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Loss of Albino3 Leads to the Specific Depletion of the Light-Harvesting System

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The chloroplast Albino3 (Alb3) protein is a chloroplast homolog of the mitochondrial Oxa1p and YidC proteins of *Escherichia coli*, which are essential components for integrating membrane proteins. In vitro studies in vascular plants have revealed that Alb3 is required for the integration of the light-harvesting complex protein into the thylakoid membrane. Here, we show that the gene affected in the *ac29* mutant of *Chlamydomonas reinhardtii* is *Alb3.1*. The availability of the *ac29* mutant has allowed us to examine the function of *Alb3.1* in vivo. The loss of *Alb3.1* has two major effects. First, the amount of light-harvesting complex from photosystem II (LHCII) and photosystem I (LHCI) is reduced >10-fold, and total chlorophyll represents only 30% of wild-type levels. Second, the amount of photosystem II is diminished 2-fold in light-grown cells and nearly 10-fold in dark-grown cells. The accumulation of photosystem I, the cytochrome b_6f complex, and ATP synthase is not affected in the *ac29* mutant. Mild solubilization of thylakoid membranes reveals that Alb3 forms two distinct complexes, a lower molecular mass complex of a size similar to LHC and a high molecular mass complex. A homolog of *Alb3.1*, *Alb3.2*, is present in Chlamydomonas, with 37% sequence identity and 57% sequence similarity. Based on the phenotype of *ac29*, these two genes appear to have mostly nonredundant functions.

INTRODUCTION

The biogenesis of the photosynthetic complexes of the thylakoid membrane depends on the concerted interactions of the chloroplast and nuclear genetic systems. Genetic and biochemical studies in Chlamydomonas, maize, and Arabidopsis have revealed a large number of nucleus-encoded factors that are involved in transcriptional and post-transcriptional steps of chloroplast gene expression (Barkan and Goldschmidt-Clermont, 2000). Once proteins of the photosynthetic apparatus have been synthesized, they need to be targeted to the thylakoid membrane, inserted into or translocated through the membrane, and assembled into functional complexes.

At least four pathways for thylakoid membrane insertion and translocation have been characterized that display unique energy and stromal protein requirements (Keegstra and Cline, 1999). Each of these pathways targets a distinct subset of proteins to the thylakoid. The Sec pathway, which requires ATP and cpSecA, is related to the Sec system of the bacterial export machinery. The presence of this system in chloroplasts is not surprising in light of the generally accepted view that chloroplasts evolved from a prokaryotic endosymbiont. Another pathway, related to the bacterial Tat pathway, uses a transthylakoid pH gradient as its sole energy source to transport proteins and does not require any stromal factor (Robinson and Bolhuis, 2001).

The light-harvesting complex protein (LHCP) integration pathway shares several features with the GTP-dependent signal recognition particle (SRP) system of the endoplasmic reticulum and bacteria (Li et al., 1995). The chloroplast SRP contains a homolog of SRP54. However, it differs from other SRPs because it lacks any SRP RNA, contains a novel 43-kD subunit, and interacts with substrates post-translationally (Schuenemann et al., 1998; Klimyuk et al., 1999). It is believed generally that the solubility of the hydrophobic LHCP is maintained in the stroma by its binding to SRP to form a targeting intermediate of 120 kD termed the transit complex.

Together with GTP and the chloroplast homolog FtsY, an *Escherichia coli* protein necessary for *E. coli* SRP function, the transit complex promotes efficient integration of LHCP into the thylakoid membrane (Tu et al., 1999). However, the fact that LHCP can insert into thylakoid membranes in the absence of functional SRP (Amin et al., 1999) suggests that alternative targeting pathways exist. Finally, some thylakoid proteins are able to insert into the thylakoid membrane without the requirement of protein factors.

Several factors specific for the assembly of photosynthetic complexes have been identified. They include BtpA

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(Bartsevich and Pakrasi, 1997; Zak et al., 1999; Zak and Pakrasi, 2000), Ycf3 and Ycf4 (Wilde et al., 1995; Boudreau et al., 1997; Ruf et al., 1997), which are required specifically for the accumulation of photosystem I (PSI), and Hcf 136, which is required for the assembly of photosystem II (PSII) (Meurer et al., 1998). However, the action of these factors at the molecular level remains poorly understood.

Studies with yeast mitochondrial protein export have shown that the inner membrane protein Oxa1p is an essential component for integrating a subset of inner membrane proteins encoded by nuclear and mitochondrial DNA (Bonnefoy et al., 1994; Hell et al., 1998, 2001). Oxa1p is homologous with the YidC protein of *E. coli* that also acts as a mediator of membrane protein assembly (Luirink et al., 2001). YidC operates either as a separate unit or in connection with the Sec YEG translocon, depending on the substrate protein that is integrated into the membrane. A homolog of Oxa1p, termed Alb3, has been found in chloroplasts (Sundberg et al., 1997). A mutant of Arabidopsis deficient in Alb3 displays an albino phenotype, indicating that this protein is required for thylakoid biogenesis (Sundberg et al., 1997).

Recently, it was shown that treatment of thylakoid membranes with an Alb3 antibody interferes with the integration of Lhcb1, the major light-harvesting chlorophyll binding protein (Moore et al., 2000). The same treatment also blocks the insertion of two other chlorophyll binding proteins, Lhcb4.1 and Lhcb5 (Woolhead et al., 2001). By contrast, preincubation of thylakoids with anti-Alb3 antibodies does not affect the thylakoid Sec or Delta pH pathways (Moore et al., 2000), nor does it block the insertion of PsbS, PsbX, PsbW, and PsbY (Woolhead et al., 2001). The latter three proteins contain signal peptides, and their insertion into the thylakoid membrane is SRP independent. These in vitro studies reveal a strict correlation between the requirements for Alb3 and SRP.

To test the role of Alb3 in vivo, we have characterized a mutant of Chlamydomonas, *ac29-3*, in which the *Alb3.1* gene is inactivated. This mutant is principally deficient in LHC. The level of PSII also is reduced, especially in dark-grown cells. The Alb3.1 protein appears to form two distinct complexes, a lower molecular mass complex of a size similar to that of LHC and a high molecular mass complex of unknown function. Chlamydomonas contains a homolog of *Alb3.1* that appears to play only a modest role in the integration of LHC in the thylakoid membrane, based on the phenotype of the *ac29* mutant.

RESULTS

Characterization of *ac29* Mutants

The first two mutants affected at the *AC29* locus of Chlamydomonas, CC-44 and CC-45, were identified as yellow, leaky, acetate-requiring mutants (Levine and Goodenough, 1970). This locus is linked tightly to that of the *MATING-TYPE* (*MT*), which was cloned and characterized previously (Ferris and Goodenough, 1994; Ferris, 1995). Several new mutants affected at the *AC29* locus were isolated during a search for large deletions in the *MT* locus (P. Ferris, unpublished results).

