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The *AtCAO* gene, encoding chlorophyll *a* oxygenase, is required for chlorophyll *b* synthesis in *Arabidopsis thaliana*

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ABSTRACT **Chlorophyll *b* is synthesized from chlorophyll a and is found in the light-harvesting complexes of prochlorophytes, green algae, and both nonvascular and vascular plants. We have used conserved motifs from the chlorophyll *a* oxygenase (*CAO*) gene from *Chlamydomonas reinhardtii* to isolate a homologue from *Arabidopsis thaliana*. This gene, *AtCAO*, is mutated in both leaky and null *chlorina1* alleles, and DNA sequence changes cosegregate with the mutant phenotype. *AtCAO* mRNA levels are higher in three different mutants that have reduced levels of chlorophyll *b*, suggesting that plants that do not have sufficient chlorophyll *b* up-regulate *AtCAO* gene expression. Additionally, *AtCAO* mRNA levels decrease in plants that are grown under dim-light conditions. We have also found that the six major Lhcb proteins do not accumulate in the null *ch1-3* allele.**

In nonvascular and vascular plants, green algae, and prochlorophytes, chlorophyll (Chl) *b* is one of the light harvesting pigments that function to bring photons to the reaction centers. Chl *b* binds and stabilizes many of the light-harvesting complex (LHC) proteins but is not associated with the reaction center or the core Chl-binding proteins of photosystem (PS)I and PSII. Chl *b* is not essential for growth, and many mutants that lack Chl *b* have been isolated; one of the best studied is the *chlorina f2* mutant of barley. Paper chromatography was first used to demonstrate a loss of Chl *b* in this yellow-green mutant (1), and later a near complete reduction of Chl *b* was shown by using HPLC (2). Studies of LHCII biogenesis in *chlorina f2* demonstrated that *Lhcb1* gene transcription, translation, chloroplast import, and insertion into the thylakoid membrane all occurred without Chl *b*; however, the insertion was transient, and the Lhcb1 was rapidly degraded (3, 4). Etioplast membranes supplemented with Zn-pheophorbide *b* were able to integrate thermolysin-resistant Lhcb1 (5), providing biochemical evidence that stable insertion of Lhcb proteins requires Chl *b*. It is possible that the synthesis of Chl *b* could contribute to the regulation of the size of LHCII, and for this reason, we are interested in the regulation of Chl *b* synthesis in higher plants.

More recent studies involving the use of monospecific LHC antibodies have shown that some LHC proteins are indeed stable in *chlorina f2* despite the 100-fold reduction in Chl *b* (6–10). These include the LHCI proteins Lhca1, Lhca2, and Lhca3 and the LHC II protein Lhcb5. Lhcb2, Lhcb3, and Lhcb4 are partially stable, whereas Lhca4, Lhcb1, and Lhcb6 do not accumulate. Independent studies confirmed this work, also demonstrating the stability of Lhcb3 in *chlorina f2* (11, 12).

The role of Lhcb1, the major LHCII protein that binds 40% of the total Chl (13) in different aspects of photosynthesis has been evaluated by studying *chlorina f2.* The mutant has an increased PSII/PSI ratio to compensate for the loss of photons that would normally be harvested by Lhcb1 (14). Even so, a redox-controlled thylakoid kinase is not activated in *chlorina f2* because the plastoquinone pool is never fully reduced (15). The *chlorina f2* PSII has a decreased quantum yield under increased temperatures and light intensities, which is probably because of the loss of the 33-kDa extrinsic oxygen-evolving protein under extreme conditions (16). The mutant does form grana stacks, albeit at lesser amounts (17), and the fluidity of normal and mutant thylakoid membranes is similar (18), despite the loss of a major protein component.

