Supporting Information

Yagi et al. 10.1073/pnas.1119403109

SI Materials and Methods

Antibodies. A fragment of OsrpoA (amino acid residues 8–337) was cloned into the pQE-30 vector and its recombinant protein was expressed in *Escherichia coli* and purified with a Ni-NTA column (detail in ref. 1). We also cloned a fragment of *At*pTAC3 corresponding to amino acid residues of 433–600 into pGEX5-1 to raise an anti-pTAC3 antibody. This region is highly conserved among all pTAC3 orthologs in land plants including wheat (Fig. S1). The recombinant protein was purified with a glutathione sepharose column (GE Healthcare) following the manufacturer's instructions and used as an antigen. The PEP α-subunit and pTAC3 antibodies were raised in a rabbit against their recombinant proteins. Detailed information on the anti-GS2 antibody is described in ref. 2.

Phylogenic Analysis of Land Plant pTAC3 Orthologs. *Arabidopsis thaliana* pTAC3 (At3g04260) was analyzed using the blastp and tblastn algorithms in National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/) to identify pTAC3 orthologs in land plants. Phylogenetic analysis was performed using the default setting of NJPlot. GeneBank accession numbers are as follows: XP_002522027.1 (*Ricinus communis*), XP_002268094.2 (*Vitis vinfera*), XP_002325363 (*Populus trichocarpa*), XP_003535382.1 (*Glycine max*), XP_003571848.1 (*Brachypodium distachyon*), AAN05531.1 (*Oryza sativa*), and XP_001759689.1 (*Physcomitrella patens*).

We searched for cDNA clones homologous to AtpTAC3 in the wheat (*Triticum aestivum*) EST library (http://wheat.pw.usda.gov/wEST/), and found three fragments (CO346801, CJ621287, and CJ725188) that correspond to the downstream of the C-terminal SAP domain. The internal protein-coding sequence of wheat pTAC3 containing the SAP domain was amplified from leaf cDNA with forward (5′- TGCGTTGTTAGTGGAATGCTT -3′) and reverse (5′- ATAACTGGACTCCTATCATTTGAAG -3′) primers that were designed by referring to an *OspTAC3*-coding sequence (A336–F342) and EST information, respectively. The amplified fragment was cloned into the pTA2 vector (Toyobo), and its sequence was determined. The extended sequence and ESTs were assembled into a contiguous DNA sequence corresponding to A328–A890 of AtpTAC3 covering the SAP domain and C-terminal region (Fig. S1).

Identification of pTAC3-Deficient Mutants. We identified plants containing a T-DNA insertion by PCR using pTAC3-specific primers fw1 (5′-TCACGCGCTTAACGGCGACG -3′) and rv1 (5′-TGTTGTGGCCATTCTACCAGC-3′), and a T-DNA specific primer T (5′-TGGTTCACGTAGTGGGCCATCGC-3′). The insertion site was confirmed by sequencing the amplified fragment. Identified lines (Salk_110045) were homozygous for the T-DNA insertion and named *ptac3-1*.

Transient Expression of GFP Fusion Proteins in Protoplasts of *Arabidopsis*. Full-length pTAC3 cDNA was amplified with primer pairs, 5′-ctgacttctagaATGTCACTCTTGTTCCTCAATCC-3′ and 5′-catgactgtacaAGAGCTTACTGTTGATGCTGG-3′ from the *Arabidopsis* cDNA library, and were subsequently cloned between the CaMV35S promoter and sGFP in the pTM vector with XbaI and BsrGI restriction sites. Methods for protoplast preparation and transient transformation are described in ref. 3. GFP fluorescence and chlorophyll autofluorescence images were detected using confocal microscopy. For DAPI staining, 20 μL of transformed protoplasts were disrupted in 10 μL of TAN buffer (20 mM Tris-HCl (pH 7.6), 0.5 mM EDTA (pH 7.0), 1.2 mM spermidine, 7 mM 2-mercaptoethanol, 1% (vol/vol) glutaraldehyde, and 0.4 mM PMSF), and then 10 μL of 1 μg/mL DAPI was added. After incubation for 10 min, protoplasts were observed by confocal microscopy.

