

Eukaryotic-type plastid nucleoid protein pTAC3 is essential for transcription by the bacterial-type plastid RNA polymerase

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Plastid transcription is mediated by two distinct types of RNA polymerases (RNAPs), bacterial-type RNAP (PEP) and phage-type RNAP (NEP). Recent genomic and proteomic studies revealed that higher plants have lost most prokaryotic transcription regulators and have acquired eukaryotic-type proteins during plant evolution. However, *in vivo* dynamics of chloroplast RNA polymerases and eukaryotic-type plastid nucleoid proteins have not been directly characterized experimentally. Here, we examine the association of the α -subunit of PEP and eukaryotic-type protein, plastid transcriptionally active chromosome 3 (pTAC3) with transcribed regions *in vivo* by using chloroplast chromatin immunoprecipitation (cpChIP) assays. PEP α -subunit preferentially associates with PEP promoters of photosynthesis and rRNA genes, but not with NEP promoter regions, suggesting selective and accurate recognition of PEP promoters by PEP. The cpChIP assays further demonstrate that the peak of PEP association occurs at the promoter-proximal region and declines gradually along the transcribed region. pTAC3 is a putative DNA-binding protein that is localized to chloroplast nucleoids and is essential for PEP-dependent transcription. Density gradient and immunoprecipitation analyses of PEP revealed that pTAC3 is associated with the PEP complex. Interestingly, pTAC3 associates with the PEP complex not only during transcription initiation, but also during elongation and termination. These results suggest that pTAC3 is an essential component of the chloroplast PEP complex. In addition, we demonstrate that light-dependent chloroplast transcription is mediated by light-induced association of the PEP–pTAC3 complex with promoters. This study illustrates unique dynamics of PEP and its associated protein pTAC3 during light-dependent transcription in chloroplasts.

Plastids are DNA-containing organelles unique to plant cells and are thought to have originated from an ancestral cyanobacterial endosymbiont. Whereas cyanobacteria contain over 3,000 genes, the plastid genome in higher plants consists of small, circular, double-stranded DNA (120–150 kbp) encoding ~120 genes for photosynthesis and gene expression machineries (1, 2), indicating massive transfer of chloroplast genes to nuclear genome during evolution (3). Vascular plants have evolved a complex transcriptional network that is mediated by two types of RNA polymerases (RNAPs): cyanobacterium-derived plastid-encoded plastid RNA polymerase (PEP) and nuclear-encoded phage-type RNA polymerase (NEP). PEP is composed of four catalytic subunits and a promoter recognition subunit, σ -factor (4). Genes for PEP core subunits, α , β , β' , and β'' were retained in plastid genomes as *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* during plant evolution, but genes for σ -factors involved in transcription initiation, have been transferred to the nuclear genome (5), which allows the nucleus to control PEP transcription initiation in response to developmental and environmental cues (recently reviewed in ref. 6). PEP is responsible for transcription of photosynthesis genes in chloroplast in response to light. On the other hand, housekeeping genes encoding PEP core subunits and ribosomal proteins are transcribed by the phage-type NEP (7, 8).

Thus, two types of RNAP have distinct roles in chloroplast transcription in higher plants.

It has been proposed that light-dependent initiation of transcription by PEP is controlled by light-induced expression of nuclear-encoded plastid σ -factors (6). Contrarily, several evidences suggest that phosphorylated PEP may tightly bind to the promoter region to arrest transcription in the dark (9–11). Dark-induced phosphorylation of PEP and/or σ -factors by redox-regulated plastid transcription kinase (PTK) may be a key step in light-dependent plastid gene transcription. Thus, molecular mechanism of light-dependent transcription in chloroplasts still remains controversial.