Additional *chlorina* mutants lacking Chl *b* have also been isolated from many other species, including *Arabidopsis thaliana* (19, 20), rice (21), and *Chlamydomonas reinhardtii* (22). It has been hypothesized that these mutants are unable to convert Chl *a* into Chl *b* (23, 24). Chl *a* has a methyl group on the D ring of the porphyrin molecule, whereas Chl *b* has a formyl group at that position. Recently, insertional mutagenesis was used to tag and isolate the Chl *a* oxygenase (*CAO*) gene in *Chlamydomonas* (25). This gene rescued Chl-*b*-less *cbs* mutants, and its deduced amino acid sequence indicated that it was a methyl monooxygenase with a Rieske-type [2Fe–2S] cluster and a mononuclear nonheme Fe-binding site.

We have used conserved motifs from the *CAO* sequence to isolate an *Arabidopsis* expressed sequence tag (EST) that encodes the *AtCAO* gene. We show that two *ch1* alleles have mutations in the *AtCAO* gene and provide genetic evidence that the *AtCAO* gene cosegregates with *ch1* mutations. Additionally, we analyze the expression of this gene and show that a null *ch1* allele that has no detectable Chl *b* by HPLC analysis has undetectable levels of all six of the major Lhcb proteins. The majority of Chl-binding proteins that this line does contain are those of the PSI and PSII core antennae.

MATERIALS AND METHODS

***Arabidopsis* Lines.** *ch1-2* and *ch1-3* were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University in Columbus, OH. Both lines were isolated after x-ray mutagenesis by G. P. Rédei (ABRC seed catalogue). The *conditional chlorina* mutant, *cch*, was selected for its light-green phenotype under moderate light intensity at California State University, Long Beach, and will be described in detail elsewhere. All lines are in the Col-0 ecotype. Plants were grown at low (60 μmol·m^{-2}·sec^{-1}) or moderate (150 μmol·m^{-2}·sec^{-1}) photosynthetically active radiation in Percival growth chambers (Percival, Boone, IA) under continuous illumination by using Sunshine soil mix 1 (Sungro, Bellevue, WA). Dim-light conditions (5 μmol·m^{-2}·sec^{-1}) were obtained

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Abbreviations: Chl, chlorophyll; *a*/*b* ratio, Chl *a* to Chl *b* ratio; *CAO*, Chl *a* oxygenase; *cch*, *conditional chlorina; ch1*, *chlorina1*; EST, expressed sequence tag; LHC, light-harvesting complex; wt, wild type.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. AF177200).

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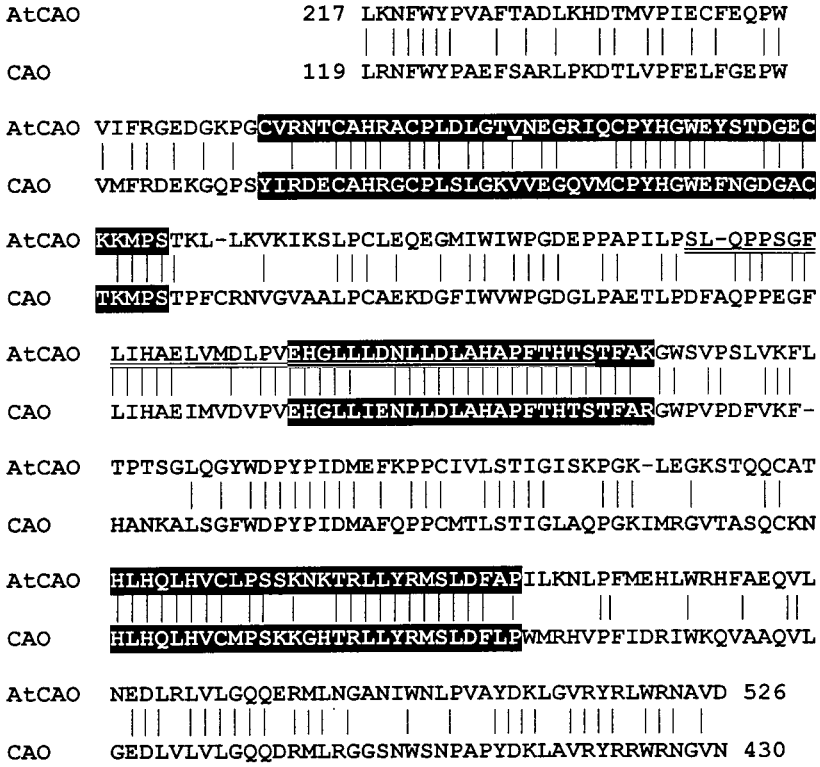

