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# *ALBINO3*, an Arabidopsis Nuclear Gene Essential for Chloroplast Differentiation, Encodes a Chloroplast Protein That Shows Homology to Proteins Present in Bacterial Membranes and Yeast Mitochondria

**Eva Sundberg,[a,b,1] Jacoba G. Slagter,[a,b] Ingela Fridborg,[a] Suzanne P. Cleary,[c] Colin Robinson,[c] and George Coupland[b]**

[a] Department of Physiological Botany, Uppsala University, Villavagen 6, S-752-36 Uppsala, Sweden
[b] Molecular Genetics Department, John Innes Centre, Colney Lane, Norwich NR4 7UJ, United Kingdom
[c] Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

**The *albino3* (*alb3*) mutant of Arabidopsis forms white or light yellow cotyledons and leaves and when germinated on soil does not survive beyond the seedling stage. The chloroplasts of the mutant are abnormal, as determined by electron microscopy, and contain reduced levels of chlorophyll. However, the chloroplasts of *alb3* mutants are sufficiently differentiated to enable the expression of two nuclear genes whose transcription requires the presence of chloroplasts. The *ALB3* gene was isolated by transposon tagging with the *Activator/Dissociation* transposable element system. *ALB3* is a novel plant gene whose product shows homology to a bacterial membrane protein previously identified in five bacterial species and to a yeast protein, OXA1, and its human homolog. OXA1 is required in the mitochondria for proper assembly of the cytochrome oxidase complex. ALB3 does not have a function identical to OXA1 because mitochondrial cytochrome oxidase activity is not affected in the mutant, and immunogold labeling as well as chloroplast import experiments performed in vitro demonstrated that the ALB3 protein is present in chloroplast membranes. *ALB3* might have a function related to that of *OXA1* and be involved in the assembly of a chloroplast enzyme complex.**

## INTRODUCTION

Chloroplast biogenesis and assembly of thylakoid photosynthetic complexes involve a complex interplay of regulatory signals between the nucleus, mitochondria, and plastids. Differentiation of plant cells therefore involves coordinated expression of genes encoded by the nuclear and organellar genomes. Because of the limited coding capacity of the plastid genome, most of the chloroplast proteins that have a structural or regulatory function are encoded in the nucleus and synthesized in the cytosol as precursors. These precursor proteins bind specifically to the chloroplast surface and are then imported into the chloroplast, where they are processed and assembled into functional complexes or act as factors regulating expression of specific chloroplast genes (Keegstra and von Heijne, 1992). The stable assembly of photosynthetic complexes requires that all core components be synthesized. Failure to produce any of the core components usually leads to rapid degradation of the other subunits of the complex (Rochaix, 1992). Mutations of a nuclear gene encoding chloroplast proteins with a structural or regulatory role can therefore interfere with chloroplast function or biogenesis (Rédei, 1973; Taylor et al., 1987).

Nuclear mutations that cause albino or pale green phenotypes because of reduced levels of chlorophyll in the chloroplasts have been found frequently in higher plants (von Wettstein et al., 1971; Coe et al., 1988; Hudson et al., 1993). Along with loss of chlorophyll, these mutants also typically show defects in chloroplast ultrastructure and composition. Synthesis of chlorophyll and development of the organelle appear to be interdependent (von Wettstein et al., 1971), and the accumulation of chlorophyll precursors, which has been found in some mutants, may therefore be the result of defects in plastid development. Chloroplast development is also arrested in maize and barley mutants blocked in chlorophyll biosynthesis (von Wettstein et al., 1971; Mascia and Robertson, 1978). Because of such pleiotropic effects, it has been difficult to elucidate whether a particular pigment mutant is impaired in chlorophyll biosynthesis or chloroplast biogenesis and function.

The isolation of genes affected in these mutants should greatly increase our understanding of how the mutations disrupt pigmentation and chloroplast function; however, to date, only a few of these genes have been cloned (Koncz et

[1] To whom correspondence should be addressed. E-mail eva.sundberg@fysbot.uu.se; fax 46-18-559885.

al., 1990; Hudson et al., 1993; Reiter et al., 1994). Previously, we used a transposon mutagenesis strategy based on the maize transposable element system *Activator/Dissociation* (*Ac/Ds*) to isolate the *albino3* (*alb3*) mutant of Arabidopsis (Long et al., 1993a). In this study, we characterize a pigment-deficient mutant, and our results indicate that the mutation blocks chloroplast biogenesis. We have cloned and characterized the wild-type *ALB3* gene and predicted the protein product based on its sequence. Our results suggest that ALB3 is a nuclear-encoded chloroplast protein that exhibits significant similarity with a protein described in five bacterial species. This protein is involved in sporulation of *Bacillus subtilis* and also shares significant similarity with a yeast mitochondrial protein that is required for the assembly of the cytochrome oxidase complex.

## RESULTS

### *alb3* Causes Reduced Chlorophyll Content and Affects Chloroplast Morphology

The *alb3* mutation is recessive and causes seedling lethality when individuals homozygous for the mutation are grown on soil; under these conditions, the plants do not develop beyond the cotyledon stage. However, when grown in vitro on medium that provides a carbon source (germination medium [GM]; Valvekens et al., 1988), *alb3* mutants produce >12 leaves and occasionally flower, but they are infertile.

Leaves of homozygous *alb3* plants cultured in vitro are primarily yellowish to white, as shown in Figure 1A (see Methods), although young leaves can be slightly pale green. To quantify the reduction in chlorophyll caused by the mutation, the chlorophyll contents of 4-week-old *alb3* mutants and wild-type plants were measured. The *alb3* plants contained an average of 0.08 mg of chlorophyll per g of leaves (SE ± 0.008; $n = 15$), which was only 5% of that of the wild-type siblings (1.65 mg of chlorophyll per g of leaves; SE ± 0.13; $n = 15$).

To study the effect of the mutation on chloroplast morphology, leaf sections from wild-type and *alb3* plants were examined by transmission electron microscopy. As shown in Figures 1D and 1E, this analysis indicated that chloroplasts of the mutant are far less organized, with very few thylakoid membranes and barely any grana stacking. Starch grains are also absent from *alb3* chloroplasts. The mitochondria of *alb3* mutants appeared to be identical to those of wild-type plants.

### Isolation of the *ALB3* Gene and Molecular Analysis of the *alb3* Mutation

The *alb3* mutation was previously shown to be caused by insertion of a *Ds* transposon carrying a hygromycin resistance

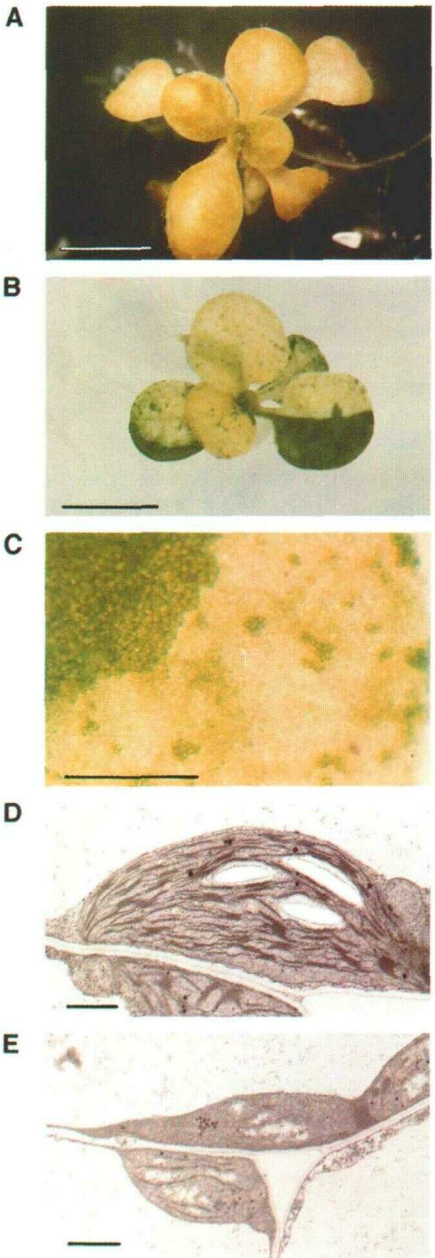


**Figure 1.** The *alb3* Phenotype.

**(A)** A 5-week-old *alb3* plant cultured on GM. Bar = 0.5 cm.

**(B)** Revertant green sectors occur on *alb3* plants carrying the *Ac* transposase (TPase) gene and are caused by excision of *Ds* from its position within the *ALB3* gene. Bar = 0.5 cm.