A diploid having a prototrophic green mt^- phenotype was constructed from CC-1336 (*nic7 ac29-2* mt^- *pf14*) and CC-1067 (++ mt^+ +). Gamma irradiation of the diploid generated yellow and/or nicotinamide-requiring mutant strains carrying new mutations at these loci. Six yellow nicotinamide prototrophs (*acy9*, *acy10*, *acy20*, *acy25*, *acy27*, and *acy32*) were obtained that have DNA rearrangements in the region marked by the *ac29-2* allele in Figure 1. DNA gel blot analysis of four of these mutants and their diploid parental strain is shown in Figure 2A.

The DNAs were digested with BglII and probed with the acB probe (Figure 1). The probe hybridizes to two fragments of the parent, one (1.3 kb) from the *ac29-2* mt^- chromosome and the other (2.2 kb) from the *AC29* mt^+ chromosome. The *acy9* strain shows no change within the BglII fragments but has an insertion larger than 10 kb farther downstream (Figure 1 and data not shown). The *acy27* and

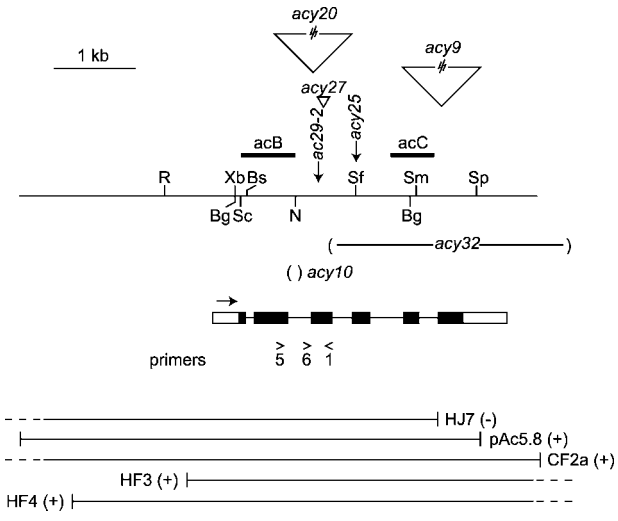

Figure 1. Map of the *AC29* Locus.

The probes acB and acC are shown. The approximate location of the point mutations, insertions (wedges), and deletions (−) of the *acy* mutants are marked. The exons and introns of the *Alb3* gene are indicated with black bars and lines, respectively. Untranslated regions are shown as open bars. Transcription proceeds from left to right. The primers used are indicated. Plasmids and phages used in transformations are shown at bottom. Rescue of the mutant phenotype is indicated by +. Restriction sites are indicated at top: Bg, BglII; Bs, BstEII; N, NsiI; R, EcoRI; Sc, SacI; Sf, SfiI; Sm, SmaI; Sp, SpeI; Xb, XbaI.

acy10 strains carry a small insertion and a deletion, respectively, within the 2.2-kb BglII fragment. These changes in the two mutants localize between the BstEII and SfiI sites (Figure 1 and data not shown). The only change detectable in the *acy25* strain is the loss of the SfiI site.

Figure 2B shows DNA gel blots obtained after digestion with SacI from the parent and the *acy9*, *acy20*, and *acy32* mutants. The mt^- and mt^+ chromosomes yield fragments of 7.2 and 10.8 kb, respectively. In the mutants, the mt^+ fragment has been replaced by smaller fragments. Additional DNA gel blots revealed that *acy9* and *acy20* contain large insertions (>10 kb) with several internal SacI sites, resulting in SacI fragments smaller than in the original, whereas *acy32* carries a 3-kb deletion (data not shown). The approximate locations of the insertions and the deletion are indicated in Figure 1.

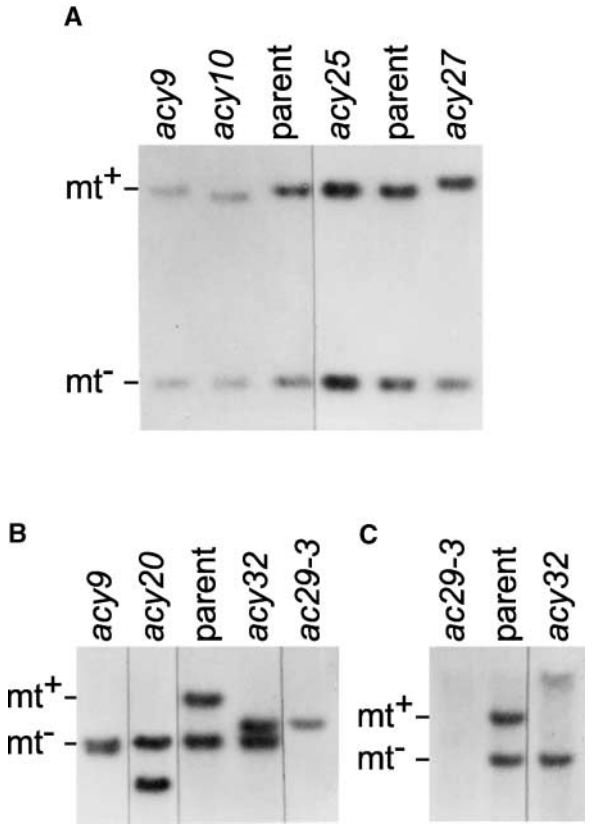

Figure 2. DNA Gel Blot Analysis of *ac29* Mutants.

DNA was isolated from the diploid parent (*nic7 ac29-2* mt^- *pf14*/++ mt^+ +) and the diploid *acy9*, *acy10*, *acy20*, *acy25*, and *acy32* mutant strains and from the haploid *ac29-3* mutant containing the mt^+ chromosome from *acy32*. The DNAs of the blots were digested with BglII **(A)** or SacI (**[B]** and **[C]**) and probed with the acB probe (**[A]** and **[B]**) or the acC probe **(C)**.

Identification and Structure of the *AC29* (*Alb3*) Gene

The sequence of 4654 bp proceeding to the right from the EcoRI site in Figure 1 was determined from the genomic copy of *AC29*. A corresponding cDNA was isolated, and its sequence was determined. Comparison of the two sequences revealed that this gene consists of six exons and five introns of 113, 313, 230, 440, and 213 nucleotides. A BLAST search (in April 2002) of the database with the protein of 495 residues predicted from the cDNA had significant sequence identity with the chloroplast Albino3 protein of Arabidopsis (46% identity and 65% similarity; Figure 3). Accordingly, the gene is called *Alb3.1*.

Chlamydomonas ESTs indicate that *Alb3.1* mRNAs are polyadenylated at multiple locations. The position of *Alb3.1* is consistent with previous transformation data (Ferris, 1995). Thus, the green phenotype of the *ac29-2* mutant was restored by transformation with the pAc5.8 plasmid, which terminates within the 3′ untranslated region, but not by transformation with the HJ7 phage, which lacks the final exon (Figure 1). The location of *Alb3.1* also is consistent with the observation that phages HF3 and CF2a were able to rescue the *ac29-2* mutant by transformation (Ferris, 1995).