FIG. 1. Amino acid alignment of AtCAO and CAO. Identical amino acids are indicated by lines between sequences. The top box is the Rieske binding site, the middle box is the mononuclear nonheme Fe-binding site, and the bottom box is the unique conserved site. The V274 that is converted to E in the *ch1-2* allele is underlined, and the region with the double underline is deleted in *ch1-3*.

by placing potted plants into a brown paper bag inside the growth chamber.

Pigment Analysis. HPLC analysis was done by Heather Rissler in the laboratory of Barry Pogson at Arizona State University (Tempe, AZ) as described in ref. 26. Chl measurements for the dim-light experiment were performed according to ref. 27, $n = 4$.

Gene Sequencing. DNA primers for sequencing were based on the 103D24 EST sequence (GenBank accession no. T22255) and the T20J22TR BAC sequence (GenBank accession no. B62146) which contained the 5′ end of the gene. Genomic DNA was isolated by using DNAzol (Life Technologies, Gaithersburg, MD). DNA primers for sequencing were synthesized by Operon Technologies (Alameda, CA). PCR fragments were generated under standard conditions, purified with a QIAquick PCR purification kit (Qiagen), and sent to the DNA Sequencing Facility at California State University, Northridge. Overlapping PCR products were sequenced by using amplification primers. Random errors by *Taq* polymerase were not detected because amplification products were sequenced directly without cloning into a plasmid vector. Kyte-Doolittle hydrophilicity profiles were performed at http://bioinformatics.weizmann.ac.il/hydroph/index.html with a window length of 17 aa.

RNA Gel Blots. RNA was isolated from leaf tissue by using the RNEasy kit (Qiagen), electrophoresed by using the standard formamide/formaldehyde method, blotted to Zetaprobe (Bio-Rad), and UV cross-linked. After transfer, blots were rinsed in 5% acetic acid, stained in 0.04% methylene blue (wt/vol), 0.5 M sodium acetate, pH 5.2 for 10 min, and destained in H_2O (28). Stained blots were scanned, and the 28S rRNA band was digitally quantified by using UN-SCAN-IT-GEL software (Silk Scientific, Orem, UT) to control for differences in loading. Northern blots that had <10% difference from the mean in rRNA levels were used in hybridizations. Blots were hybridized according to manufacturer's instructions. The final wash was 57°C for *AtCAO* and 65°C for *Lhcb1* in 0.1× SSC/0.1% SDS. The *AtCAO* probe was a 1.2-kb *Hin*dIII fragment derived from the 103D24 EST (29), and the *Lhcb1* probe was a 1.0-kb *Hin*dIII fragment from pAB140 (30). The *Lhcb1* probe recognized *Lhcb1*1*, *Lhcb1*2*, and *Lhcb1*3*. After hybridization, blots were imaged and quantified by using a Molecular Dynamics Storm PhosphorImager.