**(C)** Revertant sectors often encompass only one or a few cells. Bar = 0.1 mm.

**(D)** and **(E)** Transmission electron microscopy of leaves of wild-type and *alb3* plants, respectively. Reduced internal membrane structures, almost no grana, and no starch grains are visible in the chloroplasts of *alb3* mutants. Bars = 1 μm.

gene (*Ds*(Hyg)), because a copy of *Ds*(Hyg) cosegregated with the mutation, and excision of the transposon restored activity of the gene (Long et al., 1993a). To study the molecular and biochemical basis of the *alb3* phenotype, the gene was cloned by using transposon sequences to isolate the mutant allele, and the gene was sequenced. Inverse polymerase chain reaction had already been used to isolate the *ALB3* DNA adjacent to both termini of the *Ds*(Hyg) element in the mutant (Long et al., 1993a). These flanking DNA fragments (F1 and F2) of 200 and 600 bp were used to screen an Arabidopsis λ genomic library (Whitelam et al., 1993). This resulted in the repeated isolation of identical genomic clones (E1) containing 16 kb of DNA. A restriction enzyme map of these clones is shown in Figures 2A and 2B. Gel blot analysis showed that the F1 and F2 fragments hybridized with a 1.8-kb EcoRI-NotI fragment (palb1.8) adjacent to one of the λ arms. Because the F1 and F2 fragments were close to one end of the 16-kb fragment, it was possible that the whole *ALB3* gene was not present in the λ clone. Therefore, the palb1.8 fragment together with a 2.4-kb EcoRI fragment (palb2.4) adjacent to it were used to screen a second genomic library. In this way, an overlapping clone containing 20 kb of DNA was obtained, and this was shown to extend the region within the E1 clone to a total of 32 kb, as shown in Figure 2B.

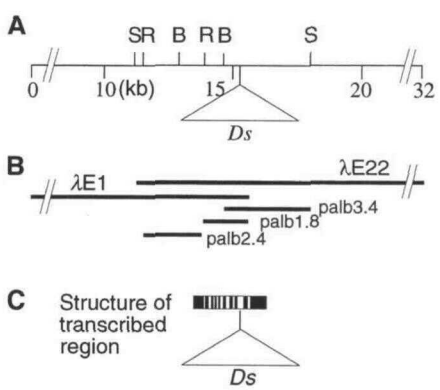


**Figure 2.** Restriction Map of the Genomic Region Containing the *ALB3* Gene and Structure of the Gene.

**(A)** A restriction map of a 10-kb region containing the *ALB3* gene and within the 32 kb cloned in overlapping λ clones. The position of the *Ds* element in the *alb3* mutant is shown. The restriction enzyme sites shown are SStI (S), EcoRI (R), and BamHI (B).

**(B)** Relative positions of the Arabidopsis DNA present in the λ and plasmid clones discussed in the text. The diagrams of these clones are positioned directly below their positions in the restriction map shown in **(A)**.

**(C)** Structure of the *ALB3* gene. Exons are shown as black boxes, and introns are shown as white boxes. The position of the *Ds* insertion is indicated.

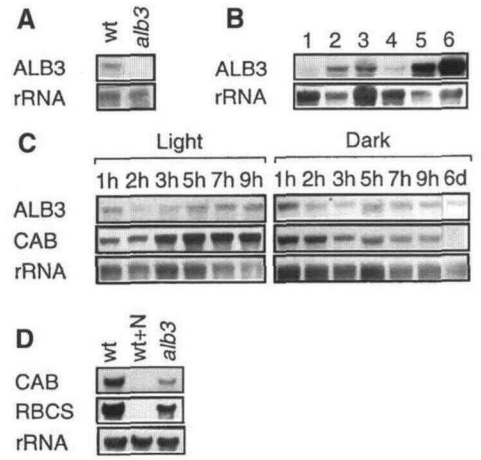


**Figure 3.** Gel Blot Analysis of *ALB3* mRNA Abundance in *alb3* and Wild-Type Plants in Response to Different Treatments.

**(A)** Abundance of *ALB3* mRNA and rRNA in *alb3* mutants and wild-type (wt) plants after 7 hr of light treatment in a 12-hr-day/12-hr-night cycle.

**(B)** Abundance of *ALB3* mRNA and rRNA in different organs of wild-type plants. The RNA used in each lane was obtained from pods (lane 1), stems (lane 2), flowers (lane 3), roots (lane 4), mature leaves (lane 5), and seedlings (lane 6).

**(C)** Abundance of *ALB3* and *CAB2* mRNA and of rRNA at different stages in a 12-hr-light/12-hr-dark cycle and after growing in continuous darkness for 6 days (6d). Plants were harvested at the indicated time points after lights on in the light samples and after lights off in the dark samples. h, hour.

**(D)** Abundance of *CAB* and *RBCS* mRNA and of rRNA in *alb3* mutants, untreated wild-type plants (wt), and wild-type plants treated with norflurazon (wt + N). RNA was extracted 7 hr into the light period in a 12-hr-day/12-hr-night cycle.

RNA was purified, subjected to gel electrophoresis, blotted to filters, and hybridized sequentially with the probes indicated at left in **(A)** to **(D)**.

To identify cDNAs corresponding to the *ALB3* gene, the palb1.8 and palb2.4 fragments were used to screen a cDNA library (described in Methods). One clone was identified that contained a 1.7-kb cDNA that hybridized strongly with both the palb1.8 and palb2.4 fragments. This cDNA was used to screen a second cDNA library (see Methods), and an additional seven cDNAs, which were all slightly shorter than the first one, were identified.

RNA gel blot analysis demonstrated that the isolated cDNA was derived from *ALB3.* As shown in Figure 3A, the longest cDNA detected a 1.7-kb transcript in RNA isolated from wild-type plants, but no transcript was found in RNA extracted from the *alb3* mutants. This cDNA was therefore very likely to have been derived from *ALB3.*

The longest cDNA clone was sequenced, as were 2.3 kb of genomic DNA that hybridized with the cDNA. The *ALB3* gene contains 10 exons and nine introns, as shown in Figure 2C.

