Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. Wild-type and *pab* mutant plants (ecotype Columbia) were grown on Murashige and Skoog medium containing 1% sucrose under short-day conditions (10-h light/14-h dark cycles) with a photon flux density of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 22 °C. To ensure synchronized germination, the seeds were incubated in darkness for 48 h at 4 °C before sowing. The *pab* mutants were isolated from a collection of T-DNA–mutagenized *Arabidopsis* lines based on their high–chlorophyll-fluorescence phenotypes. After growth for 3 wk on separate plates, the WT and *pab* mutant plants were then transferred onto one same plate for taking photographs and chlorophyll fluorescence images. The chlorophyll fluorescence images were captured in planta at room temperature using a chlorophyll fluorescence imaging system (CF imager; Technologica).

Complementation of the *pab-1* Mutant. The cDNA containing the *PAB* coding region was amplified by PCR and cloned into the pSN1301 vector under the control of the cauliflower mosaic virus 35S promoter. The construct was transformed into heterozygous *pab-1* plants by the floral dip method (1). The transgenic plants were selected on Murashige and Skoog medium containing 40 μg/mL hygromycin and grown in a greenhouse to produce seeds. Genotyping of the progeny was performed by PCR using gene-specific primers, and successful complementation was confirmed by chlorophyll fluorescence analysis.

Subcellular Localization of GFP-Fused Proteins. DNA encoding the full-length PAB protein was amplified and ligated into the GFP fusion vector pUC18-35s-sGFP with GFP as a reporter. The controls for nuclear, chloroplastic, and mitochondrial localization were constructed according to previously described methods (2). The resulting fusion constructs and the control vectors were introduced into *Arabidopsis* mesophyll protoplasts according to the PEG-mediated method (3). Fluorescence analysis was performed on an LSM 510 Meta confocal laser scanning system (Zeiss).

Antiserum Production. The nucleotide sequence encoding the mature PAB protein was amplified by RT-PCR using specific primers containing NdeI and XhoI restriction endonuclease sites and cloned into the pET28a vector (Novagen). Antigens were expressed as recombinant proteins containing an N-terminal His tag in *E. coli* strain DE3 (BL21) (Novagen). Total protein was extracted under urea-denaturation conditions and the recombinant proteins were purified on nickel-nitrilotriacetic acid agarose (Novagen) according to the manufacturer's instructions. The polyclonal antibody was raised in rabbit with purified antigen.

Protein Isolation and Immunoblotting. Total proteins were prepared as previously described (4). *Arabidopsis* high–chlorophyll-fluorescence leaves were homogenized in 125 mM Tris·HCl (pH 8.8), 1% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM $Na_2S_2O_5$. After centrifugation at 12,000 × *g* for 10 min, the supernatant containing total protein was collected. Thylakoid membrane proteins were isolated essentially as described (5). The concentration of proteins was determined with a DC Protein Assay Kit (Bio-Rad). The proteins resolved by SDS/PAGE were blotted onto nitrocellulose membranes and incubated with specific antibodies, and the signals were detected using the Pro-Light HRP Chemiluminescent Kit (Tiangen Biotech).

In Vivo Radiolabeling of Chloroplast Proteins. The high–chlorophyll-fluorescence leaves of 10-d-old plant growth on Petri dishes were preincubated in 20 mg/mL cycloheximide for 30 min and radiolabeled with 1 μCi/mL [^{35}S]Met (specific activity >1,000 Ci/mmol; Amersham Pharmacia Biotech) in the presence of 20 mg/mL cycloheximide for 20 min at 25 °C. Pulse labeling of the leaves was followed by a chase in the presence of 10 mM unlabeled Met. After labeling, the thylakoid membranes were isolated and subjected to 15% (wt/vol) SDS/PAGE followed by autoradiography.