Plastid DNAs are densely packed into protein–DNA complexes called “plastid nucleoids,” as well as bacterial nucleoids. However, higher plants and moss have lost prokaryotic major nucleoid proteins including Hu during evolution (12, 13), whereas several eukaryotic-type proteins such as PEND (14), MFP1 (15), SiR (16), and CND41 (17) have been identified as major components of nucleoids in higher plants. Furthermore, recent proteome analysis identified eukaryotic-type chloroplast nucleoid proteins including putative DNA/RNA-binding proteins as components of a chloroplast-derived DNA–protein complex termed pTAC (plastid transcriptionally active chromosome) (18) and a blue native (BN)-PAGE separated basic PEP complex (19). These findings suggest that chloroplasts have lost most of prokaryotic nucleoid proteins involved in DNA packaging, replication, transcription, and translation and acquired eukaryotic-type chloroplast nucleoid proteins during evolution (20). Because vascular plants lack prokaryotic transcription regulators such as DNA-binding proteins and transcription elongation factors except for σ -factors, chloroplast transcription might be mediated by a unique hybrid system of prokaryotic-type RNA polymerase and eukaryotic-type accessory factors. However, the role of the nonprokaryotic nucleoid proteins in plastid transcription remains largely unknown.

Earlier studies on pTAC proteins and PEP-associated proteins (PAPs) have focused mainly on *in planta* analyses using mutant and transgenic plants [e.g., pTAC2, pTAC6, and pTAC12 (18); PAP3/pTAC10, PAP6/FLN1, and PAP7/pTAC14 (19); ET1 (21); Trx-z (22)]. However, mutations in plastid transcription-related genes occasionally give rise to drastic pleiotropic phenotypes such as albino or pale green plants due to reduced accumulation of plastid rRNA and tRNAs, which are mainly transcribed by PEP, and impaired translation of chloroplast-encoded essential proteins. Thus, it is sometimes difficult to characterize the

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molecular function of pTAC proteins in chloroplast transcription using null mutants. Chromatin immunoprecipitation (ChIP) assays have been widely used to study protein–DNA interactions and map the localization of proteins to specific DNA sequences in the cell nucleus and bacterial nucleoids. However, very little work has been done using ChIP to analyze the association of PEP and its accessory proteins with transcribed regions in chloroplast genomes. Here, we examine the association of the α -subunit of PEP and pTAC3 with transcribed regions in vivo by using chloroplast chromatin immunoprecipitation (cpChIP) assays. A pTAC3 has been identified in various PEP-containing chloroplast fractions (18, 19, 23, 24). The pTAC3 contains a SAP DNA-binding domain (Pfam 02037), which is found in matrix attachment region binding protein (25) (Fig. 1A). In this study, cpChIP assays demonstrate that pTAC3 associates with the PEP complex not only during transcription initiation, but also during elongation and termination. In addition, we demonstrate light-

dependent association of the PEP–pTAC3 complex with PEP promoters. This study provides unique insight into the role of the eukaryotic-type PEP accessory protein pTAC3 in PEP-dependent transcription in chloroplasts.

Results

pTAC3 Is a Land Plant-Specific Protein That Is Localized to Plastid Nucleoids. To investigate whether pTAC3 is an evolutionally acquired component of plastid transcription, we examined its evolutionary history. BLAST and position-specific iterative (PSI)-BLAST searches revealed that pTAC3 is conserved among land plants including moss and higher plants, but not in cyanobacteria and algae (Fig. 1B and Fig. S1), suggesting a functional conservation in embryophytes. TargetP and PSORT, subcellular localization prediction programs, suggest that AtpTAC3 (At3g04260) contains a putative transit peptide of 29 amino acid residues at the N terminus. To examine the subcellular localization of pTAC3, we expressed GFP-tagged pTAC3 in *Arabidopsis*. Full-length *AtpTAC3* cDNA was fused to the GFP coding region and transiently expressed under the control of the constitutive CaMV35S promoter in protoplasts prepared from *Arabidopsis* mesophyll cells (Fig. 1C). It has been reported that GFP-fused plastid DNA-binding proteins such as PEND localized to plastid nucleoids (26). As shown in Fig. 1D, GFP-tagged AtpTAC3 appeared in small dot-like structures that were observed throughout chloroplasts and colocalized with DAPI-stained nucleoid DNA, suggesting that pTAC3 is a chloroplast nucleoid-associated protein. Homozygous T-DNA insertion mutants of *AtpTAC3* [SALK110045 (*ptac3-1*)] displayed an albino phenotype (Fig. 2A