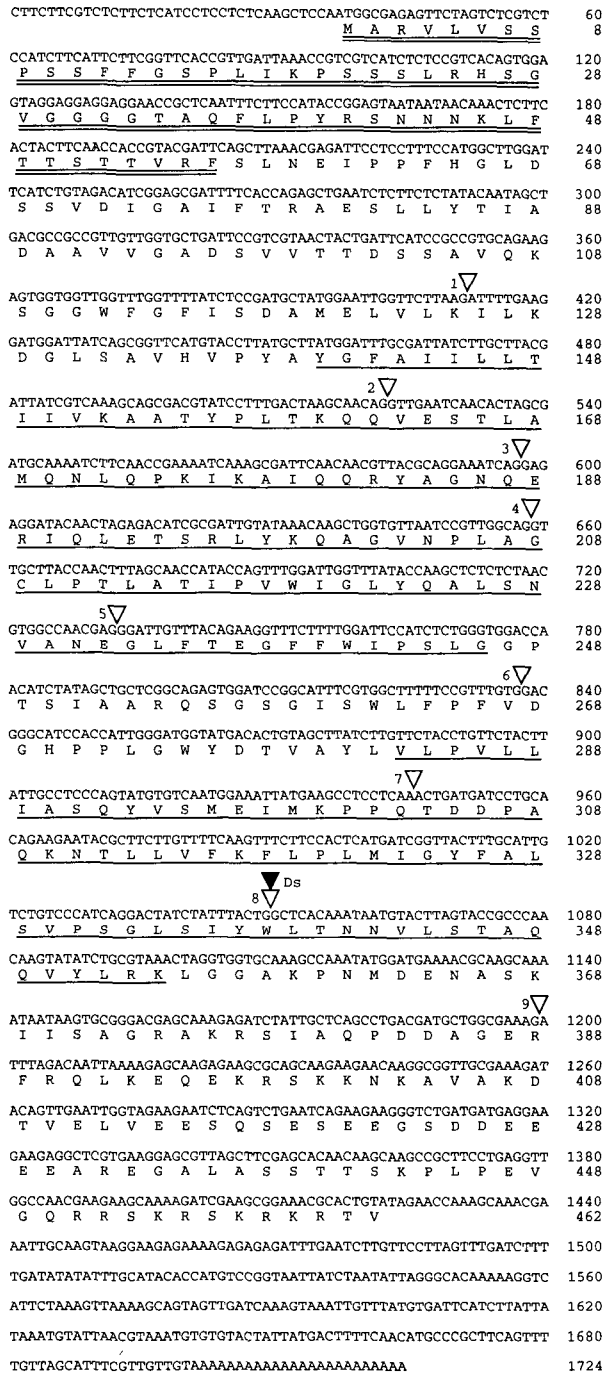

**Figure 4.** DNA Sequence and Derived Amino Acid Sequence of *ALB3*.

The amino acid sequence is presented in single-letter code below the nucleotide sequence, starting with the first in-frame methionine and ending at the first stop codon. An N-terminal region showing similarity to chloroplast transit peptide sequences is double underlined, and the regions (amino acids 140 to 246 and 283 to 354) showing similarities with other proteins are underlined with a single line. The positions of introns are marked with open triangles, and the location of the *Ds* insertion in the mutant allele is indicated by the filled triangle. The nucleotide sequence has GenBank accession number U89272.

The exons vary in length from 61 to 525 bp, and the introns are from 77 to 130 bp. All of the splice junctions conform to the consensus for Arabidopsis introns (donor site A/TG:GT and acceptor site AG:G/AT/A; Goodall and Filipowicz, 1991), except for the acceptor sites of intron 5 (AG:GG) and intron 8 (AG:GC) and both splice junctions of intron 7 (AA:GT and AG:AC). When the genomic sequence was compared with the sequence previously demonstrated to flank the *Ds*(Hyg) element in the *alb3* mutant (Long et al., 1993a), we found that the transposon is inserted in intron 8. Our demonstration that *Ds* is inserted in this intron confirms that the sequenced cDNA is derived from the *ALB3* gene and is consistent with the absence of a transcript in the mutant and the high frequency of phenotypic reversion detected in the presence of the transposase (TPase; see below and Long et al., 1993a).

## The ALB3 Protein Shows Similarity to a Bacterial Membrane Protein and to a Yeast Mitochondrial Protein

The longest *ALB3* cDNA that was isolated was 1694 bp long, and its sequence is shown in Figure 4. It contains a long open reading frame encoding 462 amino acids with 40 and 302 bp of untranslated DNA at the 5′ and 3′ ends, respectively.

Analysis of the hydrophobic and hydrophilic properties of the whole protein gave no strong indication whether ALB3 is a soluble protein or is localized in the membrane. Hydrophobicity plots (Kyte and Doolittle, 1982) using a window size of 19 residues revealed five hydrophobic regions (residues 75 to 109, 119 to 154, 206 to 226, 279 to 294, and 314 to 339). The C-terminal end of ALB3 forms a very hydrophilic domain (residues 350 to 460) in which 42% of the amino acids are charged. According to the Chou-Fasman and Robson-Garnier methods, a hydrophilic α helix is likely to be formed by ~53 residues (387 to 440) in this hydrophilic domain (Chou and Fasman, 1974; Robson and Suzuki, 1976; Garnier et al., 1978).

The predicted amino acid sequence of ALB3 shows significant similarity and identity with a bacterial inner membrane protein over two regions spanning 108 amino acids and 72 amino acids. These regions are marked in Figure 4, and comparisons of the sequences are shown in Figure 5. The genes encoding this protein have been characterized in five bacterial species. The homologous *60K* bacterial genes of *Pseudomonas putida*, *Escherichia coli* (Ogasawara and Yashikawa, 1992), and *Coxiella burnetti* (Suhan et al., 1994)

and *SPOIIIJ* of *B. subtilis* (Errington et al., 1992) are located close to the replication origin of the respective chromosomes. An alignment over the 108–amino acid region of ALB3 and the four bacterial proteins reveals that 64 amino acids (59%) are identical in ALB3 and the bacterial protein from at least one of the four species. Seventeen of these amino acids are strictly conserved (Figure 5). ALB3 is also 39% identical to a membrane protein from cyanobacterium *Synechocystis* sp strain PCC6803 over a 44–amino acid segment within these 108 amino acids. SPOIIIJ, the protein most similar to ALB3, has 41 (37%) amino acids that are identical and 76 (69%) that are similar to ALB3 in this region. Outside of this region, ALB3 shows no significant similarity to the bacterial proteins. The function of the *P. putida*, *E. coli*, *C. burnetti*, and *Synechocystis* PCC6803 proteins are at present unknown. Inactivation of the *B. subtilis SPOIIIJ* gene is not lethal but blocks sporulation after completion of prespore engulfment (stage III; Errington et al., 1992).

A yeast protein, OXA1, required for mitochondrial cytochrome oxidase biogenesis and its human homolog also show similarity to the SPOIIIJ and 60K bacterial proteins (Bonnefoy et al., 1994a, 1994b) in the same 108–amino acid region as ALB3. However, this similarity is less pronounced (12% identity, 47% similarity, and 11 gaps) than the similarity between the bacterial protein and ALB3. ALB3 also shows similarity to OXA1 in this region (22% identity, 49% similarity, and 12 gaps) and to the human OXA1 homolog (22% identity, 46% similarity, and 11 gaps) (Figure 5). The human protein also shows 21% identity (with no gaps) to ALB3 in the 72–amino acid region of homology (Figure 5). Protein subsequence analysis of ALB3, using the MacVector program, revealed no longer conserved protein motifs.

## Cytochrome Oxidase Activity Is Not Affected in *alb3* Mutants

The OXA1 protein is involved in the biogenesis of cytochrome oxidase, and *oxa1* mutants lack the activity of this enzyme complex (Bonnefoy et al., 1994a). Impaired cytochrome oxidase activity in the mitochondria can have an indirect effect on chloroplast biogenesis (Newton et al., 1990; Roussell et al., 1991; Gu et al., 1993). To test whether ALB3 is involved in the biogenesis of cytochrome oxidase, activity of the complex in mitochondria from *alb3* mutants and wild-type plants was measured. Another mitochondrial enzyme, fumarase, was used as a standard. Three independent experiments were conducted; in all cases, the relationship between cytochrome oxidase and fumarase activity did not differ significantly between *alb3* mutants and wild-type plants. In the three experiments, the ratios of cytochrome oxidase activity to fumarase activity in *alb3* mutants were 12.4:1, 26:1, and 2.7:1. In wild-type plants, the ratios were 16.5:1, 11.4:1, and 5.1:1. These results suggest that the cytochrome oxidase complex is functional in *alb3* mutants and not impaired as in the *oxa1* mutant of yeast.