Stability of Thylakoid Proteins. The high–chlorophyll-fluorescence leaves from 4-wk-old wild-type and *pab* plants grown on Murashige and Skoog plates with 1% sucrose were vacuum-infiltrated with 100 μg/mL lincomycin and 20 μg/mL cycloheximide, as indicated, to block chloroplast- and nucleus-encoded protein biosynthesis, respectively. After a 30-min incubation, the leaves were illuminated under 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 0, 2, and 4 h. The thylakoid membranes were isolated and the contents of the PSII, PSI, Cyt *b6/f*, and ATP synthase proteins were determined by immunoblot analysis.

Chloroplast Fractionation. Intact chloroplasts were isolated according to a previously described method (6) with minor modifications. Briefly, 10 g of 4-wk-old *Arabidopsis* leaves was homogenized in 50 mL isolation buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM Hepes/KOH, pH 8.0, 10 mM $NaHCO_3$). After filtration through a double layer of Miracloth, the homogenate was centrifuged at 1,000 × *g* for 5 min at 4 °C and the pellet was resuspended in isolation buffer. The resuspended chloroplasts were loaded onto a Percoll gradient (40/70%) and centrifuged at 3,000 × *g* for 30 min in a swing-out rotor. The intact chloroplasts were recovered from the lower band and washed in 50 mM Hepes/KOH (pH 8.0), 0.3 M sorbitol, 5 mM $MgCl_2$.

The chloroplast fractionation was performed as described (7). Briefly, chloroplasts (~150 μg chlorophyll) were frozen in liquid nitrogen and rapidly thawed on ice, and this was repeated three times. After centrifugation at 12,000 × *g* for 10 min, the supernatant containing the stroma fraction and the pellet containing thylakoids were recovered. The concentration of proteins was determined with a DC Protein Assay Kit (Bio-Rad).

Yeast Two-Hybrid Assays. The yeast two-hybrid assays were performed using the Matchmaker Two-Hybrid System Kit (Clontech). The sequences encoding the mature $CF_1\alpha$, β, γ, δ, and ε subunits were individually cloned into pGADT7 as prey, whereas the PAB protein coding sequence was inserted into pGBKT7 DNA-BD as bait. The prey and bait plasmids were cotransformed into Y2H Gold yeast strain (Clontech) according to the manufacturer's instructions. Protein interaction was investigated via observing the growth of the yeast transformants on synthetic dropout medium containing 40 μg/mL X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) but without tryptophan, leucine, and histidine.

Pull-Down Assays. PAB-GST, PAB/$CF_1\gamma$-MBP, and His-$CF_1\alpha$/β/γ/δ/ε fusion proteins were purified using GST-Bind resin (Novagen), Amylose-Bind resin (NEB), and Ni-NTA His-Bind resin (Novagen), respectively, according to the manufacturer's instructions. Ten micrograms of PAB-GST proteins was coupled to 100 μL GST-Bind resin [a 50% (vol/vol) suspension in PBS]. Then, 5 μg His-$CF_1\alpha$, β, γ, δ, or ε fusion protein was added. After incubation for 2 h at 4 °C, the beads were washed five times with ice-cold PBS plus 0.5% Nonidet P-40 and the bound proteins were eluted with

SDS/PAGE sample buffer at 95 °C. The eluted proteins were resolved by SDS/PAGE and subjected to immunoblot analysis.

For the pull-down assay between the refolding $CF_1\gamma$ and PAB proteins, the recombinant His-$CF_1\gamma$ was denatured in 6 M urea. Then, 0.03 μM His-CF1γ was added in the refolding buffer containing 20 mM Tris·HCl (pH 7.5), 0.1 M NaCl, 2 mM ATP, 0.3 μM Cpn60/Cpn20 at 30 °C. After incubation for 0, 30, 60, or 90 min, 200-μL mixtures were collected and centrifuged at 18,000 × *g* at 4 °C. A 100-μL supernatant fraction was used in the pull-down assay with the PAB-MBP proteins.