Plastid Gene Expression Analysis. Total RNAs were extracted from 8-d grown WT and *ptac3-1* plants using the RNeasy plant mini kit (Qiagen). For macroarray analysis, 3 μg of total RNA was labeled with ^{33}P-dCTP in RT reaction mixture containing 10× RT buffer, each of 2.0 mM dATP, dTTP, and dGTP mix, 2.5 mM $MgCl_2$, 0.2 pmol random primers, and 5 units of AMV reverse transcriptase. ^{33}P labeled cDNA was hybridized to a nitrocellulose membrane, spotted with 257 of 500 bp DNA fragments covering all *Arabidopsis* plastid genome, in hybridization buffer containing 0.5 M church buffer (pH 7.2), 1 mM EDTA, and 7% (wt/vol) SDS for 16 h at 65 °C, and then washed two times with 0.1 × SSC including 0.1% (wt/vol) SDS. For RNA gel blotting, 5 μg of total RNA was separated by 1% (wt/vol) agarose gel and blotted onto a Hybond-N nylon membrane. RNA gel blots were hybridized to ^{32}P-labeled DNA probes encoding *psbA*, *psbD*, *psaA*, *rbcL*, *rrn16*, *rrn23*, *rpoB*, *clpP*, and *accD*. Membranes were washed with 0.2× SSC and 0.1% (wt/vol) SDS at 65 °C for 30 min. Radioactive signals were detected by autoradiography or using BAS 1800 (Fuji).

CpChIP Assay. The wheat chloroplast ChIP assay was performed following the protocol described in ref. 4 with one modification. For establishment of ChIP protocol, there are two points to be considered: (*i*) how to cross-link DNA–protein complex and (*ii*) how to share chloroplast DNA. The cross-linking step is performed on isolated crude chloroplasts in the presence of 1% (vol/vol) formaldehyde as described in ref. 4. On the other hand, DNA was shared by sonication in a buffer containing 1% (vol/vol) Triton X-100 in our protocol, instead of MNase digestion of DNA in the presence of 2% (vol/vol) Nonidet P-40 (4). The details are described below.

Wheat leaves grown for 5–6 d (1 g) were harvested and immediately cooled on ice. Harvested leaves were minced with a blender in 50 mL of ice-cold chloroplast isolation buffer containing 25 mM Hepes-NaOH (pH 7.6), 350 mM sorbitol, 2 mM EDTA (pH 7.0), and 2 mM sodium ascorbic acid, in a cold room. The homogenates were filtrated with three layers of miracloth. Crude chloroplast pellets were collected by centrifugation at 4,000 × *g*, 4 °C, for 10 min. For cross-linking, the resulting pellets were suspended in 1 mL of chloroplast isolation buffer containing 1% (vol/vol) formaldehyde and incubated at 25 °C for 10 min with rotation. After incubation, 150 μL of 1 M glycine was added to the crude chloroplasts fraction and further incubated at 25 °C for 5 min with rotation to stop the cross-linking reaction. Cross-linked chloroplasts were pelleted by centrifugation and washed with chloroplast isolation buffer. The resulting pellets were stored at −80 °C until use.

Cross-linked chloroplast pellets were suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 1 mM EDTA (pH 7.6), 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.1% (wt/vol) sodium deoxycholate, and protease inhibitor mixture (Nacalai Tesque). Subsequently, chloroplast DNA was sheared to 0.2–1 kbp by sonication (30% output power, 15 times, 30 s with 30-s interval). The supernatant was collected by centrifugation at 20,000 × *g*, 4 °C, for 10 min, and incubated at 4 °C with rotation for 1 h, followed by dilution with 2 mL of lysis buffer containing 2 μg/mL RNase (Nacalai Tesque) and 10 μg/mL

herring sperm DNA (Invitrogen). The resulting extracts were aliquotted and stored at −80 °C until use.