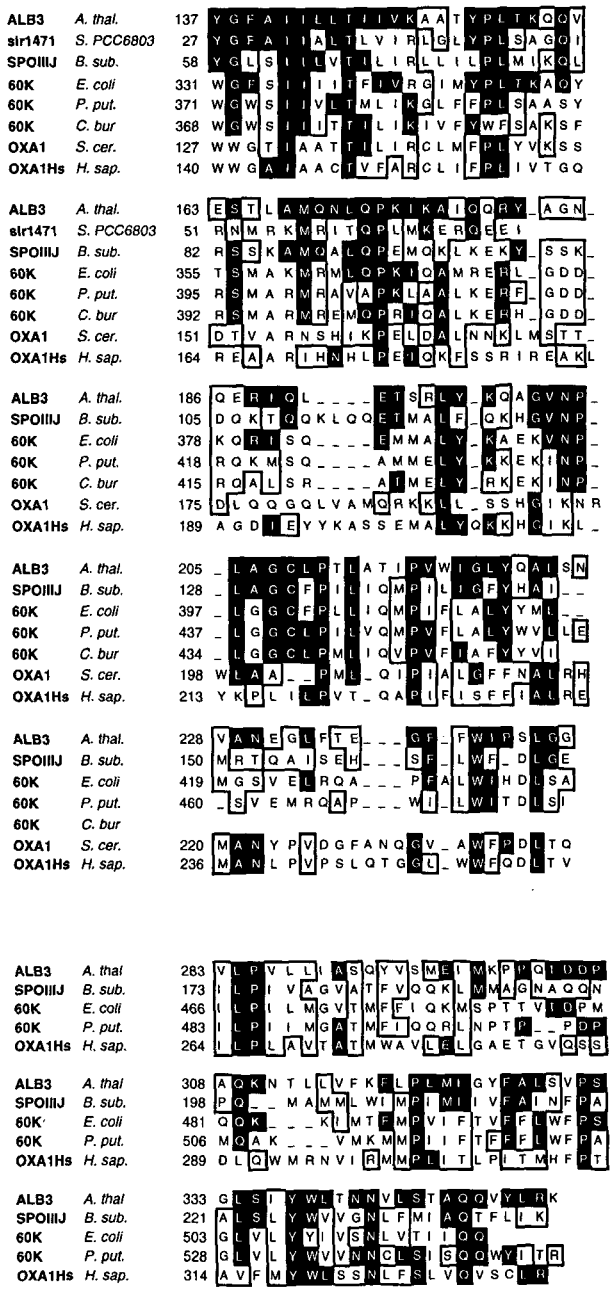


**Figure 5.** Alignment of the ALB3 Protein Sequence with Those of Similar Proteins.

Shown is a comparison of two segments of the Arabidopsis (*A. thal.*) ALB3 protein sequence with proteins from five bacterial species (*Synechocystis* PCC6803 [*S. PCC6803*], *B. subtilis* [*B. sub.*], *E. coli*, *P. putida* [*P. put.*], and *C. burnetti* [*C. bur.*]) and with OXA1 from *Saccharomyces cerevisiae* (*S. cer.*) and humans (*H. sap.*). Amino acids identical to those of ALB3 are shown in black boxes; similar amino acids are shown in open boxes. The amino acids of the homologous proteins are numbered as they were in the original publications. Gaps introduced to improve the alignments are indicated by dashes.

### The ALB3 Protein Is Localized in Chloroplasts

The effect of the *alb3* mutation on chloroplast morphology suggested that the ALB3 protein might be transported into the chloroplast. To test this, an immunogold labeling experiment was performed with thinly sectioned wild-type and *alb3* mesophyll cells, using antibodies raised against the C-terminal part of the ALB3 protein. Eighteen times more gold particles per square millimeter were observed in chloroplasts compared with the background levels detected in cytoplasm and vacuoles when thin sections of wild-type leaves labeled with anti-ALB3 antibodies were examined by using transmission electron microscopy (Figure 6A). When preimmune serum was used (Figure 6B), we found no significant difference between cell compartments, and the chloroplast signals were similar to the background. These in vivo results strongly suggest that the ALB3 protein is localized in chloroplasts. Results from the immunogold labeling experiment also suggest that ALB3 is localized in or attached to thylakoid membranes. Approximately 75% of the gold particles in chloroplasts labeled with the anti-ALB3 antibodies were found on thylakoid membranes; the others were found in stroma or attached to the inside of the chloroplast envelope. A membrane location for ALB3 was also suggested by using anti-ALB3 antibodies for protein gel blot analysis with proteins extracted from chloroplast membrane fractions. An increase in signal intensity of ~10-fold was obtained in a wild-type chloroplast membrane fraction compared with a total chloroplast fraction.

These in vivo data were strongly supported by in vitro experiments performed to test whether isolated chloroplasts import and process the predicted ALB3 precursor protein. The primary translation product was synthesized by transcription of the cDNA in vitro, followed by translation in a wheat germ cell-free system in the presence of radiolabeled methionine. Figure 7 shows that this procedure generates a translation product of ~60 kD (lane Tr) that is imported by pea chloroplasts and processed to an ~48-kD protein. The imported protein is localized almost exclusively in the membrane fraction (lane M). As a control for the import and fractionation procedures, we used the precursor of the 23-kD photosystem II protein (23K); the bottom gel in Figure 7 shows that this thylakoid protein is also imported and localized in the membrane fraction, as was expected (Mould and Robinson, 1991). We conclude from these data that ALB3 is a plastidic protein that is synthesized with a cleavable targeting signal. The in vitro experiments also indicate that ALB3 is targeted to a membrane location, but they do not distinguish between the thylakoid or envelope membranes. When ALB3 was subjected to a program for prediction of protein localization sites in cells (Nakai and Kanehisa, 1992), it was predicted to be a chloroplast thylakoid membrane protein (0.635) of a type that has several transmembrane $\alpha$ helices. As already mentioned, ALB3 has five hydrophobic regions—two of them in the part that show homology to the bacterial membrane proteins.

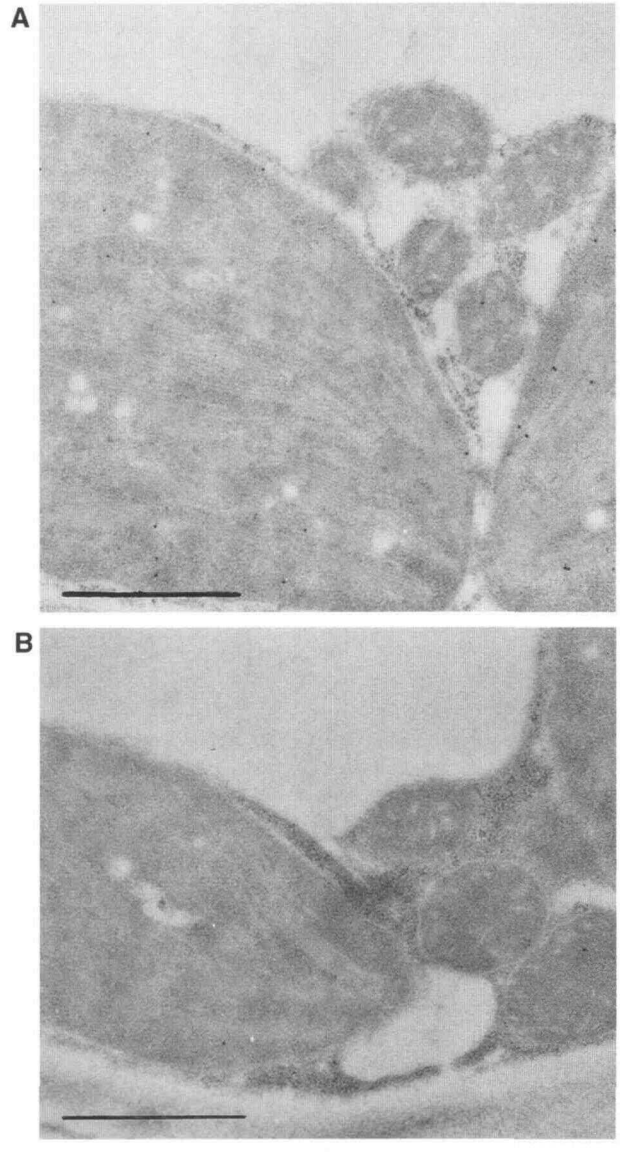


**Figure 6.** Immunolocalization of ALB3 in Thinly Sectioned Wild-Type Mesophyll Cells.

**(A)** Labeling was with anti-ALB3 antibodies raised against the C-terminal part of the ALB3 protein. Gold particles are found mainly in chloroplasts.
**(B)** Labeling was with preimmune serum.
Bars = 1 μm.