Coimmunoprecipitation Assay. For immunoprecipitation, 0.05 g of 4-wk-old leaves was homogenized in PBS buffer containing 1% (wt/vol) dodecyl-β-D-maltopyranoside. After centrifugation at 12,000 × *g* for 10 min, the supernatant containing the total protein was collected. Antibodies against PAB were added to the total protein, which was incubated overnight at 4 °C and then precipitated with protein A/G agarose. After washing the beads with PBS containing 0.2% Nonidet P-40 four times, the bound proteins were eluted with 0.1 M glycine (pH 3.0), separated by SDS/PAGE, and analyzed by immunoblot.

Bimolecular Fluorescence Complementation Assays. BiFC assays were performed according to Walter et al. (8). Full-length cDNA of PAB was cloned into pSAT4A-cEYFP-N1, and full-length cDNAs of $CF_1\alpha$, β, γ, δ, and ε subunits were cloned into pSAT4A-nEYFP-N1. The plasmids were cotransformed into protoplasts. Yellow fluorescent protein (YFP) was imaged using a confocal laser scanning microscope (LSM 510 Meta; Zeiss).

Reconstitution of the ATPase CF_1 Active Core. The cDNAs of $CF_1\alpha$ (ATcg00120), $CF_1\beta$ (ATCG00480), $CF_1\gamma$ (AT4G04640), Cpn20 (AT5G20720), Hsp40 (AT1G80030), Cpn60α (at2g28000), and Cpn60β (at1g55490) from *Arabidopsis* and GroES from *E. coli* were cloned into the pET28(a) expression vector (Novagen). The cDNA of Hsp70 (AT4G24280) from *Arabidopsis* was cloned into pET SUMO (Invitrogen). All constructs were introduced into *E. coli* expression strain BL21 (DE3). The expression of protein $CF_1\alpha$, $CF_1\beta$, and $CF_1\gamma$ was induced with isopropyl β-D-thiogalactoside (IPTG) for 7 h at 37 °C in inclusion bodies, and the BL21 cells were harvested and resuspended in 500 mM NaCl, 6 M urea, 20 mM Tris·HCl (pH 7.5). The overexpression of Cpn20, Hsp70, Hsp40, Cpn60α, Cpn60β, and GroES was induced with IPTG for 16 h at 17 °C, and the BL21 cells were harvested and resuspended in 500 mM NaCl, 20 mM Tris·HCl (pH 7.5). The bacterial cells were sonicated 90 times for 3 s each. The fusion proteins were purified on an Ni-NTA agarose resin matrix.

For the proteinase K assay, Cpn60 was reconstituted by Cpn60α and Cpn60β according to previously described methods with some modification (9). Cpn60α and Cpn60β (15 μM each) were mixed in the incubation buffer (50 mM Tris·HCl, pH 7.5, 0.3 M NaCl, 10 mM $MgCl_2$, 16 mM KCl, 2 mM DTT) at 30 °C for 3 h. For the reconstitution assay, 30 μM Cpn60α and Cpn60β were mixed in the incubation buffer (50 mM Tris·HCl, pH 7.5, 0.3 M NaCl, 10 mM $MgCl_2$, 16 mM KCl, 2 mM DTT) with the addition of 15 μM GroES at 30 °C for 1.5 h. After centrifugation at 8,000 × *g* for 30 min, the supernatant fraction was collected. Reconstituted Cpn60 was isolated by gel-filtration chromatography.

Denatured $CF_1\alpha$ subunit (0.087 μM), denatured $CF_1\beta$ subunit (0.087 μM), and denatured $CF_1\gamma$ subunit (0.026 μM) were incubated together in reconstitution buffer including 50 mM KCl, 5 mM $MgCl_2$, 2 mM MgATP, 0.1 mM DTT, 5% (vol/vol) glycerol, 25 mM Tris·chloride (pH 7.5) at 23 °C in the presence of Cpn60/Cpn20, Hsp70/Hsp40, and/or PAB. The reconstitution reaction with chloroplast stroma (100 μg) was used as a positive control. The molar ratio of total CF_1 core subunits/chloroplast molecular chaperones was about 1:1. The reconstitution reaction was conducted at 25 °C for 1 h. After centrifugation at 15,000 × *g*, the supernatants were isolated for ATPase activity assays.