Diluted extracts (200 μL) were incubated with or without 5 μL of OsRpoA antibody for 2 h at 4 °C with rotation, and then 20 μL of μMACS protein A microbeads (Miltenyi Biotec) coated with 0.2 μg of herring sperm was added to the extracts. After 1-h incubation at 4 °C with rotation, the resulting solution was mixed with 0.4 mL of 5% (wt/vol) skim milk in PBS and then applied to a magnetic column (Miltenyi Biotec). The column was washed once with lysis buffer, three times with low salt buffer [50 mM Tris-HCl (pH 7.6), 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, and 150 mM NaCl], three times with high salt buffer [50 mM Tris-HCl (pH 7.6), 1% (vol/vol) Triton-X 100, 0.1% (wt/vol) SDS, and 1 M NaCl], three times with LiCl buffer [20 mM Tris-HCl (pH 7.6), 1% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate, 1 mM EDTA (pH 7.0), and 250 mM LiCl], and three times with TE [10 mM Tris-HCl (pH 7.6), 10 mM EDTA (pH 7.0)]. The DNA–protein complex was eluted by 200 μL heated elution buffer containing 1% (wt/vol) SDS and 0.1 M $NaHCO_3$. For reverse cross-linking, 8 μL of 5 M NaCl and 2 μL of 10 mg/mL Proteinase K (NEB) were added to the elution fraction and 200 μL input sample, and incubated at 65 °C overnight. Immunoprecipitated DNA was purified with a PCR purification kit (Qiagen) according to manufacturer instructions.

Quantitative PCR. Immunoprecipitated DNA was analyzed with quantitative PCR (Light Cycler; Roche Diagnostics) using SYBR green dye. The reaction mixture (20 μL) contained 2 μL of sample DNA, 10 μL of Thunderbird SYBR qPCR mix (Toyobo), and 6 pmol each of forward and reverse primers. The primers were designed to amplify the promoter of *psbA*, *psbD*, *psaA*, *rbcL*, *rrn16*, *trnEY*, and *rpoB* spacer and coding regions of *psbA* and *rpoA*, termination region of *psbA*, and two *matK* loci, and to yield 80–120 bp fragments (Table S1 and Fig. S4). A standard curve was generated with a dilution series of input samples (6.25×10^{-2}, 1.56×10^{-2}, and 3.9×10^{-3}), and used to assess the PCR efficiency and calculate the percentage of recovery of the input. The cpChIP results obtained by three independent replicate experiments are represented as percentage of input (%IP).

Immunoblotting Analysis. Intact chloroplasts were isolated by centrifugation through a 40–80% (vol/vol) Percoll gradient and stored at −80 °C until use. Intact chloroplasts were lysed with a lysis buffer containing 50 mM Tris-HCl (pH 7.6), 1% (vol/vol) Triton X, 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl, 10% (vol/vol) glycerol, and protease inhibitor (Nacalai Tesque). Ten micrograms of proteins was mixed with an equal volume of sample buffer containing 0.1 M Tris-HCl (pH 6.8), 4% (wt/vol) SDS, 12% (vol/vol) 2-mercaptoethanol, 20% (vol/vol) glycerol, and BPB and separated by SDS/PAGE. pTAC3 and the PEP α-subunit were detected by incubation with primary antibody (1:500 for pTAC3 and 1:1,000 for the PEP α-subunit) and then with an AP-linked chemiluminescent reagent system.

Glycerol Density Gradient Centrifugation. For protein complex analysis by glycerol density gradient centrifugation, total proteins from wheat intact chloroplasts (800 μg) were loaded onto a 10–30% (vol/vol) linear glycerol gradient (each: 4.5 mL) and separated by centrifugation at 30,000 rpm (SW40Ti, Beckman), 4 °C, for 16 h. Thirteen fractions were collected from top to bottom. After TCA precipitation, samples were separated by SDS/PAGE and detected with specific antibodies.

Immunoprecipitation Analysis. One milligram of wheat total chloroplast protein was precleared by adding 100 μL of 50% (vol/vol) slurry of protein A beads (GE) and incubated for 1 h at 4 °C with rotation. After removing the beads by centrifugation at 4,000 rpm, 4 °C, for 10 min, the supernatant was divided into two aliquots for pTAC3 and PEP α-subunit detection. Subsequently, 5 μL of anti-pTAC3 antibody was added to the precleared chloroplast extracts. After 2 h of incubation at 4 °C with rotation, 50 μL of 50% (vol/vol) slurry of protein A beads was added to each extract and incubated for 2 h. The beads were washed five times with lysis buffer and boiled in 50 μL of SDS sample buffer. The immunoprecipitated proteins were separated by SDS/PAGE and transferred to a PVDF membrane using a semidry blotting system.