Further evidence that the *AC29* locus corresponds to the *Alb3.1* gene is provided by the *ac29-2* mutation in the third exon of *Alb3.1*. Restriction fragment length polymorphism analysis had revealed polymorphic BglII-XbaI tandem sites in the *ac29-2* allele of CC-44. A PCR product spanning the mutant site obtained with primers 1 and 5 (Figure 1) was cloned and sequenced. The mutation consists of a single C-to-T base change that produces a stop codon and creates two novel restriction sites, BglII and XbaI (Figure 4).

It is possible that some other changes in the *ac29-2* allele also are responsible for the phenotype, because the entire allele was not sequenced. However, examination of three green revertants by DNA gel blot analysis revealed that they had retained the BglII site but had lost the XbaI site. The only change in the sequence of the revertants relative to *ac29-2* was within the XbaI site that restored the *Alb3.1* open reading frame (Figure 4). Analysis of the *acy27* mutant revealed that it contains a 78-bp insertion that apparently resulted from the duplication of adjoining sequences in exon 3 (data not shown).

To determine whether the *acy32* deletion (referred to as the *ac29-3* allele) confers a more stringent acetate-requiring phenotype than the *ac29-2* nonsense mutation, it was first necessary to create a haploid strain for the *NIC7 ac29-3* mt^+ chromosome. Such an *ac29-3* strain was constructed by crossing the *acy32* diploid to a green *nic7* mt^+ strain and identifying a prototrophic yellow mt^+ strain among the progeny. DNA gel blot analysis (Figure 2B) demonstrated that the strain carried only the *ac29-3* chromosome and failed to hybridize with the acC probe predicted to lie in the deleted region (Figures 1 and 2C).

The haploid *ac29-3* strain, like *ac29-2*, is not strictly acetate requiring. It grows normally on Tris-acetate phosphate

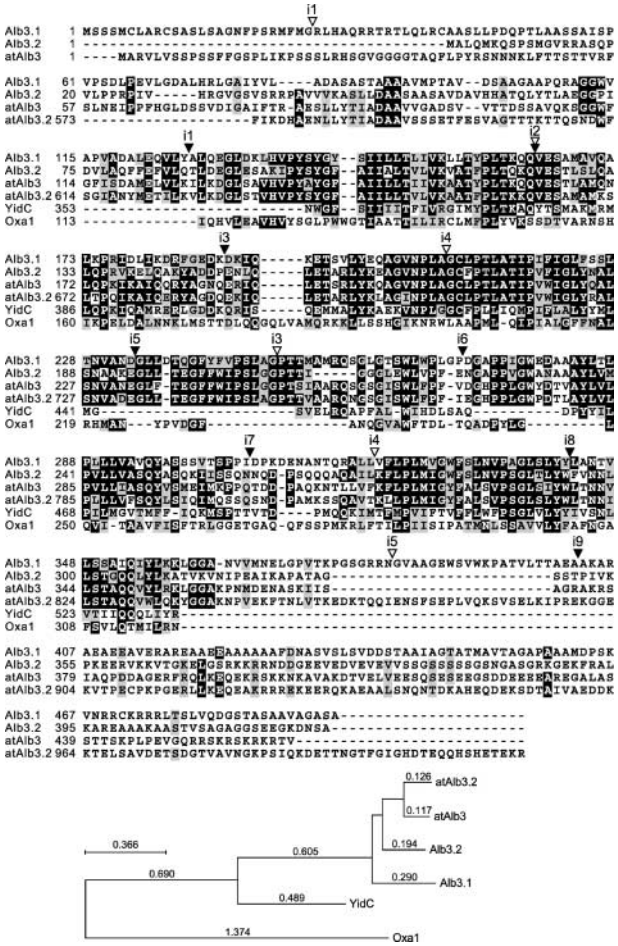

Figure 3. Sequence Comparison of the Alb3 Protein with YidC and Oxa1p.

The protein sequences were aligned using ClustalW (Thompson et al., 1994) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Alb3.1 and Alb3.2 are from Chlamydomonas; atAlb3 (Sundberg et al., 1997) and atAlb3.2 are from Arabidopsis; YidC is from *E. coli* (Luirink et al., 2001); Oxa1p is from *S. cerevisiae* (Bonnefoy et al., 1994). The sites corresponding to the introns are indicated by open and closed wedges for Alb3.1 of Chlamydomonas and atAlb3 of Arabidopsis, respectively. In the phylogenetic tree at bottom, the numbers of amino acid substitutions per alignment site are indicated on the branches. The phylogenetic analysis was performed using the PHYLO_WIN program (Galtier and Gouy, 1996).

(TAP) medium under high light. However, its growth on minimal medium or on TAP medium under low light is retarded significantly (Figure 5). It grows more slowly on high-salt medium (HSM) plates than *ac29-2* (data not shown). Transformation of *ac29-3* was performed with linearized pAc5.8. Only 2×10^6 cells were transformed, and they were spread onto 18 HSM plates. Several green sectors were isolated, and ultimately, 11 independent green transformants were isolated after subcloning. These results indicate that the yellow color of *ac29-3* is caused by the deletion in the *AC29* locus. This mutant was used for further studies.

The N-terminal part of the Alb3.1 protein of Chlamydomonas is rich in Ser, Thr, and Arg, a typical feature of chloroplast transit peptides, and is predicted by the Chloro P program to contain a chloroplast transit sequence (Emanuelsson et al., 1999). This is consistent with the location of the Arabidopsis Alb3 protein in the chloroplast (Sundberg et al., 1997) and with the finding that the Alb3.1 protein of Chlamydomonas is associated with thylakoid membranes (see below).

Besides its similarity to the Alb3 protein of Arabidopsis, two regions of the Chlamydomonas protein, residues 128 to 232 and 284 to 358, show significant sequence identity to the Oxa1p mitochondrial protein of *Saccharomyces cerevisiae* and to the inner membrane YidC protein of *E. coli* (Figure 3). The C-terminal region contains a long stretch of Ala residues and a short highly positively charged region of seven residues.

Figure 3 also indicates the positions of the Alb3 residues of Chlamydomonas and Arabidopsis that correspond to the intron insertion sites. It can be seen that among the five introns of Chlamydomonas and the nine introns of Arabidopsis, only intron 2 is inserted at the same site in both proteins. This finding suggests that, in contrast to the other introns, the second intron of *Alb3* was present before the divergence of plants and green algae during evolution.