### Expression of *ALB3* mRNA and the ALB3 Protein

To determine in which organs the *ALB3* gene is expressed, total RNA was extracted from leaves, roots, stems, and flowers, transferred to filters, and hybridized with an *ALB3* probe. *ALB3* mRNA is mainly expressed in organs, such as

leaves, flower buds, and stems, that contain green tissues. The gel blots shown in Figure 3B illustrate that the mRNA is also present in roots and mature pods but is ~20-fold less abundant than in leaves. This pattern of *ALB3* expression was confirmed by constructing a fusion of the *ALB3* promoter to the *uidA* (β-glucuronidase [*GUS*]) gene and introducing it into wild-type plants by Agrobacterium-mediated transformation. Seedlings derived from these transformants were stained with X-gluc to detect GUS activity, and enzyme activity was detected in leaves and stems but not in roots (Figure 8; see Methods). To test whether the organ specificity of the *ALB3* promoter is reflected in the location of the ALB3 protein, protein gel blotting was performed using an anti-ALB3 antibody and protein extracts made from leaves, stems, flowers, pods, and roots. The ALB3 protein was detected in leaf extracts but not in those made from stems or roots (Figure 9).

Because the major phenotypic effect of the *alb3* mutation is on chloroplast development, we wanted to determine whether transcription of the *ALB3* gene is induced by light. The result of one of these experiments is shown in Figure 3C. The level of *ALB3* expression in RNA samples of wild-type plants taken at 14 different time points during a 24-hr cycle (12 hr of light and 12 hr of darkness) did not vary significantly. Furthermore, the abundance of the *ALB3* mRNA remained at the same level even after 6 days in darkness, and exposure to light after several days of dark treatment did not increase the abundance of the mRNA.

Hormone treatment did not affect the expression of the *ALB3* gene either. The level of expression in seedlings cultured on media supplemented with indoleacetic acid, abscisic acid, kinetin, or gibberellin $A_3$ was the same as for seedlings cultured without hormones (E. Sundberg, unpublished data). However, ALB3 transcripts were found in germinating seeds 6 hr to 4 days after seed imbibition (E. Sundberg, unpublished data).

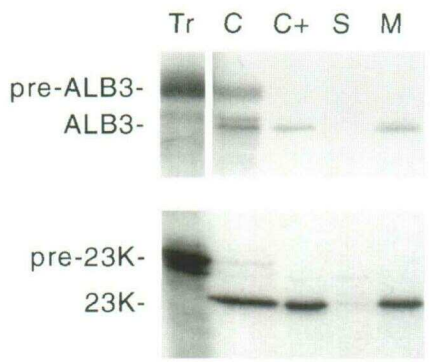


**Figure 7.** ALB3 Is Synthesized as a Larger Precursor and Imported into Pea Chloroplasts.

ALB3 was synthesized by transcription/translation, as detailed in Methods, and incubated with isolated intact pea chloroplasts. After incubation, samples of the chloroplasts (lane C), protease-treated chloroplasts (lane C+), and the stromal (lane S) and membrane (lane M) fractions recovered after lysis of the chloroplasts were analyzed. At the same time, incubation using the precursor of the wheat 23K protein (pre-23K) as substrate was conducted. Tr, translation product; 23K, mature 23K protein; ALB3, mature ALB3 protein.

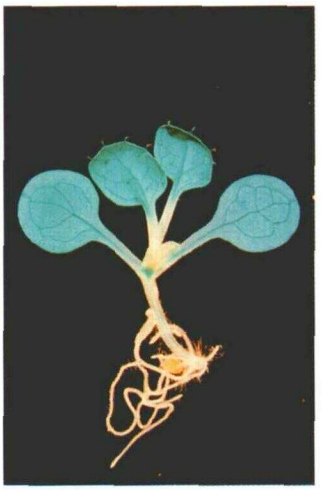

**Figure 8.** An Arabidopsis Seedling Carrying a *GUS* Reporter Gene Driven by the *ALB3* Promoter.

Shown are leaves and stems in which the *ALB3* promoter drives expression of the *GUS* reporter gene. *GUS* is not expressed in the roots.

### *alb3* Does Not Prevent Expression of the Genes Encoding the Chlorophyll *a/b* Binding Protein or the Small Subunit of Ribulose Bisphosphate Carboxylase, and It Acts Cell Autonomously

The presence of chloroplasts is required for the transcription of the nuclear *CAB* and *RBCS* genes, which encode the chlorophyll *a/b* binding protein of the photosystem II light-harvesting complex and the small subunit of ribulose bisphosphate carboxylase, respectively. Herbicides that cause severe damage to the chloroplasts block transcription of these genes (Simpson et al., 1986; Chamovitz et al., 1991), whereas *gun* mutations appear to overcome this repression (Susek et al., 1993). To test whether the chloroplasts present in the *alb3* mutants are still capable of inducing transcription of these genes, *CAB* and *RBCS* probes were hybridized with a blot containing total RNA extracted from leaves of *alb3* mutants, wild-type plants, and wild-type plants treated with the herbicide norflurazon. This herbicide destroys chloropasts by inhibiting carotenoid biosynthesis and thereby the protection against chlorophyll-mediated photooxidation (Oelmüller and Mohr, 1986). As shown in Figure 3D, *CAB* and *RBCS* mRNAs

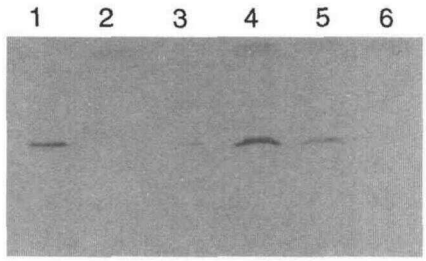


**Figure 9.** Immunoblot Analysis of the ALB3 Protein Abundance in Different Tissues of the Wild Type and *alb3* Mutant.

Anti-ALB3 antibodies were used to detect the levels of ALB3 in protein extracts from different plant organs. Lane 1 contains wild-type leaves; lane 2, wild-type roots; lane 3, wild-type stems; lane 4, wild-type flowers; lane 5, wild-type pods; and lane 6, *alb3* leaves.

were absent from the norflurazon-treated plants but were present in the *alb3* mutants, although at a reduced abundance when compared with the wild-type controls. The chloroplasts of *alb3* mutants are therefore still capable of inducing expression of the *CAB* and *RBCS* genes.

Excision of *Ds*(Hyg) from within the *ALB3* gene causes reversion to the wild-type phenotype. In the presence of a fusion of the cauliflower mosaic virus 35S promoter to the *Ac* (TPase) gene (35S::TPase), *Ds*(Hyg) excision occurs both early and late during leaf development, with the result that large and small green sectors are seen on mature leaves. Some of these sectors are shown in Figures 1B and 1C. The smallest sectors encompass only one or two cells; therefore, excision must have occurred close to the end of leaf cell division. It is thought that plastids arise by division rather than by de novo synthesis (Possingham et al., 1988), that differentiated leaf tissues contain only mature chloroplasts, and that proplastids are restricted to vegetative meristems (Mullet, 1988). Therefore, the small green sectors produced by *Ds*(Hyg) excision late in leaf development are probably not the result of the development of chloroplasts from proplastids. The green sectors could be caused by the completion of the development of the chloroplasts found in the *alb3* mutants when the expression of the gene is restored or by the degradation of the membranes within the mutant chloroplasts and the synthesis of new membranes after reversion of the mutation. In any case, the sharp boundaries of these revertant sectors indicate that the product of the *ALB3* gene and its effect on chloroplast development are cell autonomous because the wild-type cells have no effect on the phenotype of adjacent mutant cells.

### *ALB3* Is Located on Chromosome 2

*ALB3* is a single-copy gene, as demonstrated by low-stringency DNA gel blot hybridizations, using the *ALB3* cDNA as a probe (E. Sundberg, unpublished data). Restriction fragment length polymorphism (RFLP) mapping of recombinant inbred lines (Lister and Dean, 1993) showed that *ALB3* is located on chromosome 2, ~9.5 centimorgans (cM) below the *erecta* locus, 15.1 cM below RFLP marker g6842 (Nam et al., 1989), and 2.1 and 11.0 cM above RFLP markers m220 and m323, respectively (Chang et al., 1988). Therefore, *ALB3* represents a gene different from the previously mapped *alb1* and *alb2* mutations, because these are located on chromosomes 1 and 5, respectively (Koornneef et al., 1983). The original position of the *Ds*(Hyg) element was not on chromosome 2. It is located on chromosome 4 and is 2.9 cM below *AGAMOUS* (*AG*) (Long et al., 1997). The tagging of *ALB3* therefore required an interchromosomal transposition.