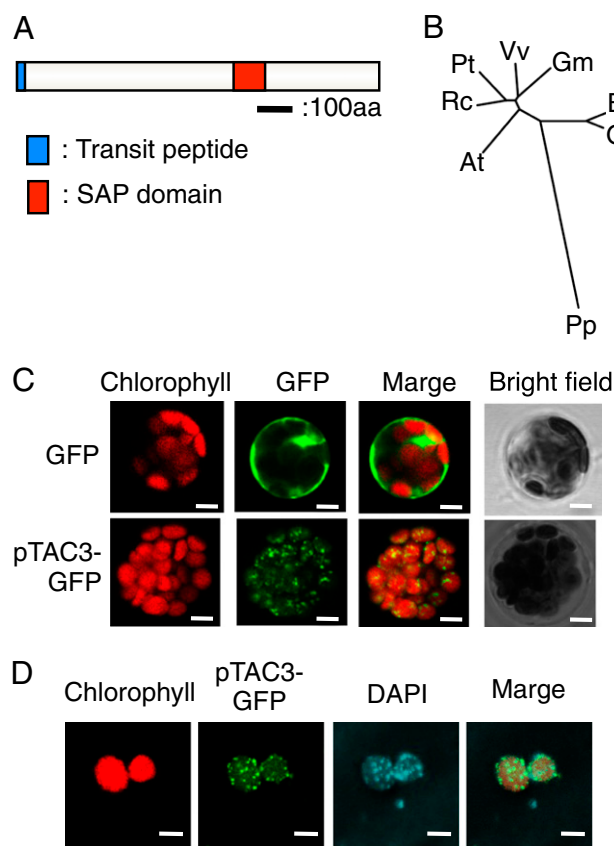


Fig. 1. pTAC3 is a chloroplast nucleoid protein. (A) Schematic representation of pTAC3 proteins. Black bar corresponds to 100 aa in length. Blue and red boxes represent the chloroplast transit peptide and SAP domain, respectively. (B) Phylogenetic tree of pTAC3 homolog proteins. Phylogenetic analysis was conducted using ClustalW based on amino acid sequences of pTAC3 proteins including At, *Arabidopsis thaliana*; Rc, *Ricinus communis*; Gm, *Glycine max*; Vv, *Vitis vinifera*; Pt, *Populus trichocarpa*; Os, *Oryza sativa*; Bd, *Brachypodium distachyon*; Pp, *Physcomitrella patens*. (C) Localization analysis of pTAC3–GFP in a protoplast transient expression system. Protoplasts prepared from wild-type *A. thaliana* leaves were transformed with expression plasmids harboring the GFP gene (control) or the full-length *AtpTAC3* fused to GFP at its N terminus under the control of the CaMV 35S promoter. GFP fluorescence and chlorophyll autofluorescence of transformed protoplasts were observed by confocal microscopy. (Scale bar, 5 μ m.) (D) DAPI staining of protoplasts expressing pTAC3–GFP. Protoplasts were disrupted by osmotic pressure, stained with DAPI, and observed by confocal microscopy. (Scale bar, 5 μ m.)