1. Kusumi K, Yara A, Mitsui N, Tozawa Y, Iba K (2004) Characterization of a rice nuclear-encoded plastid RNA polymerase gene OsRpoTp. *Plant Cell Physiol* 45:1194–1201.
2. Kozaki A, Takeba G (1996) Photorespiration protects C3 plants from photooxidation. *Nature* 384:557–560.
3. Tsunoyama Y, et al. (2004) Blue light-induced transcription of plastid-encoded psbD gene is mediated by a nuclear-encoded transcription initiation factor, AtSig5. *Proc Natl Acad Sci USA* 101:3304–3309.
4. Newell CA, Gray JC (2010) Binding of lac repressor-GFP fusion protein to lac operator sites inserted in the tobacco chloroplast genome examined by chromatin immunoprecipitation. *Nucleic Acids Res* 38:e145.

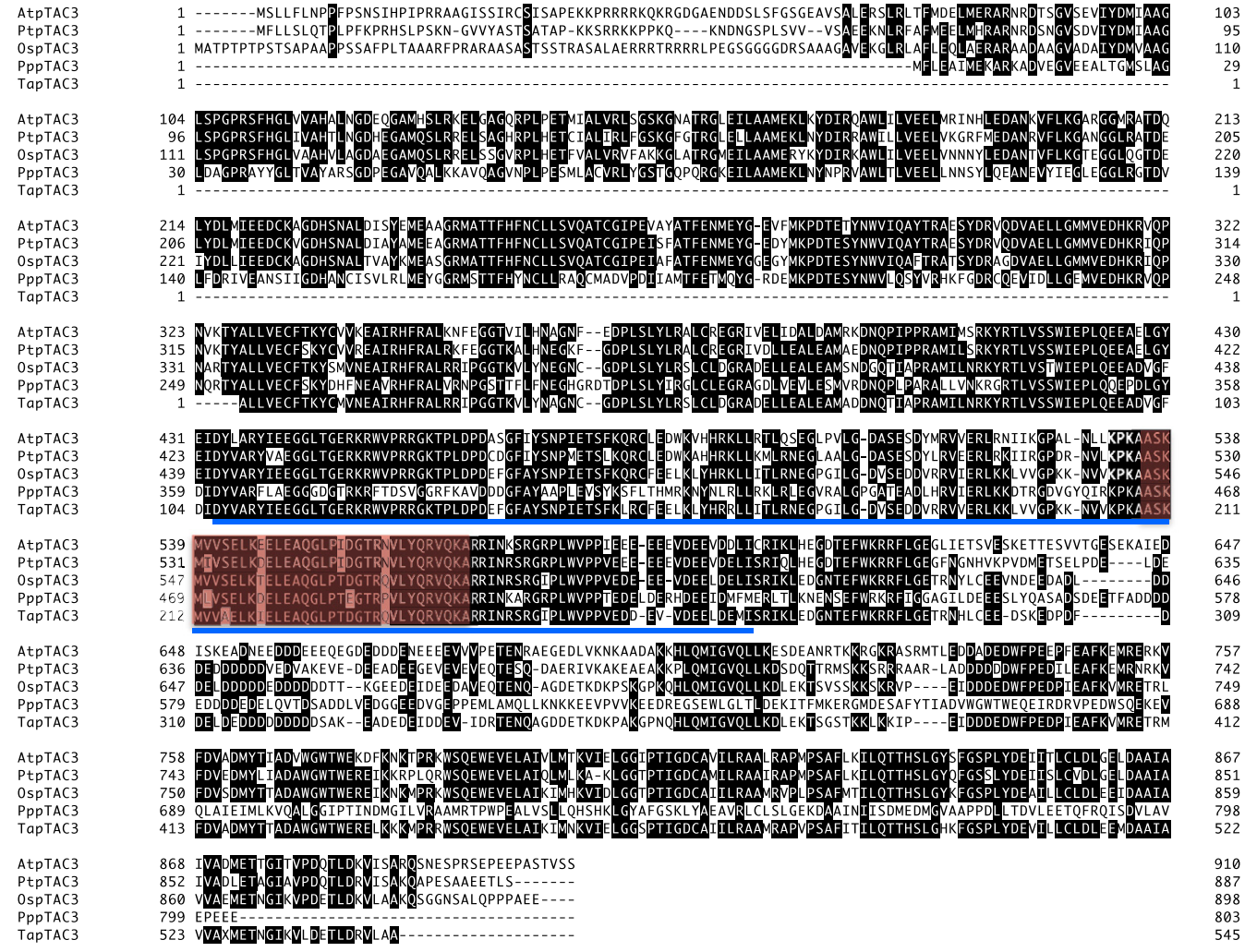

Fig. S1. pTAC3 homolog proteins in land plants. Alignment of amino acid sequence of pTAC3 homologs in land plants. AtpTAC3, *Arabidopsis thaliana*; PtpTAC3, *Populus trichocarpa*; OspTAC3, *Oryza sativa*; PppTAC3, *Physcomitrella patents* (partial sequence); and TapTAC3, *Triticum aestivum* (partial sequence). For wheat pTAC3, a partial *Tap*TAC3 fragment was amplified from wheat leaf cDNA with primer sets designed by reference to OspTAC3 nucleotide sequence and a partial