Localization of Alb3.1

Attempts to produce an antiserum against Alb3.1 were unsuccessful. Therefore, to localize the Alb3.1 protein within

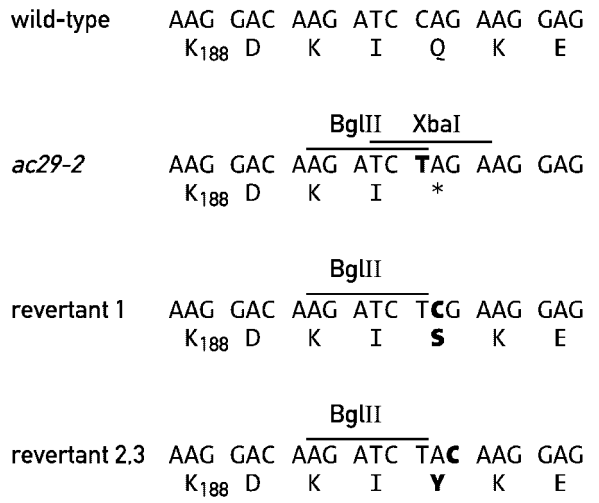

Figure 4. Sequence Analysis of the Region of *Alb3.1* Affected in the *ac29-2* Mutant and Three Revertant Strains.

Only the sequence surrounding the BglII site of *ac29-2* is shown.

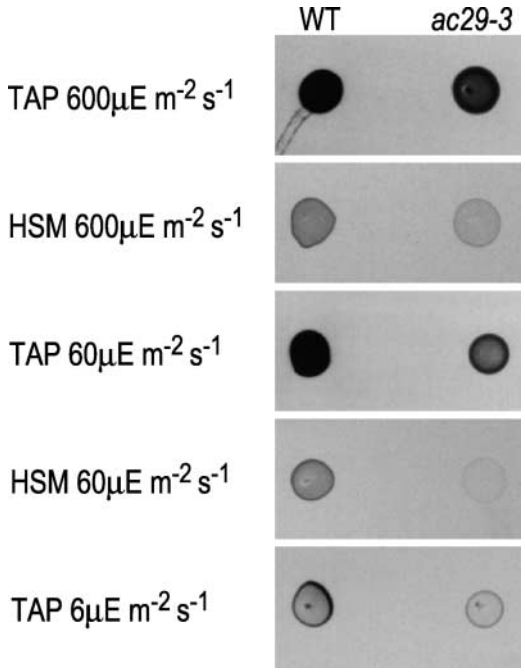

Figure 5. Growth Patterns of the Wild Type (WT) and *ac29-3*.

Five microliters from an exponentially growing culture was spotted on TAP or HSM (minimal) under different light conditions as indicated.

the chloroplast, the protein was tagged with an epitope. This was achieved by inserting the coding sequence of the triple hemagglutinin (HA) epitope at the 3′ end of the *Alb3.1* cDNA fused to the promoter and the first exon of the *Alb3.1* gene. This construct was inserted into the nuclear genome of the *ac29-3* mutant by cotransformation using the *Cry1* gene, which confers resistance to emetine, as a selectable marker (Nelson et al., 1994).

The drug-resistant transformants were screened for expression of the tagged protein by immunoblotting with an HA antibody. Among 15 transformants tested, two (T1 and T2) expressed the Alb3-HA protein to detectable levels (Figure 6A). As expected, the tagged Alb3.1 protein was found in the thylakoid membrane fraction (Figure 6B). The purity of the fractions was tested with antibodies against RbcL and PsaE.

To determine the amount of *Alb3.1* mRNA in the wild type and the rescued *ac-29* strain, total RNA was isolated and examined by RNA gel blot analysis. Because the amount of RNA was undetectable by this method, reverse transcriptase–mediated (RT) PCR was performed using appropriate *Alb3.1*-specific oligonucleotides (see Methods). A fragment of the expected size was present in similar amounts in the wild-type and rescued strains and was absent, as expected, in the *ac-29* mutant (Figure 6C, lanes 2 to 4). However, because the RT-PCR method used is semiquantitative, we were not able to measure accurately the relative levels of expression in the two strains.

The Alb3 Protein Is Required for the Normal Accumulation of LHC

The *ac29* mutant has a pale phenotype, indicating reduced accumulation of chlorophyll. The amount of chlorophyll in light-grown *ac29-3* mutant cells is 31% relative to wild-type cells; in dark-grown cells, the corresponding value is only 16% (Table 1). The reduced accumulation of chlorophyll in the dark is attributable to the *ac29-3* mutation, because mutant cells rescued with the wild-type copy of the *Alb3.1* gene by transformation accumulated similar chlorophyll levels in both the light and the dark (Table 1). The rescued cells accumulated only 60% of the chlorophyll that wild-type cells accumulated, possibly because the HA-tagged version of *Alb3.1* lacks introns, which are known to be required in most cases for efficient gene expression in Chlamydomonas (Fischer and Rochaix, 2001). Another possibility is that the insertion of the HA tag diminishes the stability of the Alb3.1 protein or affects its function.

To study the role of the Alb3.1 protein in the biogenesis of the photosynthetic apparatus, the levels of the different photosynthetic complexes in the thylakoid membranes of the wild type and the *ac29-3* mutant were determined. Figure 7 shows the results obtained by immunoblotting of thylakoid membrane extracts and reacting the blots with antisera raised against specific subunits of PSI (PsaE), PSII (D1), the ATP synthase complex (α), and the cytochrome b_6f complex (Cyt*f*). In all of these cases, no significant difference was observed between mutant and wild-type cells grown in the light, with the exception of the level of PSII, which was reduced twofold in *ac29-3* cells.

The effect of the *ac29-3* mutation on PSII was more pronounced in dark-grown cells, in which this complex was reduced nearly 10-fold. The major effect of the loss of *Alb3.1* was the strong reduction of the LHC polypeptides (Figure 7). Quantitative immunoblot analysis with the LHCI-specific P14.1 and the LHCII-specific P11 antibodies indicated that LHCI accumulates to $<$5% and that LHCII accumulates to 5 to 10% of wild-type levels in light-grown mutant cells, in agreement with the chlorophyll measurements. This observed decrease of LHC is compatible with the chlorophyll remaining in *ac29-3*, 30% relative to the wild type.

The chlorophyll antennae from PSII and PSI of Chlamydomonas have been estimated at 320 and 290 chlorophyll molecules, respectively (Polle et al., 2000). If one takes into account the chlorophylls that are bound to the core complexes of PSII (36 molecules) and PSI (100 molecules), one can estimate that a 90% reduction of total LHC would lead to a 70% reduction of total chlorophyll. The chlorophyll *a*/*b* ratio was increased significantly in the *ac29-3* mutant, as expected from a deficiency in LHC (Table 1).