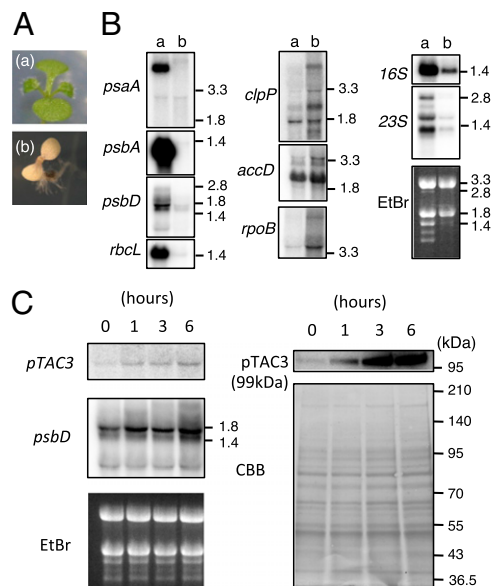


Fig. 2. Analysis of pTAC3-deficient mutants. (A) Phenotype of *ptac3-1*. WT (a) and *ptac3-1* (b) were grown on 1/2 MS medium containing 1% (wt/vol) sucrose for 8 d. (B) RNA gel blotting analysis of plastid gene expression in WT and *ptac3-1*. Five micrograms of RNA extracted from 8-d-old WT (a) or *ptac3-1* (b) was separated on 1% (wt/vol) agarose gels, and blots were hybridized with 32 P-labeled probes. Sizes determined by the position of 16S, 18S, mature 23S, and 25S ribosomal RNA, which correspond to 1.4, 1.8, 2.8, and 3.3 kbp in length, respectively. (C) RNA gel blotting (Left) and Western blotting analysis (Right) of pTAC3 expression during greening of etiolated seedlings. Wild-type *Arabidopsis* plants were grown in continuous darkness for 4 d, and then the etiolated plants were exposed to light for 1, 3, or 6 h. Total RNA and protein were extracted and analyzed by RNA gel blotting and Western blotting, respectively. EtBr and CBB staining image are shown below. *psbD* light-dependent expression is also shown.

and Fig. S2 A and B). To analyze the role of pTAC3 in plastid transcription, we examined global plastid gene expression patterns in *ptac3-1* by using a plastid DNA macroarray. Expression of PEP-dependent photosynthesis genes, which are actively transcribed in chloroplasts, is significantly reduced in 8-d-old *ptac3-1* mutant plants (Fig. S3). By contrast, knockout of pTAC3 resulted in increased accumulation of several low abundance gene transcripts, including NEP-dependent genes such as *accD* and PEP core subunit genes (*rpoA*, and the *rpoB-C1-C2* operon). RNA gel blot analysis confirmed the reduced accumulation of PEP-dependent photosynthesis and *rm* transcripts, and the up-regulation of NEP-dependent transcripts in *ptac3-1* (Fig. 2B). This is a typical plastid gene expression pattern of mutant plants with impaired PEP transcription. Furthermore, we analyzed the developmental expression of *AtpTAC3* during greening of etiolated seedlings. Wild-type *Arabidopsis* plants were grown in continuous darkness for 4 d and subsequently exposed to light for 1, 3, or 6 h. Both *AtpTAC3* mRNA and AtpTAC3 protein are induced by light within 1 h. (Fig. 2C). The transcript levels of *psbD*, which are a marker for light-dependent plastid transcription, are also increased by light. These data suggest that pTAC3 may play a crucial role in PEP-dependent transcription and be essential for light-induced chloroplast development.

cpChIP Analysis of pTAC3 and PEP Association with Wheat Chloroplast DNA. To understand the role of pTAC3 in PEP-dependent transcription in chloroplasts, we designed a modified cpChIP assay using wheat chloroplasts. First we evaluated the association of PEP α -subunit with specific regions of plastid DNA, including PEP promoters (*psbA*, *rbcL*, *psaA*, *rm16*, *psbDLRP*, and *trnEY*), a NEP promoter (*rpoB*), the coding region of a NEP-dependent gene (*rpoA*), and the noncoding region between *rps12* and *rm16* (Spacer) (Fig. S4 and Table S1). The immunoprecipitated DNA was analyzed by quantitative PCR and quantified using standard curves based on a dilution series of input samples. The data were analyzed as a percentage of the input sample (details in *Materials and Methods*). The cpChIP assay showed binding of PEP α -subunit to the promoter regions of PEP-dependent photosynthesis genes and the *rm* operon, but not to those of NEP-dependent genes or the spacer region in vivo (Fig. 3A). By contrast, the cpChIP assay using an antibody against the non-DNA-binding chloroplast protein, glutamine synthetase 2 (GS2) resulted in a lower cpChIP-QPCR signal at the *psbA* promoter comparable to that at the *rpoB* promoter (Fig. S5). These results indicate that the cpChIP analysis can be used to detect the specific association of DNA-binding protein with chloroplast DNA. Furthermore, cpChIP signal of PEP α -subunit characteristics vary markedly among PEP promoters, suggesting that the relative association of PEP with promoters may be dependent on promoter architecture. The highest signal was detected at the *psbA* promoter, which is known as the most active promoter in the chloroplast genome. We further examined the distribution of PEP α -subunit along the *psbA* gene including the promoter, coding, and termination regions (Fig. 3B). The peak association with PEP α -subunit was localized in the promoter region, and the level decreased slightly toward the termination region of *psbA*, whereas the signal in the *trnK-matK* region, which is located upstream of *psbA*, was significantly lower. Less intensive ChIP signals in the coding region compared with the promoter region were also observed in *rbcL* and the *rm* operon (Fig. S6), suggesting that PEP density decreases gradually along the transcription unit. This cpChIP assay provides the first direct evidence that PEP exclusively recognizes PEP promoters and initiates transcription in vivo, suggesting that PEP- σ complexes have high binding affinity to PEP promoters.