EST sequence of *T. aestivum* (detailed information is described in *SI Materials and Methods*).The aligment was constructed using the ClustalW program with default settings. Red box in the alignment represents SAP domain. An anti-pTAC3 antibody was raised against a peptide corresponding to amino acid residues of 433D-600I of AtpTAC3 (shown as a thick blue line under sequences).

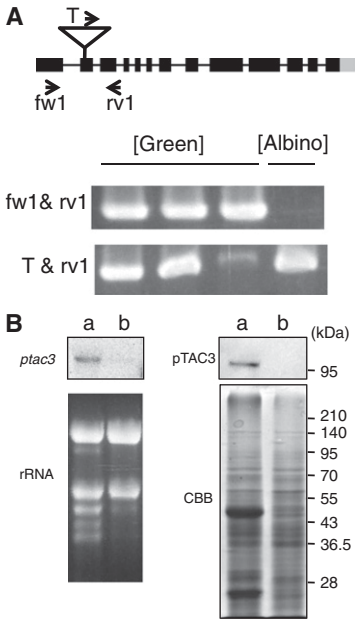

Fig. S2. Analysis of pTAC3-deficient *Arabidopsis thaliana*. (*A*) Schematic map of position of T-DNA insertion in *ptac3-1*. T-DNA region is inserted in second exon of *ptac3-1* (Salk_110045). Homozygous plants were identified by PCR analysis with specific primer sets (fw1 and rv1, T and rv1). Homozygous plants grown on 1/2 MS containing 1% (wt/vol) sucrose under normal light condition, has albino cotyledons and true leaves, and displayed lethal phenotype at vegetative stage (Fig. 2*A*). However, heterozygous plants did not show any visible phenotype. (*B*) RNA and total proteins were prepared from WT (a) and *ptac3-1* (b) and analyzed by RNA gel blotting and Western blotting with ptac3-specific probe and pTAC3 antibody, respectively. EtBr and CBB staining are shown below.