ATPase Activity Assay. The ATPase activity was determined by measuring phosphate release in a mixture containing 50 mM Tris·HCl (pH 8.0), 50 mM NaCl, 4 mM ATP, 50 mM Na_2SO_3, 20% (vol/vol) methanol, 2 mM $MgCl_2$ incubated with 0.2 mL reconstitution mixture for 5 min at 37 °C. After the reaction was stopped by adding 40 μL trichloroacetic acid, the supernatant was collected and the released Pi was determined by a microcolorimetric method (10). ATPase activity was calculated based on the amount of core subunit protein added initially into the assay, and the background activity of the chaperones was subtracted from the observed results.

Proteinase K Protection of Chaperonin–Substrate Complexes. Ternary complexes were obtained in two ways that differed in the absence (Fig. 4*A*, *Left*) or presence (Fig. 4*A*, *Right*) of a cochaperonin in the incubation mixture containing Cpn60 and unfolded substrate $CF_1\gamma$. The Cpn60 (0.8 μM) and the unfolded substrate (0.65 μM) were incubated in buffer B (50 mM Tris·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$) for 10 min at 25 °C in the presence or absence of Cpn20 (1.9 μM) and ADP (1 mM). Protease treatment of the various protein mixtures was performed by addition of proteinase K to a final concentration of 1.2 μg/mL. After incubation for 0, 5, 10, 15, and 20 min at 25 °C with rotary shaking, the proteolysis was stopped by adding PMSF (1 mM). Protein mixtures were analyzed by SDS/PAGE and immunoblotting.

Preparation of CF_1 and Subunit-Deficient CF_1 Complexes. The intact CF_1 was prepared according to previously described procedures (11). The $CF_1\alpha\beta$ complex was prepared from intact CF_1 by removing the ε, δ, and γ subunits (12).

Substitution Assay Between $CF_1\alpha\beta$ and the PAB–$CF_1\gamma$ Complex. The PAB–$CF_1\gamma$ complex was prepared by incubating N-terminal MBP fusion protein $CF_1\gamma$-MBP and His-PAB protein after removal of unbound proteins as described for the pull-down assays. The $CF_1\alpha\beta$ complex was then added to the PAB–$CF_1\gamma$ complex and incubated at 4 °C for various times as shown in Fig. 5*B*. After centrifugation for 15 min at 10,000 × *g*, the supernatant and amylose resin fractions were collected and subjected to SDS/PAGE and immunoblot analysis.

1. Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16(6):735–743.
2. Cai WH, et al. (2009) LPA66 is required for editing psbF chloroplast transcripts in *Arabidopsis*. *Plant Physiol* 150(3):1260–1271.
3. Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc Natl Acad Sci USA* 97(6):2940–2945.
4. Martinez-Garcia JF, Monte E, Quail PH (1999) A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J* 20(2):251–257.
5. Zhang LX, Paakkarinen V, van Wijk KJ, Aro EM (1999) Co-translational assembly of the D1 protein into photosystem II. *J Biol Chem* 274(23):16062–16067.
6. Aronsson H, Jarvis P (2002) A simple method for isolating import-competent *Arabidopsis* chloroplasts. *FEBS Lett* 529(2-3):215–220.
7. Keegstra K, Yousif AE (1988) Isolation and characterization of chloroplast envelope membranes. *Methods Enzymol* 118:316–325.
8. Walter M, et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40(3):428–438.
9. Dickson R, et al. (2000) Reconstitution of higher plant chloroplast chaperonin 60 tetradecamers active in protein folding. *J Biol Chem* 275(16):11829–11835.
10. Taussky HH, Shorr E (1953) A microcolorimetric method for the determination of inorganic phosphorus. *J Biol Chem* 202(2):675–685.
11. Andreo CS, Patrie WJ, McCarty RE (1982) Effect of ATPase activation and the δ subunit of coupling factor 1 on reconstitution of photophosphorylation. *J Biol Chem* 257(17): 9968–9975.
12. Patrie WJ, McCarty RE (1984) Specific binding of coupling factor 1 lacking the δ and ε subunits to thylakoids. *J Biol Chem* 259(17):11121–11128.