We also examined the association of pTAC3 with chloroplast DNA via cpChIP assays. As in the case of PEP α -subunit, pTAC3 preferentially binds to promoter regions of PEP-dependent

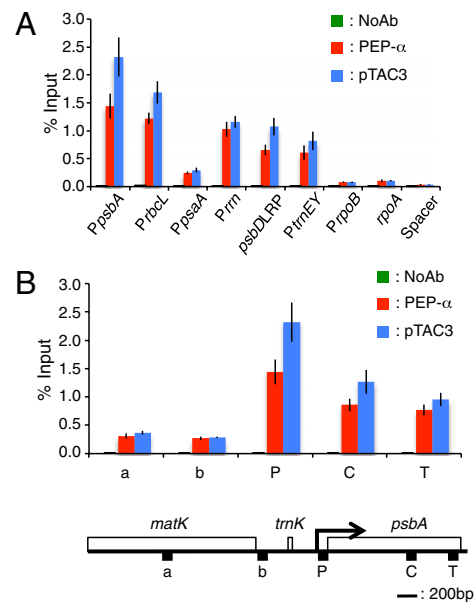


Fig. 3. Association of PEP α -subunit and pTAC3 with chloroplast DNA. (A) Association of PEP α -subunit and pTAC3 with PEP promoter regions (*PpsbA*, *PrbcL*, *PpsaA*, *Prm16*, *psbDLRP*, *PtrnEY*), a NEP promoter region (*PrpoB*), the coding region of the *rpoA* gene (*rpoA*), and a noncoding spacer region located between *rps12* and *rm16* (Spacer) was analyzed by ChIP assay. Chloroplasts were prepared from wheat seedlings grown for 5 d in the light and subjected to ChIP assays using antibodies against PEP α -subunit and pTAC3. NoAb, no antibody control. Enriched DNA was quantified by qPCR. The amount of immunoprecipitated DNA in each sample is presented as a percentage of the total input chromatin. Mean values and SDs of three independent experiments are shown. (B) Spatial association of α -subunit and pTAC3 along the *psbA* transcription unit. Data are shown as in A. Schematic gene map of the *matK-psbA* region is shown below. Horizontal black bar represents 200 bp. Arrow indicates the transcription start site of the *psbA* gene and the direction of transcription. DNA regions corresponding to the *psbA* promoter (P), coding region (C), terminator (T), and two units (a and b) in loci upstream of *psbA* are shown.

genes, but not of NEP-dependent genes, suggesting a role for pTAC3 in the PEP complex (Fig. 3A and Fig. S3). We further examined the local patterns of spatial association of pTAC3 with the *psbA* transcription unit (Fig. 3B). We found that pTAC3 binds not only to the promoter region but also the transcription elongation region during transcription, suggesting that pTAC3 as well as PEP is associated with chloroplast DNA along the *psbA* transcription unit. Thus, it is unlikely that pTAC3 binds to specific sequences in the *psbA* promoter; rather it may be an important component of the PEP complex in chloroplasts.

To examine further the interaction between pTAC3 and PEP, we isolated the PEP complex by glycerol density gradient centrifugation and probed for the presence of pTAC3 by Western blotting. The molecular weight of the PEP complex from wheat chloroplasts was estimated as around 700 kDa, as reported previously in mustard (27). pTAC3 was detected in two fractions, corresponding to a peak containing PEP α -subunit (fraction 8) and a lower molecular weight fraction (roughly estimated to 200–400 kDa; fractions 4 and 5) (Fig. 4A). These results suggest that pTAC3 in wheat chloroplasts is associated mainly with the PEP complex, although a portion of the pTAC3 may form another complex without PEP. Furthermore, immunoprecipitation assays using pTAC3 antibody with wheat chloroplast extracts demonstrated the presence of α -subunit in the pTAC3 immunoprecipitated complex (Fig. 4B). These results demonstrate that pTAC3 has a direct function in PEP transcription.