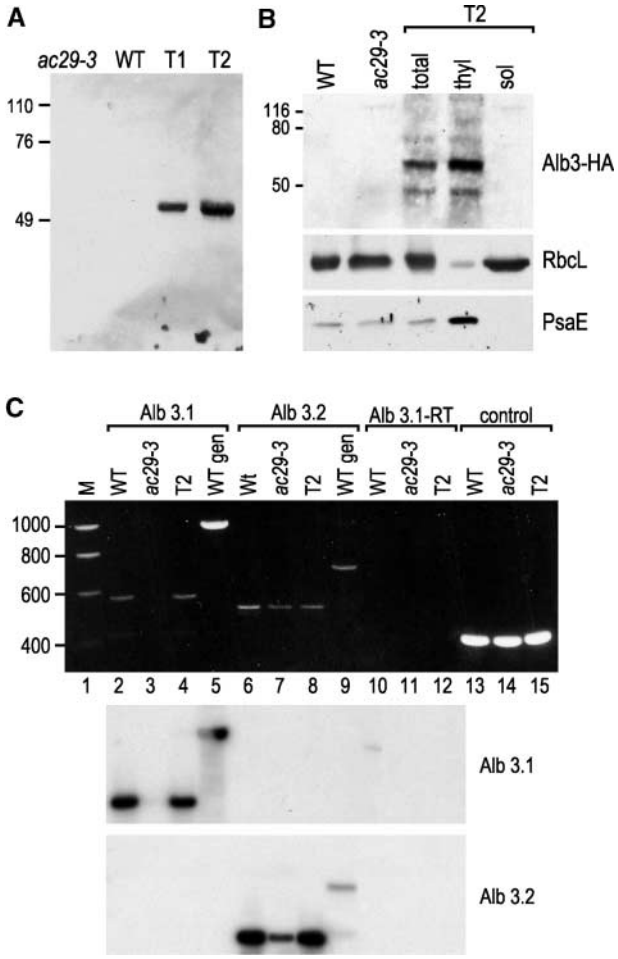

Figure 6. Localization of the Alb3-HA Protein and Expression of the *Alb3.1* and *Alb3.2* Genes.

(A) Immunoblot analysis of total cell extracts from the *ac29-3* mutant, the wild type (WT), and two *ac29-3* transformants (T1 and T2) rescued with the HA epitope–tagged *Alb3* gene. The blot was decorated with HA antibody.

(B) Cells from the T2 transformant were lysed and fractionated into a thylakoid membrane fraction (thyl) and a soluble fraction (sol).

(C) At top, total RNA from wild-type, *ac29-3*, and T2 transformants was subjected to RT-PCR, and the products were fractionated by agarose gel electrophoresis using primers specific for *Alb3.1* (lanes 2 to 4), *Alb3.2* (lanes 6 to 8), and the G-protein β-subunit gene (Schloss, 1990) (control; lanes 13 to 15). The same reaction with the *Alb3.1* primers without reverse transcriptase did not yield any signal (lanes 10 to 12). The same primers for *Alb3.1* and *Alb3.2* were used for PCR on wild-type genomic DNA (lanes 5 and 9, respectively). Lane 1 contained molecular mass markers (M). At bottom, the DNA fragments were blotted onto filters and hybridized with probes specific for *Alb3.1* and *Alb3.2* as indicated.

Alb3.1-HA Is Associated with Two Complexes

To determine whether Alb3.1 is associated with a large molecular mass complex, purified thylakoid membranes were solubilized with dodecyl maltoside and fractionated by Suc density gradient centrifugation. An equal aliquot of each fraction was separated by PAGE and used for immunoblot analysis with antibodies against HA, LHCII, the PSII subunit PsbB, and the PSI subunit PsaE. Figure 8 shows that the Alb3-HA protein fractionates in two distinct regions of the gradient.

The first peak of Alb3.1, with a size in the 60- to 80-kD range, cofractionates with LHCII, and the second peak corresponds to a high molecular mass complex, in the 600- to 700-kD range, that is slightly larger than the PSI complex. However, because the multiple forms of these complexes have been observed with the HA-tagged Alb3.1 protein, and because the tag could alter the properties of Alb3.1, it remains to be determined whether the untagged protein also is associated with two complexes.

Chlamydomonas Contains Two Alb3-Related Genes

Screening of the Chlamydomonas ESTs with the *Alb3.1* sequence revealed a second *Alb3*-related gene named *Alb3.2* that encodes a protein of 422 amino acids and that shows 37% sequence identity and 54% sequence similarity to Alb3.1 over its entire length (Figure 3). As for Alb3.1, the Alb3.2 mRNA is poorly expressed and was detected only by RT-PCR (Figure 6C, lanes 6 to 8). Because the method used is not quantitative, we have not been able to measure the relative levels of these two transcripts. The central region of both proteins, comprising ~260 residues, is significantly more conserved (46% identity and 65% similarity).

Alb3.2 displays 53% sequence identity and 71% sequence similarity to the Alb3 protein of Arabidopsis. The

Table 1. Chlorophyll Accumulation in *ac29-3*

Strain	Chlorophyll Concentration (μg/μg protein)	*ac29-3* Concentration (%)	Chlorophyll *a/b* Ratio
Wild-type L	0.091 ± 0.001	100	2.4
Wild-type D	0.077 ± 0.016	84	2.2
ac29-3 L	0.028 ± 0.004	31	4.6
ac29-3 D	0.015 ± 0.001	16	5.1
ac29-3-HA L	0.072 ± 0.007	78	2.3
ac29-3-HA D	0.057 ± 0.015	62	2.3

In all cases, the percentage was determined by comparison of the mutant with the wild-type cells grown under the same light or dark conditions. Chlorophyll concentrations were determined according to Porra et al. (1989). Measurements were performed five times, and the average is indicated with the standard deviation. D, darkness; L, light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$).

relatedness between Alb3.1 and the Arabidopsis protein is slightly smaller (46% identity and 65% similarity). These three Alb3 proteins have significantly more sequence similarity between themselves than to the yeast mitochondrial Oxa1p protein (19% sequence identity and 38% sequence similarity to Alb3.1, 18% sequence identity and 37% sequence similarity to both Alb3.2 and Alb3 of Arabidopsis). The relatedness to the bacterial YidC protein also is similar for Alb3.1 (27% sequence identity and 47% sequence similarity), Alb3.2 (31% sequence identity and 50% sequence similarity), and Alb3 of Arabidopsis (30% sequence identity and 48% sequence similarity).

The relationship between these different proteins is illustrated by the dendrogram at the bottom of Figure 3, which shows that Alb3.2 is related more closely to Alb3 of Arabidopsis than to Alb3.1. The Alb3 proteins of Chlamydomonas and Arabidopsis contain very similar hydrophobicity profiles, with five common putative transmembrane domains (data not shown). However, Alb3.1 appears to contain an additional transmembrane domain in its C-terminal region (data not shown). The Alb3.2 protein is likely to be a chloroplast protein, based on the prediction of the ChloroP program (Emanuelsson et al., 1999), although this assignment remains to be tested with appropriate cellular localization studies.