Thylakoid Preparation. Ten grams of *Arabidopsis* leaves were placed in a Waring blender in 1/3 vol of cold grinding buffer (0.4 M sorbitol/20 mM Tricine, pH 7.8/20 mM NaCl/5 mM $MgCl_2$/250 μM PMSF), blended for 2 pulses (5 sec each), filtered through Miracloth, and then centrifuged at 2,000 × *g* for 4 min at 4°C. The pellet was homogenized in 15 ml of wash buffer (10 mM Tricine, pH 7.8/10 mM NaCl/10 mM $MgCl_2$), and a 20-μl sample was removed for Chl determination in 1 ml of 80% acetone. The remainder was placed in an Oak Ridge tube, wash buffer was added to fill the tube, and the sample was centrifuged at 9,000 × *g* for 5 min at 4°C. The pellet was resuspended in storage buffer (7 mM Tris base/53 mM glycine/10% glycerol) to a final concentration of 1.1 mg/ml Chl and quick-frozen in liquid nitrogen. Thylakoid preparation for the immunoblotting was done on a small scale by using 1 g of tissue. Plants were ground in 1 ml of grinding buffer for 1 min by using a mortar and pestle on ice. Miracloth filtering was done in an open P1000 blue tip, and thylakoid pelleting was done in microcentrifuge tubes (Beckman) at 2,900 × *g*. Plastic blue pestles (VWR) in microcentrifuge tubes were used to homogenize pellets, and washes were centrifuged at 8,000 × *g*.

Green-Gel and Second-Dimension Electrophoresis. Green-gel electrophoresis was performed as described (31) with the following modifications. Our surfactant stock was a 5% *n*-decyl β-D-maltoside (Sigma), 5% *n*-undecyl β-D-maltoside (Anatrace, Maumee, OH), and *ch1* membranes were solubilized in a 5:1 ratio of membrane/surfactant. Second-dimension electrophoresis was as described (31) except that a 13.5% acrylamide gel was used, and excised green-gel slices were

incubated in denaturing buffer for 60 min at RT and then for 15 min at 30°C.

Antibodies and Western Conditions. The CP43 antibody was obtained from Daryl Morishige and John Mullet at Texas A&M University (College Station, TX) (32). Lhcb1, 2, 3, 5, and 6 antibodies were obtained from Janet Meehl and L. Andrew Staehelin at University of Colorado (Boulder, CO) (6, 7). The Lhcb4 antibody was obtained from David Simpson at the Carlsberg Research Laboratories in Copenhagen, Denmark (8). Fifty micrograms of thylakoid protein was loaded onto each lane of a 13.5% acrylamide minigel, and alkaline phosphatase was used to detect antibody binding.

RESULTS

The *AtCAO* Gene. We searched GenBank for methyl monooxygenase genes that also contained Rieske-binding sites and found a spinach choline monooxygenase gene (accession no. U85780). When the amino acid sequence of this gene was used to search the *Arabidopsis* gene bank, we recovered a highly homologous EST, 103D24. This EST hybridized to wild-type (wt) genomic DNA but not to two *ch1* deletion lines (gifts of Kris Niyogi at University of California, Berkeley; data not shown). This gene was named *AtCAO*.

Genomic DNA from wt plants was PCR-amplified and sequenced, and *AtCAO* was found to contain eight introns and a 283-bp 3′ UTR (GenBank accession no. AF177200). A large region of the predicted AtCAO amino acid sequence was found to be 60% identical to that predicted by *Chlamydomonas reinhardtii CAO*, and Fig. 1 shows the alignment of these two amino acid sequences. Regions of high similarity include the Rieske binding site, the mononuclear nonheme Fe-binding site, and a unique conserved site that is not found in any other GenBank nonredundant sequences other than the *CAO* genes. The amino-terminal 36 amino acids of AtCAO were predicted to be a chloroplast import leader (33), because Chl biosynthesis occurs within the chloroplast (34). The hydropathic profile of AtCAO suggested that there were no transmembrane helices.