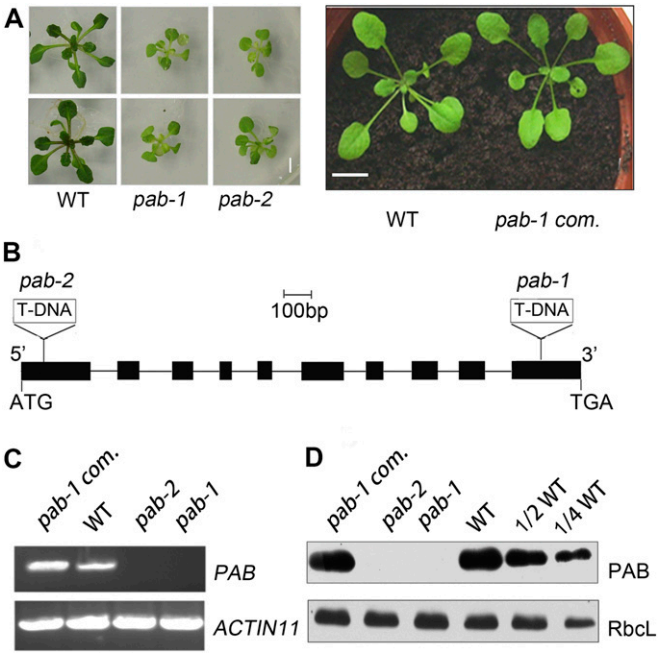

Fig. S1. Identification of *pab* mutants. (*A*) Phenotypes of *pab* and wild-type plants. (*Left*) Photographs of *pab-1* and *pab-2* mutants and wild-type plants grown for 3 wk on Murashige and Skoog medium containing 1% sucrose. After growth for 3 wk on separate plates, the WT and *pab* mutant plants were then transferred onto one same plate for taking photographs. Dividing lines indicate noncontiguous seedlings in the picture. (*Right*) Photographs of wild-type and *pab-1* complemented plants grown for 3 wk in the growth chamber. (Scale bars, 0.5 cm.) (*B*) Schematic diagram of the PAB gene. Exons (black boxes) and introns (lines) are indicated. The positions of the T-DNA insertions of *pab-1* and *pab-2* are indicated by triangles. ATG indicates the start codon and TGA indicates the stop codon of *PAB*. (*C*) RT-PCR analysis of *PAB* expression in the *pab-1*, *pab-2*, and wild-type plants. The expression of *ACTIN11* was used as a control. The immunoblot detection limit of PAB protein was about 5% of that in wild type. (*D*) Immunoblot analysis of PAB expression in wild-type, *pab-1*, and complemented plants. Immunoblotting of RbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) is shown below to provide an estimate of gel loading.