In spite of their similar structure, it is likely that the functions of Alb3.1 and Alb3.2 differ significantly, because the *Alb3.1*-deficient *ac29-3* mutant is affected drastically in the accumulation of LHCI and LHCII. However, because small amounts of LHCII still are present in this mutant, it is possible that Alb3.2 also plays some role in the assembly of this complex. Besides *Alb3*, a BLAST search for *Alb3* homologs in Arabidopsis revealed another gene encoding a protein, named atAlb3.2, of 1013 residues that is related to Alb3 in its C-terminal half (45% identity and 67% similarity). Based on its N-terminal sequence, the atAlb3.2 protein is not predicted to be localized within the chloroplast, although the phlyogenetic tree indicates that its C-terminal part is closely related to Alb3 (Figure 3).

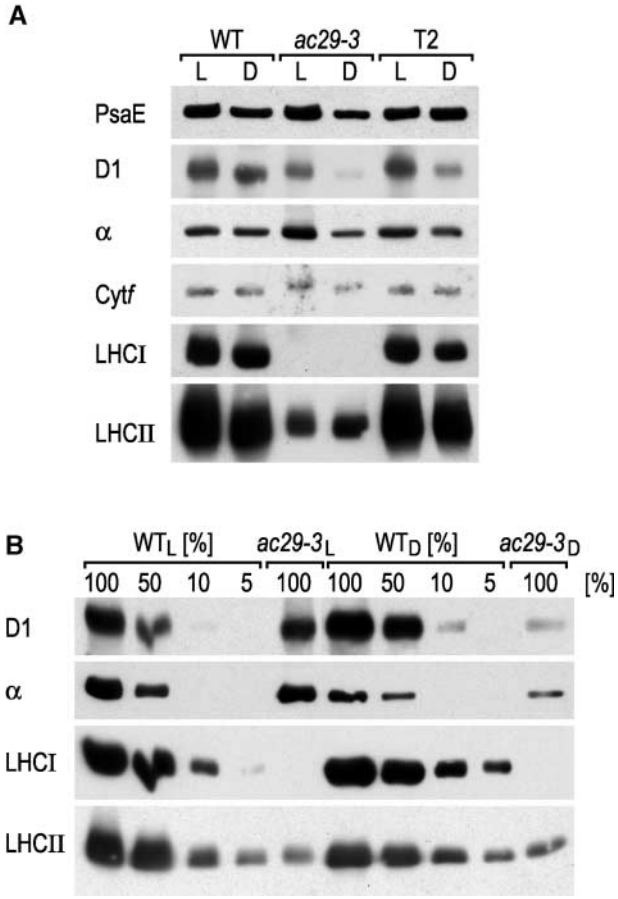

Figure 7. The Abundance of LHCI and LHCII Is Reduced in the *ac29-3* Mutant.

(A) Thylakoid proteins from the wild type (WT), *ac29-3*, and *ac29-3* rescued with a HA epitope–tagged *Alb3* gene (T2) were separated by PAGE, immunoblotted, and decorated with antibodies against PSI (PsaE), PSII (D1), ATP synthase (α), cytochrome b_6f complex (Cytf), LHCI, and LHCII.

(B) Dilutions of the protein extracts shown in **(A)** were used to estimate the amount of protein accumulated in *ac29-3*.

D, darkness; L, light.

DISCUSSION

The *AC29* Locus Encodes *Alb3.1*

In this study, we have shown that the *AC29* locus encodes the Alb3.1 protein. This assignment is based on the physical characterization of several *ac29* mutants with insertions and deletions at the *AC29* locus, on the molecular characterization of pseudorevertants of the *ac29-2* nonsense mutation that restore the open reading frame of *Alb3.1*, and on the rescue of the mutant phenotype by the transformation of *ac29* mutants with the genomic *Alb3.1* clone or with its cDNA.

The Alb3.1 protein shows a sequence identity of 42% to the Arabidopsis Alb3 protein. Furthermore, two regions of the protein display significant sequence identity to mitochondrial Oxa1p of yeast and the YidC inner membrane protein of *E. coli*. These proteins appear to be evolutionarily conserved mediators of membrane protein assembly in chloroplasts, mitochondria, and bacteria (Luirink et al., 2001; Jiang et al., 2002).

Inactivation of *Alb3.1* in Chlamydomonas

The inactivation of *Alb3.1* in Chlamydomonas leads to a drastic reduction of the LHCII and LHCI polypeptides. It is

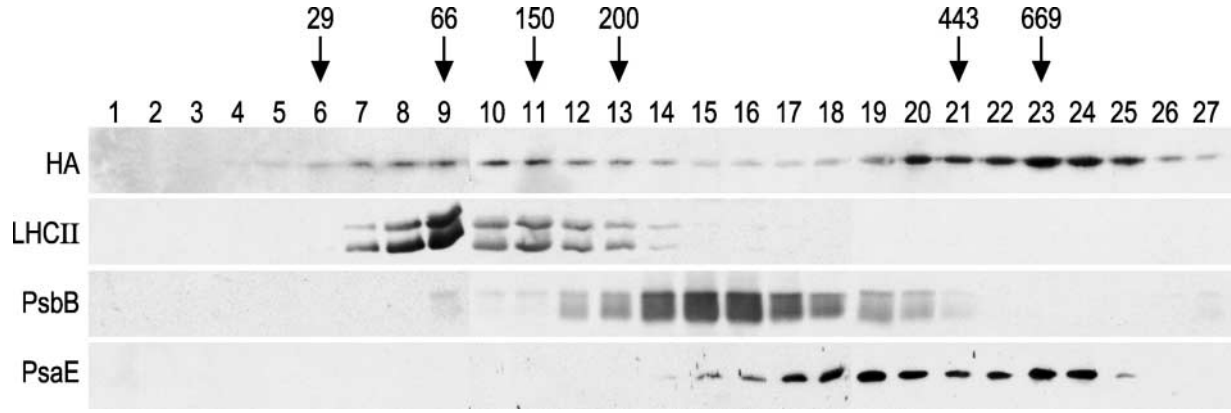

Figure 8. The Alb3-HA Protein Is Associated with Two Complexes.

Thylakoid membranes from the T2 transformant were solubilized with dodecyl maltoside and fractionated on a Suc density gradient. Fractions of 1 mL were collected, and 32-μL aliquots were used for PAGE and immunoblotting. The antibodies used are indicated at left. Antibodies specific for LHCII, PSII (PsbB), and PSI (PsaE) were used. Masses are indicated in kD and were determined by comparison with molecular mass standards provided with a molecular marker kit (Sigma). HA, HA-tagged Alb3 protein.

interesting that in this case, LHCI is more affected than LHCII, which accumulate to <5% and nearly 10% of wild-type levels, respectively. Whereas PSI, cytochrome b_6f, and ATP synthase are largely unaffected by the loss of Alb3.1, PSII is reduced twofold in light-grown cells. Surprisingly, this complex is reduced nearly 10-fold in dark-grown cells. This dark effect appears to be restricted to PSII, because the accumulation of LHCI and LHCII is the same in the light and the dark. It is interesting that ribosome nascent chain complexes of the PSII reaction center polypeptide D1 with SRP have been detected (Nilsson et al., 1999).