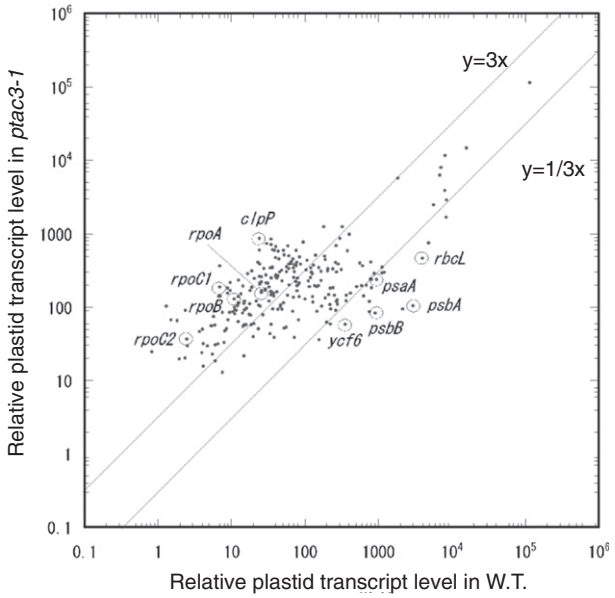

Fig. S3. Global plastid gene expression in ptac3-1 and wild type. Total RNA were prepared from 8-d-old wild type and *ptac3-1* and labeled with ^{33}P. Each labeled RNA was hybridized to plastid macroarray. Scatter plots of relative transcript levels in ptac3-1 and WT. Lines represent three times decreased and increased. Decreased (*psbA*, *psaA*, *ycf6*, *rbcL*, and *psbB*) and increased (*rpoA*, *rpoB*, *rpoC1*, *rpoC2*, and *clpP*) signals in *ptac3-1* are partially shown.

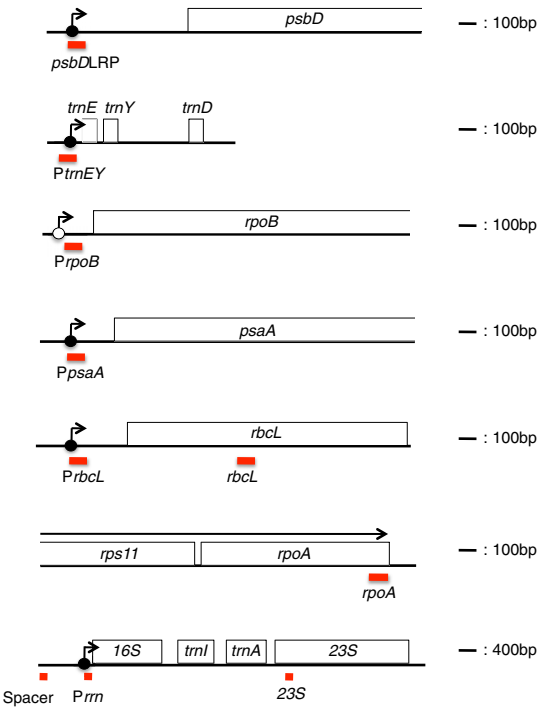

Fig. S4. Schematic representation of gene maps with primer positions for cpChIP analysis. Amplified positions for cpChIP assay corresponding to *psbD*LRP, *trnEY* promoter (P*trnEY*), *rpoB* promoter (P*rpoB*), *psaA* promoter (P*psaA*), *rbcL* promoter (P*rbcL*), *rbcL* coding region (*rbcL*), *rpoA* coding region (*rpoA*), noncoding region (Spacer), *rrn* promoter (P*rrn*), and 23S ribosomal RNA region (*23S*) are shown as red bar below each gene map. Arrowhead represents the direction of transcription. Black and white circles indicate PEP and NEP promoter, respectively. Black bars indicate 100 bp or 400 bp (for *rrn* operon).

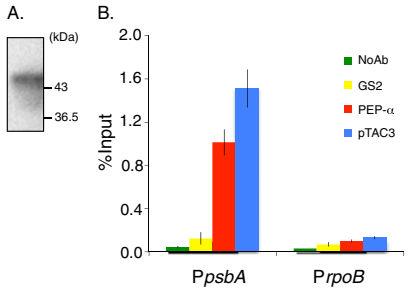

Fig. S5. CpChIP analysis with antibody against a non–DNA-binding protein, glutamine synthetase 2 (GS2). (*A*) Immunodetection of GS2 in wheat chloroplast with anti-GS2 antibody. Glutamine synthetase 2 is a chloroplast localized protein (45 kDa). Twenty micrograms of wheat chloroplast protein was loaded and then analyzed by immunoblotting with anti-GS2 antibody. (*B*) Association levels of GS2 with *psbA* promoter and *rpoB* promoter. Wheat chloroplasts were prepared from seedlings grown for 5 d in the light and subjected to cpChIP assay with antibody against GS2, PEP α-subunit (PEP-α), and pTAC3 or without (NoAb). Data are presented as in Fig. 3*A*.

