thylakoid membrane localized Alb3/Oxa1/YidC homologue is involved in proper chloroplast biogenesis in Arabidopsis thaliana

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