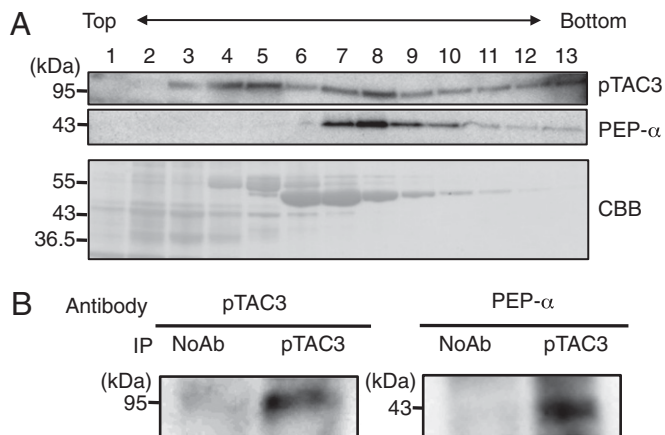


Fig. 4. Analysis of the pTAC3 protein complex. (A) Separation of the PEP complex by glycerol density gradient centrifugation. Total chloroplast proteins prepared from wheat grown under continuous light for 6 d were loaded onto a 10–30% (vol/vol) glycerol density gradient and separated by centrifugation. Thirteen fractions were collected from top to bottom and analyzed by immunoblotting with anti-PEP α -subunit or pTAC3 antibodies. Gel staining with CBB is also shown. (B) Immunoprecipitation analysis of the pTAC3 complex. Total wheat chloroplast extracts were subjected to immunoprecipitation with pTAC3 antibody or without (NoAb) and analyzed by immunoblotting with anti-PEP α -subunit and pTAC3 antibodies.

Light-Dependent Association of the PEP–pTAC3 Complex with Chloroplast DNA. PEP transcription activity is greatly stimulated by light in mature wheat chloroplasts (28). We therefore examined the light-dependent association of PEP with promoter regions of several plastid genes *in vivo*. Chloroplasts were prepared from wheat seedlings grown for 5 d in the light and then dark adapted for 24 h or seedlings reilluminated for 6 h after the 24-h dark adaptation. Immunoblot analysis revealed the constitutive accumulation of both PEP α -subunit and pTAC3 upon light illumination in wheat seedlings (Fig. S7), suggesting that the expression level of the PEP complex is not affected by light. On the other hand, the cpChIP assays showed that the relative amount of PEP α -subunit associated with the promoter region of photosynthesis genes including *psbA*, *psbD*, *psaA*, and *rbcL*, and ribosomal RNA *rml16*, was two- to fivefold higher in the illuminated chloroplasts than in the dark-adapted chloroplasts (Fig. 5A). Amounts of chloroplast DNA in input samples between both conditions were not significantly different (Fig. S8). Thus, the light-dependent cpChIP signals would be indicative of light-dependent association of PEP α -subunit and pTAC3 to chloroplast DNA. Moreover, we could not detect tight binding of PEP α -subunit to the promoter region of photosynthesis genes in the dark-adapted chloroplasts, suggesting a limited role for transcription arrest mediated by dark-induced phosphorylation of PEP subunits and σ -factors. In addition to promoter regions, we also detected light-dependent association of PEP α -subunit with coding and termination regions of *psbA* (Fig. 5B). These results demonstrate that recruitment of PEP to its target promoters is dependent on light. On the other hand, light-dependent association of PEP with NEP-dependent genes including *rpoA* and *rpoB* and the spacer region was not detected. Furthermore, ChIP analysis of pTAC3 showed that light accelerated the association of pTAC3 with not only the PEP promoter region but also the coding region in the *psbA* transcription unit (Fig. 5A and B) similar to the distribution pattern of PEP α -subunit, suggesting that pTAC3 associates with PEP-dependent transcribed regions as a component of a large PEP complex in a light-dependent manner.