To be certain that mutation of the *AtCAO* gene was responsible for the *ch1* phenotype, the *AtCAO* gene was sequenced from leaky and null *ch1* alleles. *AtCAO* from the leaky *ch1-2* allele had a T → A transversion creating a V274E mutation within the Rieske-binding site. The strong *ch1-3* allele was the result of a 213-bp deletion that removed part of exon 3, intron 4, and exon 4. At the site of the deletion was a 5-bp insertion, and the net result was a deletion of 40 aa that included most of the mononuclear nonheme Fe-binding site. (S334 through T375 were deleted and replaced by Val and Ala, respectively). The locations of these mutations are shown in Fig. 1.

The T → A transversion found in the *ch1-2* line cosegregated in six of six yellow-green F_2 sibling lines after *ch1-2* was crossed to wild-type *Arabidopsis*. The probability of an unlinked mutation cosegregating in six of six lines is $(1/4)^6$ or (1/4,096), providing genetic evidence that the *AtCAO* gene is responsible for the *ch1* mutant phenotype.

AtCAO Gene Expression. Chl *b* synthesis is required for the stable integration of Lhcb1 into the thylakoid membrane (3–5), and therefore we tested whether *AtCAO* and *Lhcb1* gene expression could be correlated to the amount of Chl *b* bound to the LHC. To obtain plants that had variable Chl *a* to Chl *b* ratios (*a*/*b* ratios) under normal growing conditions, we used the *conditional chlorina* (*cch*), *ch1-2*, and *ch1-3* mutants. *cch* is a leaky chlorophyll biosynthesis mutant with a missense mutation in the H subunit of Mg-chelatase (this mutant will be described elsewhere); under higher light conditions, the *a*/*b* ratio increases, resulting in a chlorina phenotype. RNA was extracted from plants that had been grown under low light for 1 week and then under moderate light intensity for 2 weeks. The *a*/*b* ratios were 2.4 for the wt , 11.1 for *cch*, 12.0 for *ch1-2*, and infinity for *ch1-3*.

Quantitation data for probes with similar specific activity indicated that *Lhcb1* mRNAs were ≈40-fold more abundant that those of *AtCAO*. Fig. 2 shows the relative amounts of *AtCAO* and *Lhcb1* mRNAs in the wt and the mutants. The amount of *AtCAO* mRNA increased ≈2-fold in the chlorina mutants, whereas the highly abundant *Lhcb1* mRNA increased slightly in *cch*, did not change in *ch1-2*, and decreased in the *ch1-3* mutant. These results suggest that *AtCAO*, but not *Lhcb1*, mRNA levels are being up-regulated when the *a*/*b* ratio is increased.

We have also tested *AtCAO* and *Lhcb1* mRNA levels in plants grown for 10 days under low light, transferred to dim light for 1, 2, or 3 days, and returned to low light for 3 or 6 hours. Fig. 2 shows that *Lhcb1* mRNA levels decreased 2-fold during this treatment, whereas *AtCAO* mRNA levels dropped 3-fold. Starch reserves were depleted during the dim-light treatment, as indicated by small starch pellets during RNA extraction. After 3 days, dim light-treated leaves had less Chl per gram of tissue (1.56 ± 0.093) than plants that remained in low light (1.93 ± 0.166), but *a*/*b* ratios did not change. When dim light-treated plants were returned to 60 μmol·m^{-2}·sec^{-1}, *AtCAO* mRNA levels increased rapidly whereas *Lhcb1* mRNA levels were less responsive.

Chlorophyll Binding Proteins in the *ch1-3*-Null Allele. To determine the identities of the Chl-binding proteins in *ch1-3*, the null allele that completely lacks Chl *b*, we used nondenaturing Deriphat green gels (35) to isolate pigment–protein complexes. These complexes were cut from the gel, denatured, and separated by denaturing SDS/PAGE on duplicate gels. One gel was stained with Coomassie Brilliant Blue, whereas the other was blotted to nitrocellulose and immunoreacted with an antibody that recognizes CP43, an internal antenna protein of PSII. Fig. 3 shows these data for wild-type and *ch1-3*. The wild-type complexes were similar to those described for barley, with the darkest green Chl band being composed of trimers consisting of Lhcb1, Lhcb2, and Lhcb3. The Chl-binding proteins were quite different in *ch1-3*. The darkest green band was the PSII core, which reacted strongly to the anti-CP43 antibody. Other prominent bands were the PsaA and PsaB proteins that migrated at 60 kDa. In the last lane, some LHC apoproteins were present, and their size suggested that they were LHCI proteins. These data show that the most abundant Chl-binding proteins of *ch1-3* are internal antennae of PSI and PSII, confirming that most of the light harvesting