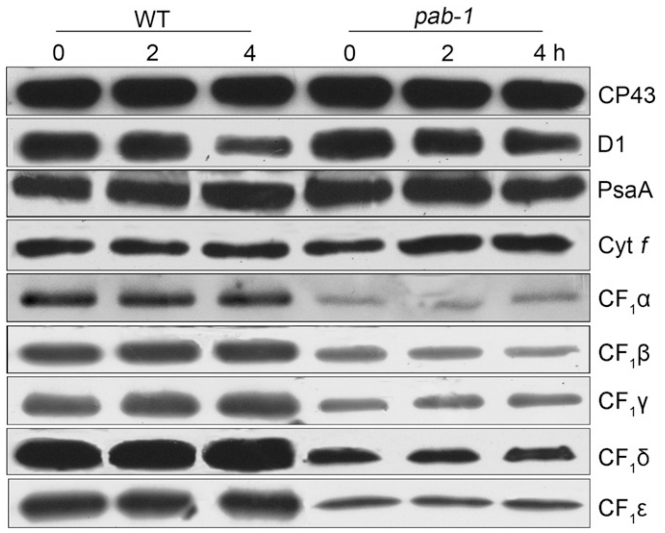

Fig. S2. Analysis of thylakoid protein stability in *pab-1*. The leaves from wild type and *pab-1* were treated with cycloheximide and lincomycin for 30 min followed by illumination for 0, 2, and 4 h. The thylakoid membranes were isolated and separated by 15% SDS/PAGE followed by immunoblot analysis.