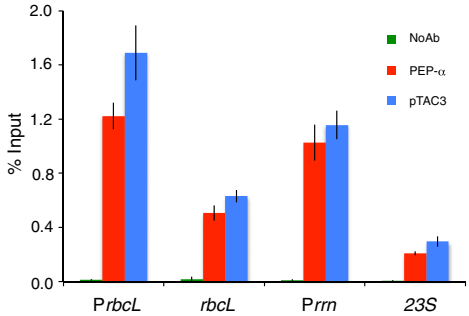

Fig. S6. Association level of a PEP-α subunit and pTAC3 with *rbcL* and *rrn* operon. ChIP was performed to determine the association level of PEP α-subunit and pTAC3 with promoter region and coding region of *rbcL* (PrbcL and *rbcL*) and the *rrn* operon (P*rrn* and *23S*), using PEP α-subunit (PEP-α), pTAC3 antibody or no antibody (NoAb). The immunoprecipitated DNA was analyzed by quantitative PCR and quantified via standard curves based on a dilution series of input samples. Each value is shown as percentage of input sample and is the mean value with SD of three independent experiments.

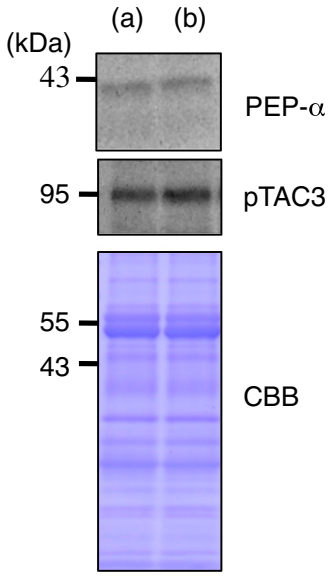

Fig. S7. Protein levels of PEP α-subunits and pTAC3 in dark and light condition. Chloroplasts were prepared from wheat seedlings grown for 5 d in the light and then dark adapted for 24 h (*A*) or seedlings reilluminated for 6 h after the 24-h dark adaptation (*B*). Same amount of each protein was separated by SDS/PAGE (7.5% gel) and analyzed by immunoblotting with anti-PEP α-subunits (PEP-α) or pTAC3 antibody. CBB staining is shown below.

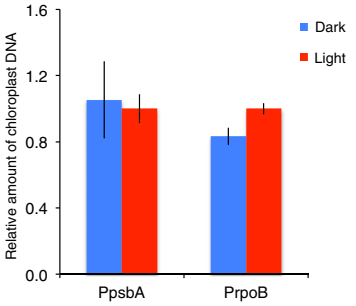

Fig. S8. Relative amounts of chloroplast DNA in dark and light input samples. Amounts of chloroplast DNA in dark and light input samples were analyzed by quantitative PCR with specific primer sets to amplify PpsbA and PrpoB loci. Each value is shown as a ratio to that in light input sample at each locus and is represented as a mean value with SD of three independent experiments.

Table S1. Primer list for cpChIP assay

	Forward (5′–3′)	Reverse (5′–3′)
P*psbA*	ccccacaggcttgtactttc	ggggtccttgcaatttgaata
C	aggaaccatgcatagcactg	aggcagagcataacatccttatg
T	tggaacttcaacagcagctaa	atcaaccgtgctaaccttgg
a	aataccgaacccaaccattg	cgactttcatgcgctagaact
b	agaaaaaccagggtacattatcc	cgtaaacaatcttcttgcttacc
P*rbcL*	attaggaattaatttgggttgcg	ttgttgttatggtaagttaacacgg
rbcL	ggacttgattttaccaaagatgatg	tcggcctgtgatttataaatagc
P*rrn*	gtgggattgacgtgataggg	cggaattgtctttccttcca
23S	aagtccagtagccgaaagca	caaggtggtccttgctgatt
P*psaA*	gcaattatatattgaagtcaatccg	caagataaacaatgtccgttaggc
*psbD*LRP	caccataaaattggaaagaagca	cgcttatacaatttcatctacatcg
P*trnEY*	tgctttggatctatttatacttttcg	ccatatacaaccgctcgtga
P*rpoB*	atgaaatacgtatgtggagttccc	gctcactcttcatcaatccctat
rpoA	tgggagatgcttctctagagtgt	ttggacctttgagtaagactga
Spacer	tagttaccgatcccggctct	tcctcggtccacagagaaaaa