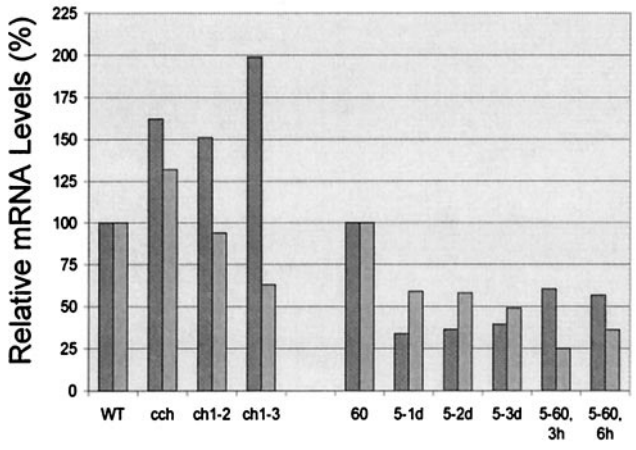

FIG. 2. Relative *AtCAO* and *Lhcb1* mRNA levels in wt plants and lines with increased *a*/*b* ratios and in wt plants grown in dim light. Quantified and normalized mRNA levels are shown relative to wt plants for each experiment. Dark gray boxes represent *AtCAO* mRNA levels, whereas the light gray boxes represent *Lhcb1* mRNA levels. "60" indicates 60 μmol·m^{-2}·sec^{-1}, "5" indicates 5 μmol·m^{-2}·sec^{-1} and "5–60" indicates samples that had been in 5 μmol·m^{-2}·sec^{-1} for 3 days and then transferred back to 60 μmol·m^{-2}·sec^{-1} for the indicated time.