Together, these results agree with the idea derived from in vitro experiments that Alb3 is connected closely to the SRP pathway (Woolhead et al., 2001). The enhanced effect of the *ac29-3* mutation on PSII in the dark cannot be attributed to an alteration in the chlorophyll synthesis pathway in the dark because the rescued mutant accumulates chlorophyll to the same level in dark- and light-grown cells. This finding indicates that the *Alb3.1* gene influences chloroplast metabolism in the dark in a manner that remains unknown.

Role of Alb3 in LHC Assembly

Moore et al. (2000) recently demonstrated that Alb3 is required for the post-translational integration of the light-harvesting chlorophyll binding proteins into thylakoid membranes, based on the observation that thylakoids treated with an anti-Alb3 antibody are unable to integrate LHCP. The same treatment did not affect protein transport by the thylakoid Sec or Delta pH pathway. Suc density gradient fractionation of solubilized thylakoid membranes reveals that a portion of Alb3.1 forms a complex of 60 to 80 kD that cofractionates with the LHC complex (Figure 8). The cofractionation could imply an interaction between LHC and Alb3.1, but direct demonstration of such an interaction by coimmunoprecipitation was not possible.

The major portion of Alb3.1 is contained within a high molecular mass (600 to 700 kD) complex that is slightly larger than PSI. The current accepted model is that LHCP bound to chloroplast SRP in a soluble transit complex represents the LHCP targeted to the thylakoid membrane. LHCP integration requires the SRP receptor homolog cpFtsY, which partitions between stroma and thylakoids (Tu et al., 1999). One possible reason for this effect is that the high molecular mass Alb3.1 complex of Chlamydomonas participates in the recruitment of FtsY to the thylakoids. Another possible explanation is that the high molecular mass Alb3.1 complex includes the Sec translocon. In this respect, it is interesting that the *E. coli* YidC protein can act independently as a translocon, but it also has been found to be associated with the Sec translocon (Valent et al., 1998; Houben et al., 2000). Likewise, yeast Oxa1p is part of an oligomeric complex (Hell et al., 1998). This complex of 170 to 180 kD is homooligomeric and forms an essential part of the mitochondrial export translocase of *Neurospora crassa* (Nargang et al., 2002).

Although our data support the view that Alb3.1 is part of a translocon system that is used specifically to integrate LHCII and LHCI into thylakoid membranes, as much as 10% of LHCII still accumulates in the absence of Alb3.1. Thus, an alternative pathway must exist for LHC integration into the thylakoid membrane. In this respect, it is interesting to note the existence of Alb3.2, a homolog of Alb3.1 that belongs clearly to the Alb3 family rather than to the Oxa1p group (Figure 3). Although this Alb3 homolog has not yet been characterized fully, it is possible that it also plays a role in LHC assembly and that it is partially redundant with Alb3.1.

This could explain why LHC accumulation is not blocked fully in the absence of Alb3.1. However, it is clear that the functions of these two Alb3 homologs differ considerably. The *ac29-3* mutant still is able to grow photoautotrophically, although at a much reduced rate.

A similar mutant with a disruption of the *Alb3* gene has been described in Arabidopsis with a phenotype that is significantly more severe (Sundberg et al., 1997). The leaves of the mutant are yellowish to white, and the overall chlorophyll content is only 5% of that of wild-type plants. There are only a few thylakoid membranes, and the mutant dies at the seedling stage. Thus, the loss of Alb3 leads to a much milder phenotype in Chlamydomonas than in Arabidopsis. In particular, the polytopic membrane proteins of PSI and PSII that are synthesized in the chloroplast are much less affected by the mutation. Because of its specific effect on LHC, this algal system offers promising possibilities for elucidating the exact function of Alb3.

METHODS

Strains and Growth Conditions

Wild-type and mutant strains of *Chlamydomonas reinhardtii* were maintained on solid Tris-acetate phosphate (TAP) medium supplemented with 4 μg/mL nicotinamide or 100 μg/mL Arg as needed. Sueoka's high-salt medium was used whenever an acetate-deficient medium was required (Harris, 1989). Crosses were performed using standard protocols (Harris, 1989). Transformations were performed using the glass beads/vortex protocol (Kindle, 1990).

DNA Gel Blot Analysis

Genomic DNA and DNA gel blots were prepared as described by Ferris (1989). Probes were derived as follows: probe acB is a 0.8-kb NsiI-SacI fragment from pAc5.8, and probe acC is a 0.6-kb SstII fragment from pAc5.8.

cDNA Cloning

The pAc5.8 plasmid was used as a probe for screening a cDNA library from Chlamydomonas as described (Perron et al., 1999).

DNA Sequencing

The genomic sequence of a 4654-bp region beginning at the EcoRI site and spanning the *Alb.3* gene was determined from plasmid subclones of the λEMBL3 chromosome walk from strain CC-621 (mt^-) (Ferris and Goodenough, 1994). The bulk of the DNA sequencing was performed on single-stranded DNA using the Sequenase kit (United States Biochemical) after cloning restriction fragments into pUC118 or pUC119.

The segment of the *ac29-2* allele from strain CC-44 that carries the BglII-XbaI polymorphism was isolated as follows. PCR was performed on 25 ng of genomic DNA from the *ac29-2* mutant using Vent DNA polymerase (New England Biolabs, Beverly, MA) primers 1 and 6 under the following conditions: 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. The resulting fragment was gel purified and ligated into HincII-digested pUC118 and sequenced.

Three potential revertants of *ac29-2* were analyzed. Revertant 1 was a culture of CC-2663 received from the Chlamydomonas Genetics Center (Durham, NC) that was green, rather than the expected yellow, at the time of receipt. Revertant 2 was a culture of *ac29-2* that has been in use in our laboratory for many years and had acquired a green color. Revertant 3 was a green colony isolated during an attempt to transform *ac29-2* with a wild-type *Alb3-1* genomic clone; it appeared not to contain any transforming DNA and therefore was assumed to be a revertant.

All three revertants, when examined by genomic DNA gel blot analysis, had retained the BglII polymorphism of the *ac29-2* mutation but had lost the XbaI polymorphism. A PCR product was cloned from each strain (twice in the case of revertant 1) by the same protocol used for *ac29-2* (except that primers 1 and 5 were used, with an annealing temperature of 66°C), and a segment of ~100 bp spanning the mutant site in *ac29-2* was sequenced. The only change in the sequence relative to *ac29-2* was within the XbaI site.