## DISCUSSION

### *ALB3* Encodes a Plant Chloroplast Protein Related to Bacterial Membrane Proteins and to OXA1 of Yeast

We believe that the gene we analyzed is *ALB3* because the *Ds*(Hyg) element is inserted in an intron of the gene and that excision of *Ds* restores *ALB3* function. The *alb3* mutation is in a nuclear gene, whereas immunogold localization and in vitro import experiments demonstrated that the ALB3 protein is imported into chloroplasts. The protein is cleaved during import, and the N-terminal region of the protein shows features previously associated with presequences of proteins imported into chloroplasts, such as a high number of serine residues and almost no negatively charged amino acids (Keegstra and von Heijne, 1992).

The ALB3 protein shows no homology to any protein previously shown to be localized in chloroplasts and represents a novel plant protein. However, ALB3 contains a 108–amino acid region that shows homology to a yeast protein, OXA1, which is imported into mitochondria, and to bacterial membrane proteins of unknown function that were previously shown to have homology with OXA1 in the same region (Bonnefoy et al., 1994a). The *oxa1* mutation was identified in a screen for mutants that reduce activity of the mitochondrial enzyme cytochrome oxidase. Apparently, the subunits of cytochrome oxidase are present in the *oxa1* mutant but fail to assemble into an active complex (Bonnefoy et al., 1994a). Although the chloroplast localization of ALB3 suggested that it is not required for cytochrome oxidase activity (cytochrome oxidase enzyme assays confirmed this), it is possible that ALB3 has a function similar to that of OXA1 in the assembly of chloroplast enzyme complexes.

### *alb3* Does Not Prevent Transcription of the *CAB* or *RBCS* Genes

The phenotype of *alb3*, pigment deficiency and arrested chloroplast development, suggests that *ALB3* is involved in pig-

ment biosynthesis or chloroplast biogenesis. The chloroplasts of *alb3* mutants have reduced grana and thylakoid membranes and no visible starch grains, and they contain very little chlorophyll. However, nuclear *CAB* and *RBCS* genes are expressed in the mutant; therefore, *alb3* chloroplasts are able to produce the signal that is postulated to be required for the transcription of these genes. Therefore, the chloroplasts of *alb3* mutants are not entirely nonfunctional. This also makes it unlikely that *alb3* causes a defect in the synthesis of carotenoids, because in bright light, carotenoid-deficient plants suffer severe free radical photooxidation, resulting in loss of chloroplast function that abolishes *CAB* and *RBCS* transcription (Anderson and Robertson, 1960; Giuliano and Scolnik, 1988; Oelmüller, 1989; Taylor, 1989).

### *ALB3* Is Probably Involved in Chloroplast Biogenesis

The phenotype of the mutant suggests that the *ALB3* gene is involved in chlorophyll biosynthesis or chloroplast biogenesis. From protein sequence similarity data, it seems most likely that *ALB3* is involved in chloroplast biogenesis. Proteins similar to ALB3 were isolated from nonphotosynthetic organisms lacking photosynthetic pigments. Therefore, because ALB3 contains a domain that is highly conserved with these other proteins, it might be involved in a more general process occurring in all of these organisms. Such processes could include membrane biogenesis or protein complex assembly rather than direct involvement in chlorophyll biosynthesis. The function of the bacterial protein is still unknown. A mutation in the *SPOIIIJ* gene of *B. subtilis* blocks sporulation at stage III; however, this block might not be the primary effect of the disruption, based on the fact that *SPOIIIJ* is expressed only during vegetative growth and that it has a homolog in nonsporogenic bacteria. It is possible that *ALB3* is involved in the biogenesis of an important thylakoid membrane complex either directly as a structural component or indirectly in the assembly of such a complex. The relatively high expression level of *ALB3*, especially in young green tissue, would support the idea that the ALB3 protein might have a structural role.

### The *alb3* Allele Is Unstable, and Sector Boundaries Suggest That the Effect of *alb3* on Chloroplast Differentiation Is Cell Autonomous

In maize and transgenic plants, *Ac* and *Ds* transposons tend to transpose to sites genetically linked to their starting position. In Arabidopsis, ~68% of *Ds* transpositions were shown to occur to linked sites, and of four unlinked transpositions in which the positions of the transposed elements were determined, three were to locations on the same chromosome and one was to a location on another chromosome (Bancroft and Dean, 1993). Therefore, a relatively low proportion of mutations induced by *Ds* in Arabidopsis would be expected to be caused by interchromosomal transposition. It is likely that *alb3* falls into this group because it is located on chromosome 2 and because the initial position of the *Ds* element used in the tagging experiment was on chromosome 4.

Somatic revertant sectors formed by the excision of transposons can be used to assess whether a gene product is cell autonomous and to determine whether the product is required transiently or for longer periods during plant growth and development (e.g., Carpenter and Coen, 1989; Hantke et al., 1995). Excision of *Ds* from the *alb3* allele at different times during plant development produces revertant sectors varying in size (Long et al., 1993a; Figure 1B). Generally, variegated plants grow to maturity on soil, whereas stable mutant plants die at the cotyledon stage. So, in the variegated plants, the carbon fixed in the green sectors is presumably sufficient to enable plant growth. Nevertheless, in these plants, sharp boundaries occur between the green wild-type sectors and neighboring albino cells. This indicates that *ALB3* influences chloroplast differentiation in a cell-autonomous manner and not by preventing the formation of molecules that can diffuse or be transported between cells.

Large and small revertant sectors occur on the leaves, cotyledons, and stems of *alb3* plants carrying 35S::TPase. For example, large leaf sectors can cause half of a leaf to be green, whereas small sectors encompass only one or two cells. Therefore, restoring *ALB3* action at any time in leaf development appears to allow the completion of chloroplast differentiation so that the gene product can act very late during leaf development.

## METHODS

### *Arabidopsis thaliana* Strains and Culture Conditions

The initial isolation of the *albino3* (*alb3*) mutation was described previously (Long et al., 1993a). Transposition of a *Dissociation* (*Ds*) element marked with a hygromycin resistance gene (*Ds*(Hyg)) was induced by a stable *Activator* (*Ac*) element comprising the *Ac* transposase (TPase) gene fused to the cauliflower mosaic virus 35S promoter. The *Ds*(Hyg) transformant as well as the *Ac* transformant were described previously and designated *Ds*(Hyg) transformant B and 35S::TPase transformant A, respectively (Long et al., 1993b). A stable *alb3* line was obtained by removing the *Ac* element by genetic segregation. Because the stable homozygote *alb3* mutant is infertile, the mutant line is kept as a heterozygous stock segregating *alb3* mutants.

### Chlorophyll Content Measurements

Four-week-old *alb3* and wild-type seedlings cultured in vitro in a 12-hr-light/12-hr-dark cycle were used for chlorophyll content measurements, according to Schmidt and Mohr (1981). All of the leaves (approximately eight) from an individual seedling were weighed and ground in 1 mL of 80% acetone. After mixing by shaking for 1 hr at room temperature in darkness, the debris was removed by centrifugation at 8000*g* for 15 min. In the clear supernatant, absorbance at 645

and 663 nm was measured, and the chlorophyll content was calculated using the formula $(20.2 \times A_{645}) + (8.02 \times A_{663}) = \mu g$ chlorophyll/mL (Arnon, 1949).