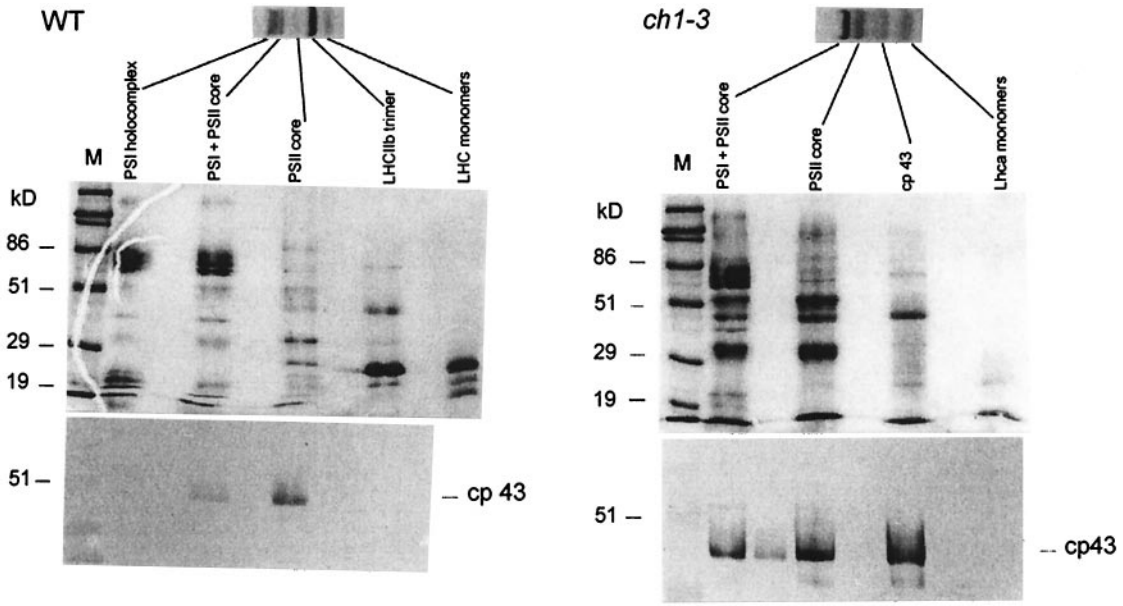

FIG. 3. Second-dimension denaturing gel electrophoreses of wt and *ch1-3* thylakoid membranes and immunoblot analysis with anti-CP43. The nondenaturing green-gel is shown at *Top*. The indicated pigmented bands were electrophoresed on denaturing gels, stained with Coomassie, and are shown in the *Middle*. *Bottom* is an immunoblot of the denaturing gels reacted with the anti-CP43 antibody.

is done by the core antennae and not by the LHC in the *ch1-3* mutant.

To determine which proteins of LHCII were present in *ch1-2* and *ch1-3*, we used a set of monospecific antibodies that recognize Lhcb1 through Lhcb6. Fig. 4 shows that all six Lhcb proteins were undetectable in *ch1-3*. *ch1-2* contains reduced amounts of Lhcb1–5, and has no detectable Lhcb6. The undetectable levels of all six Lhcb proteins in the *ch1-3* mutant of *Arabidopsis* suggest that this line has a more diminished LHCII than the *chlorina f2* mutant of barley. This is consistent with HPLC data showing a complete lack of Chl *b* in *ch1-3*, whereas a small percentage of Chl *b* was detectable in *chlorina f2* (2).

DISCUSSION

We have isolated the *AtCAO* gene from *A. thaliana*, which encodes Chl *a* oxygenase. Mutations of this gene result in reduced levels or complete absence of Chl *b*, implicating the necessity of this gene in vascular plant Chl *b* biosynthesis. The leaky *ch1-2* allele is a missense mutation in the Reiske-binding site (V274E), providing functional evidence that this conserved region is important in Chl *b* synthesis. This point mutation cosegregated in six of six *ch1-2* F_2 sibling lines, providing genetic evidence that this T → A transversion is indeed responsible for the reduced levels of Chl *b*. It is possible that the *ch1-2* mutation is linked to *AtCAO*, and the T → A transversion is only cosegregating with the mutant phenotype, but a second *AtCAO* mutation in the *ch1-3* allele makes this possibility unlikely. The *ch1-3* allele has a 40-aa deletion of *AtCAO*, which includes the mononuclear nonheme Fe-binding site. This deletion resulted in a plant that had no detectable Chl *b*, indicating that there are no alternative Chl *b* synthesis pathways and that redundant *AtCAO* genes do not exist. Protein structure prediction programs suggested that AtCAO is imported into the chloroplast and is a soluble enzyme, most likely functioning in the stroma.

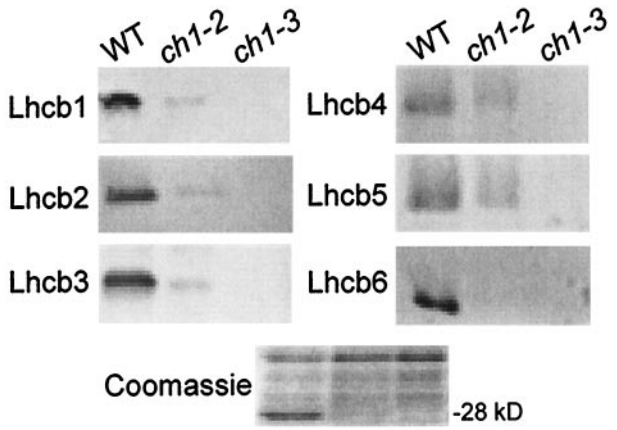

FIG. 4. Immunoblots probed with specific Lhcb antibodies. Thylakoid proteins from wt , *ch1-2*, and *ch1-3* plants were loaded on an equal protein basis and reacted with the Lhcb antibodies described in *Materials and Methods*. A Coomassie-stained gel is shown below the immunoblots as a control for protein loading.