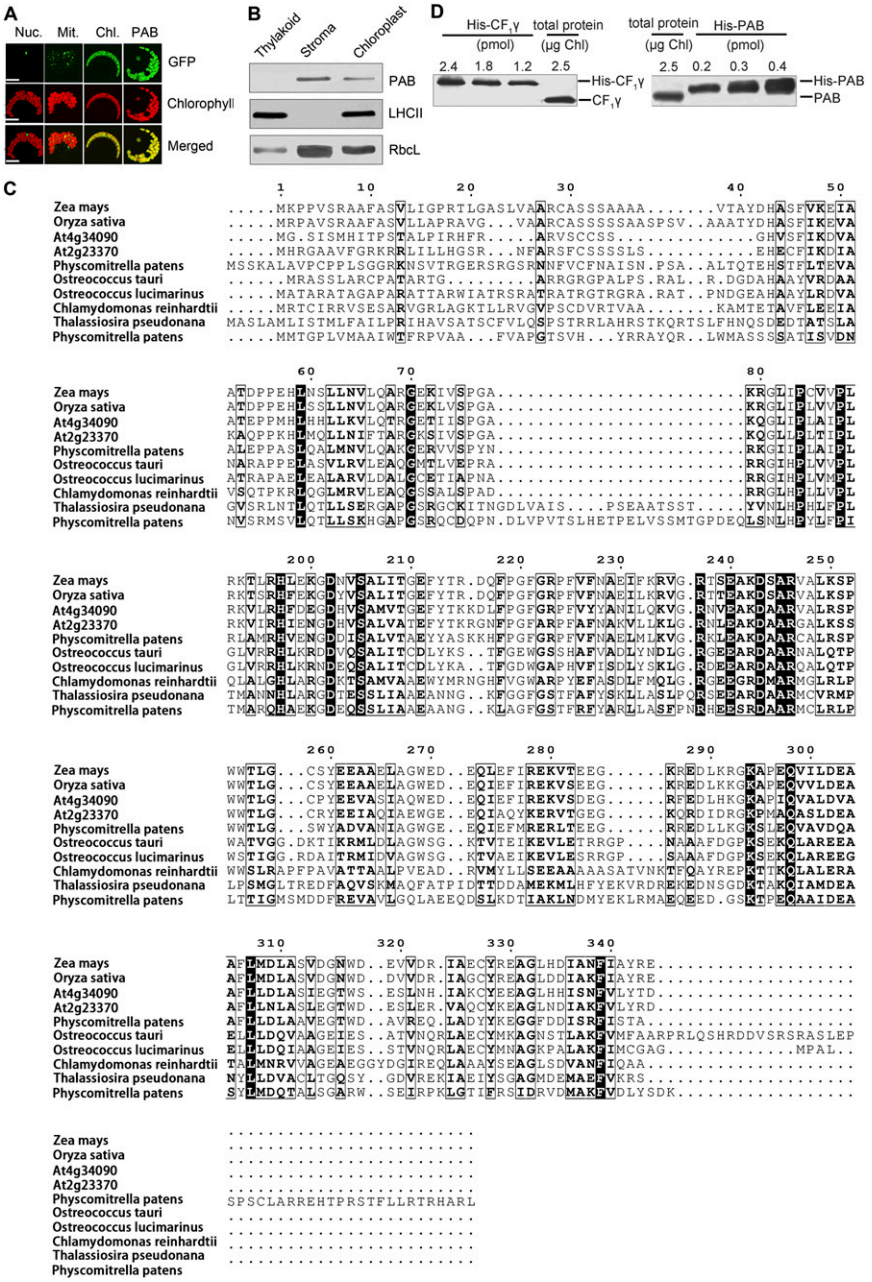

Fig. S3. Characterizations of the PAB protein. (*A*) Subcellular localization of the PAB protein by GFP fluorescence assay. Green indicates GFP, red shows chloroplast autofluorescence, and yellow shows the colocalization in the merged images. The constructs used for transformation are indicated above: Nuc., control nuclear localization signal of fibrillarin; Mit., control mitochondrial localization signal of the 18-kDa Fe-S subunit of mitochondrial NADH dehydrogenase; Chl., control chloroplast localization signal of the LPA66-GFP fusion protein (2); PAB, signal from the PAB-GFP fusion protein. (Scale bars, 0.5 μm.) (*B*) Subcellular localization of the PAB protein by immunoblot analysis. The chloroplast, stroma, and thylakoid fractions were subjected to immunoblot analysis with specific antisera against PAB, LHCII, and RbcL. (*C*) Protein sequence alignment of PAB homologs. Alignments were performed using the ESPript program (espript.ibcp.fr/ESPript/ESPript). Strictly conserved amino acids are indicated in black, and closely related residues are indicated by boxes. (*D*) Quantification of PAB and $CF_1\gamma$ protein in plants. Appropriate quantifications of the His-PAB and His-$CF_1\gamma$ fusion proteins were titrated against total proteins and subjected to immunoblot analysis. After recombination, PAB and $CF_1\gamma$ were expressed in *E. coli* and purified, and a dilution series of the recombinant PAB and $CF_1\gamma$ proteins was titrated against stroma preparations and subjected to immunoblot analysis.

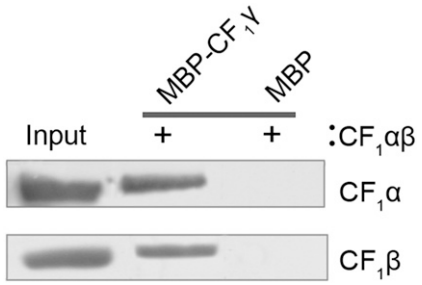

Fig. S4. Interaction of the $CF_1\alpha\beta$ subcomplex and MBP-$CF_1\gamma$ by pull-down assay. MBP-$CF_1\gamma$ fusion protein was constructed as bait, and the pull-down analysis was performed as in Fig. 2 *B* and *C*.

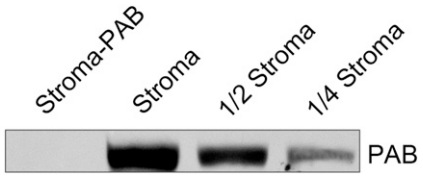

Fig. S5. Analysis of the protein level of PAB deletion from stroma. PAB-deleted stroma and total stroma were separated by 15% SDS/PAGE followed by immunoblot analysis. The immunoblot detection limit of PAB protein was about 5% of that in wild type.