### Cytochrome Oxidase Activity

A crude extract containing mitochondria and thylakoid membranes was prepared from both *alb3* and wild-type Arabidopsis seedlings. Approximately 2 g of leaf tissue from young in vitro–grown seedlings was ground with a mortar and pestle in a buffer containing 0.3 M sucrose, 50 mM Mops, and 1 mM EDTA, pH 7.0; the supernatant was filtered through four layers of Miracloth (Calbiochem, La Jolla, CA). Mitochondria were separated along with other organelles and membranes from debris on a sucrose gradient and collected from the layer between 20 and 52% sucrose after 30 min of centrifugation at 19,000*g*. This material was slowly suspended in 4 volumes of phosphate buffer with sucrose (10 mM $KH_2PO_4$, 3 M sucrose, pH 7.0) and recollected by centrifugation at 19,000*g* for 20 min. The washed mitochondria and thylakoid membranes were resuspended in phosphate buffer with sucrose. Cytochrome *c* oxidase activity was measured in a spectrophotometer (model UV-160A; Shimadzu Corporation, Kyoto, Japan) at 550 nm. A solution that consisted of 10 mM $KH_2PO_4$, 100 mM KCl, and 0.02% (v/v) Triton X-100 was added to half of the organelle preparations of *alb3* and to one-tenth of the wild-type preparations. After 1 min of incubation, 50 μM reduced cytochrome *c* was added; the change in absorbance was measured after 3 min.

Fumarase activity was used to measure the relative amount of mitochondria in the *alb3* and wild-type organelle preparations, respectively. Fumarase activity was measured spectrophotometrically at 340 nm as the steady state production of NADPH in a medium containing 25 mM Hepes, pH 7.5, 5 mM $KH_2PO_4$, 4 mM $MgCl_2$, 0.05% (v/v) Triton X-100, 0.4 mM NADP, 6 mM fumarate, 0.1 units of NADP–malic enzyme, and the second half of the *alb3* organelle preparations or one-tenth of the wild-type preparations, respectively.

### Transmission Electron Microscopy

Leaves were removed from 4- to 6-week-old *alb3* and wild-type plants cultured on germination medium (GM; Valvekens et al., 1988). Leaves were immediately fixed in 2.5% glutaraldehyde and 1% osmium tetroxide in phosphate buffer for at least 5 hr, followed by dehydration in ethanol and acetone and embedding in TAAB 812 resin (TAAB Laboratories Equipment Ltd., Reading, UK). The samples were cut into sections 40 nm thick by using an LKB Ultratom I (LKB Products, Bromma, Sweden) with a Du Pont diamond knife. The sections were stained with uranyl acetate and lead citrate and visualized by transmission electron microscopy using a Philips CM10 microscope.

### Import Studies

The precursor form of ALB3 was generated by transcription of a clone carrying the longest *ALB3* cDNA, using T3 RNA polymerase, after which capped transcripts were translated in a wheat-germ lysate in the presence of $^{35}$S-methionine, as detailed in Robinson (1993). The translation product was incubated with intact isolated pea chloroplasts (*Pisum sativum* var Feltham First), and the organelles were subsequently protease treated and fractionated, as described in Robinson (1993).

### Cloning of *ALB3*

Inverse polymerase chain reaction had already been made to isolate the DNA flanking the *Ds*(Hyg) element in the *ALB3* gene (Long et al., 1993a). Here, we used the flanking DNA fragments (F1 and F2) to screen a λ Fix II (Stratagene) library made of genomic DNA isolated from Arabidopsis tissue (Whitelam et al., 1993), resulting in the isolation of a genomic clone (E1) with a 16-kb insert. Characterization of E1 by restriction digest mapping and DNA gel blot analysis showed that a 1.8-kb EcoRI-NotI fragment next to the 9-kb λ Fix II arm strongly hybridized with the F1 and F2 probes. This fragment as well as a 2.4-kb *EcoRI-EcoRI fragment next to it were subcloned into pBluescript* II SK+ (Stratagene) to give palb1.8 and palb2.4. The inserts of these two genomic subclones were used as probes to screen a second Arabidopsis (Landsberg *erecta*) leaf genomic library constructed in a Promega pGEM11 vector (J. Goodrich, unpublished data) as well as an Arabidopsis Columbia leaf cDNA library constructed in the Stratagene λ Uni-Zap vector (supplied by the E.C. Stock Center, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany).

From this second genomic library, another genomic clone (E22) with a 20-kb insert was obtained. Restriction digest mapping and DNA gel blot analysis showed that this genomic clone not only carried almost the entire region corresponding to the 1.8-kb EcoRI-NotI and 2.4-kb EcoRI fragments of E1 but also 16.8 kb of DNA that did not overlap with the E1 clone. From the cDNA library, a 1.7-kb cDNA clone (E34) was obtained. On a gel blot, it hybridized strongly with both the 1.8- and 2.4-kb restriction fragments of the E1 genomic clone as well as with a unique 3.4-kb BamHI-SstI restriction fragment of E22. This cDNA was used as a probe to screen a second Arabidopsis Columbia cDNA library (PRL-2) constructed in λ ZipLox (supplied by the E.C. Stock Center, Max-Planck-Institut), which provided another seven cDNA clones.

### RNA Gel Blot Analysis

Four sets of material were collected for RNA extractions. The first set included leaf, stem, and shoot material that was harvested from 4- to 6-week-old *alb3* and wild-type seedlings cultured in vitro under a 12-hr-light/12-hr-dark regime. Under this light regime, leaf and stem material was harvested from the wild-type seedlings after 1, 2, 3, 5, 7, 9, and 12 hr of light, respectively, and after 1, 2, 3, 5, 7, 9, and 12 hr of dark, respectively. Green parts were also harvested from 4- to 6-week-old wild-type seedlings cultured in complete darkness for 4 or 6 days and from seedlings that after 4 days in darkness were exposed to 4 hr of light.

The second set included parts of Arabidopsis plants that were harvested separately. Open flowers were collected and bulked along with green and slightly yellowish pods. These two samples were called flowers and pods, respectively. Stems from flowering greenhouse-grown plants as well as rosette leaves were collected, and the RNA extracts were called stems and leaves, respectively. Roots were cultured in vitro in liquid medium and harvested with care to avoid contamination with leaf tissue. Seeds were imbibed in water and harvested 6, 12, and 24 hr and 2 and 4 days after imbibition.

In the third set, seedlings cultured for 3 weeks on GM and thereafter exposed to 10 mM hormones for 4 days were also harvested. The

hormones used were as follows: abscisic acid, indoleacetic acid, gibberellin $A_3$, gibberellin, abscisic acid plus gibberellin $A_3$, kinetin, and kinetin plus indoleacetic acid.

In the fourth set, wild-type plants were exposed to norflurazon by growing them on GM agar supplemented with 10 μM norflurazon (4-chloro-5-[methylamino]-2[α,α,α-trifluoro-*m*-totyl]-3[2H]-pyridazinone; SAN9789; Sandoz, Basel, Switzerland). The plants were grown under a light regime of 12 hr of light and 12 hr of dark and harvested after 7 hr of light on the 21st day. Control wild-type and *alb3* seedlings were grown under exactly the same conditions, except norflurazon was omitted from the GM.

Total RNA was then extracted, according to Stiekema et al. (1988). For gel blot analysis, 25 μg of total RNA was denatured with formamide and formaldehyde, according to Sambrook et al. (1989), separated by agarose gel electrophoresis, blotted to nitrocellulose on either Hybond N or Hybond C (Amersham) membranes, and probed with $^{32}$P-labeled *ALB3* cDNA or pCAB, respectively. Hybridizations of probes with RNA blots were performed in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M $NaH_2PO_4$, and 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.5% SDS, and 0.1 mg/mL denatured salmon sperm DNA. The filters were prehybridized for 3 hr and hybridized overnight at 65°C. The RNA gel blots were washed twice for 5 min at room temperature and once for 20 min at 65°C with 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.1% SDS. The blots were then washed once for 20 min with 1 × SCC and 0.1% SDS at 65°C and once for 20 min with 0.1 × SCC and 0.1% SDS at 65°C. Subsequently, the filters were autoradiographed with XAR-films (Kodak), using intensifying screens.