AtCAO mRNA levels were increased 2-fold in three different Chl-*b*-deficient mutants of *Arabidopsis*, whereas *Lhcb1* mRNA levels changed variably. The observed up-regulation of *AtCAO* suggests that some type of feedback regulation might be occurring in which the amount of Chl *b* in the thylakoid membrane regulates *AtCAO* mRNA levels. In wt plants, there are sufficient levels of Chl *b*, and *AtCAO* mRNA levels are low, whereas the mutants with inadequate levels of Chl *b* have higher levels of *AtCAO* mRNA. Light harvesting by PSII affects the redox poise of the plastoquinone pool, and it is possible that this is the parameter that is being communicated by the chloroplast to the cytoplasm and/or the nucleus to regulate *AtCAO* mRNA levels. A similar type of regulation has been demonstrated for transcriptional regulation of *Lhcb* genes in the alga *Dunaliella salina* (36).

AtCAO mRNA levels were also shown to decrease 3-fold and *Lhcb1* mRNA levels dropped 2-fold when plants were grown under dim light intensity. Under these light conditions, plants hydrolyzed starch reserves for respiration, and construction of LHCII was not energetically wise, as indicated by the decrease in mg of Chl per gram of tissue. A correlation between decreased *AtCAO* and *Lhcb1* transcript levels and decreased tissue Chl suggests that control of the synthesis of LHCII is in part regulated at the level of gene expression. Whether this change is transcriptional or posttranscriptional remains to be

determined. A decrease in *Lhcb1* mRNA levels at high light intensity has been observed in *Arabidopsis* plants transferred to 400 μmol·m^{-2}·sec^{-1} for 24 hr (37) and for barley plants grown at 1,500 μmol·m^{-2}·sec^{-1} (38).

Finally, *ch1-3* has been shown to be a null mutation, stronger than *chlorina f2.* Unlike *chlorina f2*, *ch1−3* had undetectable levels of all six Lhcb proteins. Complete loss of Lhcb1, Lhcb2, and Lhcb3 has been confirmed by using a monoclonal antibody, MLH9, that specifically recognizes these three proteins (ref. 39; E. J. TerKeurst and J.A.B., unpublished results). It is possible that very small amounts of the Lhcb proteins were present below our detection levels, but a clear reduction compared with wt plants was evident. Our results also showed that Lhcb6 is the most unstable Lhcb protein, becoming undetectable in the leaky *ch1-2* allele. This result could be because of Lhcb6 being at the periphery of the LHC (40) or because of a lower binding affinity for Chl *b* (41). Despite the greatly diminished LHCII, the *ch1* mutants are still green and able to perform photosynthesis because Chl *a* stabilizes the internal antennae; CP43, CP47, PsaA, and PsaB, and these chlorophyll proteins become the primary light harvesters.

In conclusion, we have shown that the *AtCAO* gene is responsible for Chl *b* synthesis in a vascular plant and that the Reiske-binding site as well as the mononuclear nonheme Fe-binding site are important for gene function. Partial deletion of *AtCAO* results in the complete absence of Chl *b*, indicating there are no redundant genes or biochemical pathways in *Arabidopsis*. We have also presented evidence suggesting that *AtCAO* mRNA abundance is regulated in response to Chl *b* levels as well as to light intensity.

Note Added in Proof. The *CAO* coding sequence from *A. thaliana* was published by Tomitani *et al*. (42) (GenBank accession no. AB021316).

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