The insertion in mutant ac*y27* was characterized as follows. PCR amplification was performed using 25 ng of ac*y27* genomic DNA and primers 1 and 5 under the following conditions: 30 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min. This resulted in the formation of two products, one of 558 bp (from the *ac29-2* allele on the mt^- chromosome) and one of 636 bp (from the new *ac29* allele on the mt^+ chromosome). The larger fragment was gel purified and ligated into HincII-digested pUC118, and the sequence of ~250 bp that includes the insertion was determined. PCR amplification and cloning were performed twice and yielded the same result. In addition to the insertion, a base change relative the wild-type genomic sequence was noted at position 1851 (A→C). This is within the second intron and represents a strain difference rather than a mutational change.

Primers

The primers used were as follows: primer 1, 5′-ATGGTGGCCAGTGTTGG-3′; primer 5, 5′-CACGTGCCCTACTCCTAC-3′; and primer 6, 5′-TCGGAAGCTATCCTGGC-3′. Note that primer 6 has 1 bp deleted relative to the actual genomic sequence.

Generation of New Mutants

A diploid was constructed (Ebersold, 1967) by mating CC-1067 (*arg2 nr-u-2-1* mt^+) to CC-1336 (*nic7 ac29-2 act2 pf14 msr1* mt^-), plating the cells onto TAP plus 3-acetylpyridine, and incubating in the light. Because 3-acetylpyridine is toxic to nicotinamide-requiring cells, only diploids and prototrophic recombinant meiotic progeny will survive, and the diploids tend to mature sooner. In addition, because *NIC7* is linked tightly to mt^+, the surviving meiotic progeny almost always will be mt^+, whereas the diploids will mate as mt^-. A strain mating as *minus* was selected, and diploidy was confirmed by showing the presence of *mt*-linked restriction fragment length polymorphisms from both parents on DNA gel blots.

The diploid strain was plated onto TAP at low density (~50 colonies on an 85-mm plastic Petri dish). When the colonies were 1 to 2 mm in diameter, the entire plate was irradiated at 422 rad/min for 12

min from a ^{137}Cs source (provided by William Wright, Washington University, St. Louis, MO). Thirty-six colonies were chosen from the irradiated plate and replated onto TAP plus nicotinamide. Mutants isolated from different colonies represent independent events. The plates were replica-plated to TAP plus 3-acetylpyridine to identify nicotinamide-requiring mutants. Any colonies that appeared yellow also were chosen and tested for nicotinamide requirement.

Transformation

Cells of *ac29-3* were transformed with the linearized pAc5.8 plasmid, and green sectors on the high-salt medium plates were isolated, from which individual transformants were obtained after subcloning. Alternatively, the cells were cotransformed with a plasmid containing the hemagglutinin-tagged *Alb3.1* gene and a plasmid containing the *Cry1* gene that confers resistance to emetine (Nelson et al., 1994). Drug-resistant transformants were screened for expression of the tagged protein by immunoblot analysis with a hemagglutinin antiserum.

Protein Extracts and Immunoblotting

Thylakoid membranes were isolated as described (Fischer et al., 1997). These membranes were solubilized with β-dodecyl maltoside and fractionated on a 0.1 to 1 M Suc density gradient as described (Fischer et al., 1997). Immunoblot analysis was performed using the chemiluminescence detection system (Supersignal; Pierce).

Isolation and Characterization of the *Alb3.2* cDNA

A BLAST search of the Chlamydomonas EST library (http://www.biology.duke.edu/chlamy_genome/EST.html) for homologs of *Alb3.1* identified the EST clone BE122079.1. Total RNA was extracted from a wild-type Chlamydomonas strain grown in TAP to midlogarithmic phase using TriReagent from Sigma, and the RNA was treated with RQ-DNase (Promega, Madison, WI) to remove residual DNA. Reverse transcription was primed with random hexamers. Primers for the amplification of a 358-bp fragment were derived from EST BE122079.1 (forward 1, 5′-CAGGTGGAGTCGACGCTG-3′; forward 2, 5′-TGGAGTCGACGCTGTCGCT-3′; reverse 1, 5′-CTGGCGTCTTGGCTCGCC-3′; and reverse 2, 5′-AGCACCGGCATCACCAGGTA-3′).

The resulting PCR fragment was A-tailed (elongated with dATP; 2 mM dATP and 1 unit of TaqPol) for cloning into pDRIVE (Qiagen, Valencia, CA). The cloned fragment was verified by sequencing. A 380-bp EcoRI fragment from this plasmid was used as a probe to screen a cDNA library. Screening of 800,000 plaque forming units resulted in four positive clones containing inserts of 1.5, 1.8, 2.1, and 2.1 kb. Inserts were cut out with EcoRI, cloned into pBluescript KS−, and sequenced.

Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described by Sambrook et al. (1989).

RNA Analysis

The amount of Alb3.1 and Alb3.2 RNA was measured using reverse transcriptase–mediated PCR. The primers used were 5′-GTTATGTCGAGCTCCATGTG-3′ and 5′-CGAAACGGTCCTTGATC-3′ for Alb3.1 and 5′-GATGCTGTGCACCACG-3′ and 5′-ACCACCAATGGTGGTTG-3′ for Alb3.2. The transcript of the gene encoding a G-protein β-subunit–like polypeptide of Chlamydomonas (Schloss, 1990) was used for reverse transcriptase–mediated PCR with primers 5′-GACGTCATCCACTGCCTGTG-3′ and 5′-CGACGCATCCTCAACACACC-3′.

Five micrograms of RNA from the wild type was reverse transcribed with 50 units of Superscript reverse transcriptase, 0.5 μg of oligo(dT), 0.5 mM deoxynucleotide triphosphate, 5 mM $MgCl_2$, 10 mM DTT, 20 mM Tris-HCl, pH 8.4, and 50 mM KCl for 50 min at 50°C in a total reaction volume of 50 μL. Three micrograms were used for PCR with Taq polymerase under the following conditions: 94°C for 4 min, then 30 cycles of 94°C for 1 min, 54°C for 45 s, and 72°C for 30 s, followed by 72°C for 4 min.

Sequence Analysis

Sequence alignments were performed using ClustalW (Thompson et al., 1994) and version 3.21 of Boxshade, written by K. Hofmann and M. Baron (http://www.ch.embnet.org/software/BOX_form.html). Potential transmembrane domains were identified using the transmembrane prediction program TMHMM 2.0 (Krogh et al., 2001) and TopPred2 (http://bioweb.pasteur.fr/seqanal/tmp/toppred/). Phylogenetic analysis was performed using the neighbor-joining method (Saitou and Nei, 1987). Distances were corrected for multiple substitutions using the percentage accepted mutations matrix of Dayhoff. The PHYLO_WIN program was used (Galtier and Gouy, 1996; http://evolution.genetics.washington.edu/phylip/software.html).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The accession number for the *Alb3.1* gene is AF492768 and for the Alb3.2 cDNA is AF514291.

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