### DNA Sequence Analysis

cDNA and genomic clones were subcloned into pBluescript II SK+ (Stratagene), and sequential deletions were generated using the Erase-A-Base system (Promega). Plasmid DNA was sequenced by the dideoxy termination method (Sambrook et al., 1989), using the Sequenase system (U.S. Biochemical). The cDNA was sequenced on both strands. The predicted ALB3 protein sequence was assembled from the cDNA, using the MacVector program (Eastman Kodak), which was also used for protein composition and structure analysis. DNA and protein sequences were used to search for homology, with sequences present in the following databases: GenBank (R) release 83.0, EMBL data library release 39.0, Cyanobase database of expressed sequence tags release 35; Brookhaven protein data bank release January 1994, SWISS_PROT release 29.0, PIR release 41.0, CDS translations from GenBank (R) release 83.0, and TFD transcription factor (protein) database release 7.0. The programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) were used.

### In Vitro Purification of ALB3, Antibody Production, and Immunological Procedures

The C-terminal part of the ALB3 protein (amino acids 327 to 462) was expressed in *Escherichia coli* and purified by using the QIAexpress Protein Expression and Purification System (Qiagen Inc., Chatsworth, CA). The truncated ALB3 protein was dialyzed to PBS and used to inoculate rabbits to raise polyclonal anti-ALB3 antibodies. Antibodies were collected by ammonium sulfate precipitation and centrifugation, according to Harlow and Lane (1988), and redissolved in PBS. The salts were dialyzed against PBS.

### Immunolocalization and Electron Microscopy

Leaf tissue from 3-week-old wild-type and *alb3* plants was fixed for 16 hr at room temperature in 2.5% glutaraldehyde, 0.05 M sodium cacodylate, and 0.05% Nonidet P-40 at pH 7.2, dehydrated stepwise at a low temperature in a series of ethanol concentrations from 30 to 100%, according to Wells (1985), infiltrated with London Resin White (London Resin Company, Basingstoke, Hampshire, UK) medium grade resin with 0.5% benzoin methyl ether at −20°C stepwise from 50 to 100%, according to Wells (1985), and polymerized by indirect UV irradiation for 24 hr at −20°C, followed by 16 hr at room temperature. Ultrathin sections (~100 nm) of leaf tissue were cut on a Reichert Ultracut E microtome (Agar Scientific Ltd., Stansted, Essex, UK) and collected on 200-square mesh gold grids coated with plastic (4% pyroxylin). The grids were incubated for 1.5 hr with 1% Aurion BSA-C (Aurion, Wageningen, The Netherlands) and 0.1% Tween 20 in PBS at pH 7.4. The sections were labeled with a 1:100 dilution of primary anti-ALB3 antibodies or preimmune sera overnight at 4°C. Antibodies had previously been cross-absorbed to nitrogen-ground wild-type roots. The grids were then washed three times for 15 min with 0.1% Tween 20 in PBS, incubated for 1 hr at room temperature with a 1:40 dilution of goat anti–rabbit antibody conjugated to 15-nm gold particles (British BioCell International, Cardiff, UK), and washed under constant agitation three times for 15 min with 0.1% Tween 20 in PBS, three times for 15 min with PBS, and twice for 15 min with distilled $H_2O$, followed by overnight incubation in distilled $H_2O$. Sections were contrast stained with 2% uranyl citrate and 0.5% lead citrate and viewed with a transmission electron microscope (model 1200 EX; Jeol Ltd., Watchmead, Herts, UK). The number of gold particles per square millimeter in individual chloroplasts and mitochondria was estimated. In each experiment, the mean number of gold particles per square millimeter of cytoplasm and vacuole was considered the level of background signal, and this number was subtracted from each individual measurement in the organelles. A covariance analysis was made between the estimated signals in the individual chloroplasts and mitochondria in each experiment.

### Expression Studies Using Protein Gel Blots and Promoter–β-Glucuronidase Fusions

#### *Protein Blots*

To collect intact chloroplasts for protein isolation, we isolated protoplasts, according to Glimelius (1984), from dark-treated (4 days) 3-week-old seedlings cultured on GM. Protoplast lysis was performed in an ice-cold solution containing 0.3 M sorbitol, 20 mM Hepes, pH 8.0, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 0.2% (w/v) BSA by squeezing the protoplasts through a needle (4 cm long and 0.7 mm in diameter) three times. By using this treatment, the plasmalemma of >90% of the protoplasts were ruptured, but the chloroplasts remained intact. The chloroplast suspension was passed through three layers of Miracloth and pelleted at 1500*g*. Half of the chloroplast pellet was used for protein isolation, and the other half was used for isolation of chloroplast membranes, according to a low-salt method (Steinback et al., 1982), with subsequent isolation of proteins. Protein was extracted from membranes, chloroplasts,

dark-treated 3-week-old seedlings, and different parts of 4-week-old wild-type and *alb3* plants, according to Stabel et al. (1990), loaded on denaturing 12% SDS–polyacrylamide gels (Sambrook et al., 1989), and transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore AB, Sundbyberg, Sweden) by electroblotting, according to the manufacturer's instructions. The membrane was incubated with anti-ALB3 antibodies, washed with PBS, incubated with horseradish peroxidase–conjugated antibodies from the horseradish peroxidase–conjugate substrate kit (Bio-Rad), and subjected to the horseradish peroxidase substrate 4-chloro-1-naphthol, according to the manufacturer's instructions.

### Promoter–β-Glucuronidase Fusions

The region spanning −1006 to +30 (where +1 is the first base after the transcription start site) of the *ALB3* gene was cloned into pBI101.2 (Clontech, Palo Alto, CA) upstream of a promoterless *uidA* (β-glucuronidase [*GUS*]) gene. The first 30 bp of the transcribed region contain no introns (Figure 4). The vector was introduced into host strain pmp90 of *Agrobacterium tumefaciens* by conjugation (Herrera-Estrella and Simpson, 1988) and then transferred to Arabidopsis ecotype Wassilewskija plants by vacuum infiltration, according to Bechtold et al. (1993). Seeds obtained by selfing of individual infiltrated plants were sown on Murashige and Skoog medium (Sigma) supplemented with 1% sucrose, 0.8% agar, and 50 μg/mL kanamycin. GUS activity of kanamycin-resistant plants was tested according to Jefferson (1987).

### *Map Position of ALB3*

Recombinant inbred (RI) lines of Arabidopsis have been generated by Lister and Dean (1993) from a cross between the ecotypes Landsberg *erecta* and Columbia. We used the F9 generation of 46 of these RI lines to map the position of *ALB3* in the Arabidopsis genome. Genomic DNA was prepared from 2 to 3 g of leaf material from 4- to 5-week-old plants of each of the two ecotypes and of the 46 RI lines by using a miniprep CTAB (cetyltrimethylammonium bromide) method (Dean et al., 1992). A restriction fragment length polymorphism (RFLP) was found between Landsberg *erecta* and Columbia when genomic DNA (2 μg) was digested with the restriction enzyme CfoI, separated on an 0.8% agarose gel, transferred to a Hybond N (Amersham) membrane, according to the manufacturer's recommendations, and hybridized with a random primed $^{32}$P-labeled insert of palb1.8. Hybridization was performed in 5 × SSC, 0.5% SDS, and 5 × Denhardt's solution. The filter was washed in 2 × SSC and 1% SDS at 65°C, followed by a wash in 1 × SSC and 1% SDS at 65°C. The filter was exposed to Kodak X-Omat XAR x-ray film for 1 day. The CfoI RFLP was used to score each individual RI line as having the *ALB3*-hybridizing CfoI fragment from Landsberg *erecta* or Columbia. This information was entered into Macintosh version 1.0 of the MAPMAKER mapping program of Lander et al. (1987), supplied by S. Tingey (Du Pont), according to Lister and Dean (1993), to give the *alb3* position relative to the known RFLP markers.

The location of the *Ds*(Hyg)-containing T-DNA that was used in the mutagenesis experiments was mapped to a position 2.9 centimorgans (cM) below *AG* on chromosome 4 by first using an inverse polymerase chain reaction to isolate a fragment of plant DNA adjacent to the T-DNA. An RFLP between Landsberg *erecta* and Columbia was then identified for this fragment, and it was mapped as described above for the RI lines of Lister and Dean (1993).

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