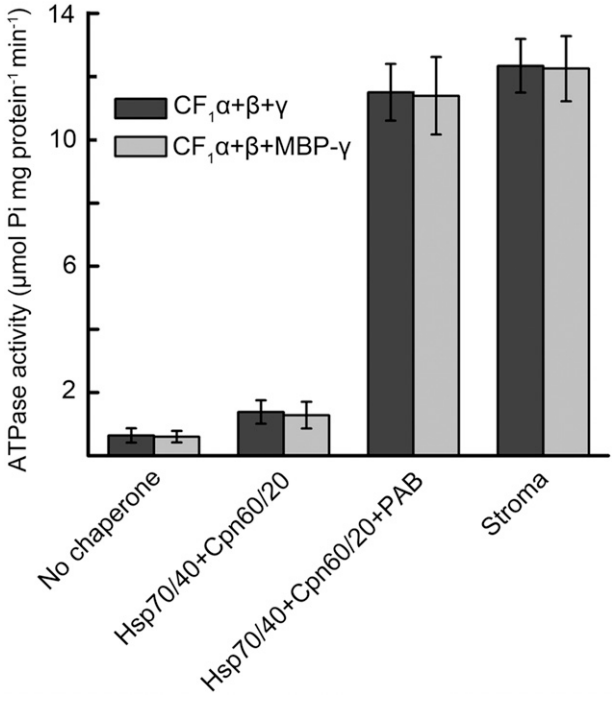

Fig. S6. Reconstitution of the ATPase CF_1 core with His-α, His-β, and MBP-γ. Individually expressed urea-solubilized His-α, His-β, and MBP-γ subunits were incubated together with different combinations of protein factors, and the magnesium-dependent ATPase activity was determined. Error bars represent SEM (n = 6).

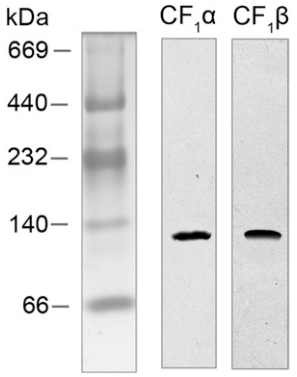

Fig. S7. Immunoblot assay for oligomerization of the $CF_1\alpha\beta$ subcomplex. After incubation of $CF_1\alpha$ and $CF_1\beta$ together, 4–12.5% (wt/vol) BN gel electrophoresis was performed followed by immunoblotting with $CF_1\alpha$ and $CF_1\beta$ antibodies, respectively.

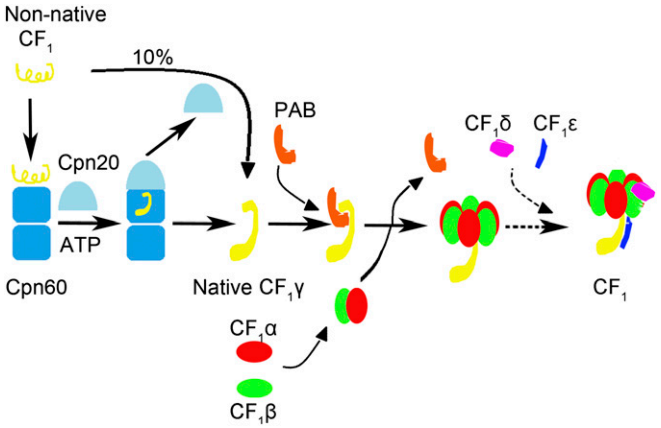

Fig. S8. Proposed model for $CF_1\gamma$ assembly into the CF_1 catalytic core with the $CF_1\alpha\beta$ complex. PAB increases the efficiency of the assembly of the ATP synthase CF_1 catalytic core by acting on folded $CF_1\gamma$ subunits following their folding mediated by Cpn60/Cpn20 (see details in *Discussion*).

Table S1. Reconstitution of the CF_1 core complex with different subunit combinations in the presence of Cpn60/20 + Hsp70/40 + PAB

Sequence of subunit into the recombination buffer	ATPase activity, μmol Pi·mg protein^{-1}·min^{-1}
$CF_1\alpha+\beta+\gamma$	0
$CF_1(\alpha+\gamma)+\beta$	0
$CF_1(\beta+\gamma)+\alpha$	0
$CF_1(\alpha+\beta)+\gamma$	12.9 ± 0.5
$CF_1(\alpha+\beta+\gamma)$	12.7 ± 0.6