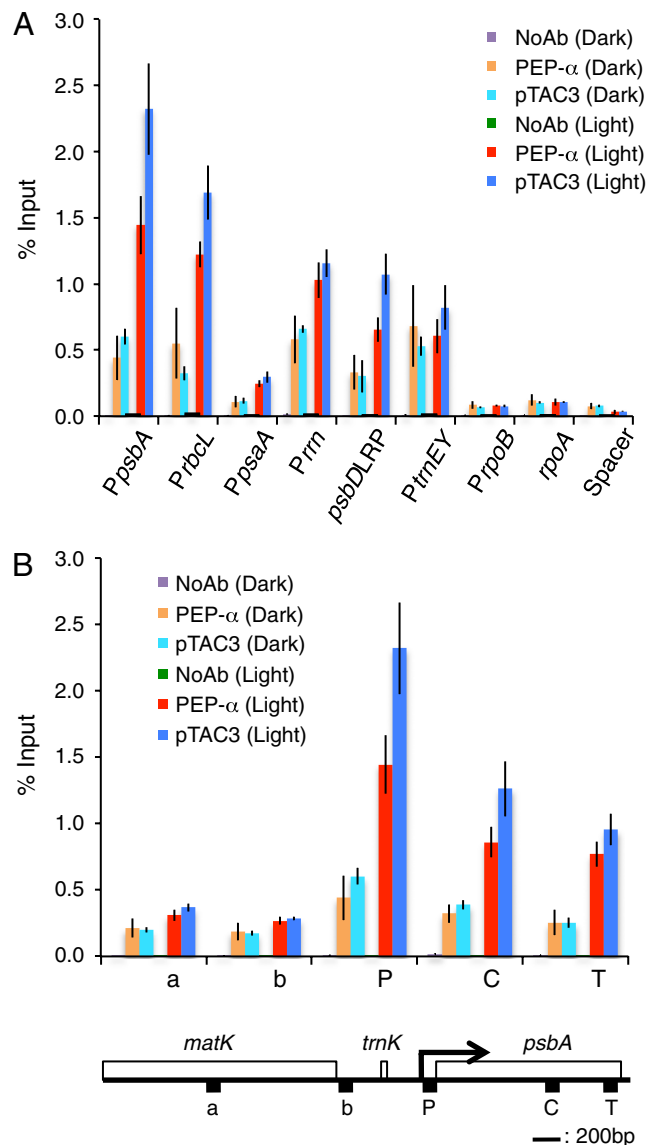


Fig. 5. Analysis of light-dependent association of PEP α -subunit and pTAC3 with chloroplast DNA. (A) Association of PEP α -subunit and pTAC3 with chloroplast DNA in response to light. Chloroplasts were prepared from wheat seedlings grown for 5 d in the light and then dark adapted for 24 h (dark) or seedlings reilluminated for 6 h after the 24-h dark adaptation (light). ChIP was performed to determine the association level of PEP α -subunit and pTAC3 with PEP promoter regions (*PpsbA*, *PrbcL*, *PpsaA*, *Prrn16*, *psbDLRP*, and *PtrnEY*), a NEP promoter (*PrpoB*), the coding region of the *rpoA* gene (*rpoA*), and a noncoding region located between *rps12* and *rml16* (Spacer), using anti-PEP α -subunit or pTAC3 antibodies 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. The amount of immunoprecipitated DNA in each sample is presented as a percentage of the total input chromatin. Mean values and SDs of three independent experiments are shown. (B) Association level of PEP α -subunit and pTAC3 with regions of the *psbA* transcription unit in response to light. Data are presented as in A. Schematic gene map of the *matK-psbA* region is shown as in Fig. 3B.

Discussion

This study reveals light-dependent associations of PEP and pTAC3 with chloroplast DNA *in vivo* using cpChIP assays. ChIP assays have been widely used to detect specific binding sites for transcription factors (29), distribution patterns of several

modified histones (30), and trafficking of RNAP and its associated proteins on genomic DNA (31). In chloroplasts, a few studies have shown the association of endogenous (Whirly1) and recombinant (LacI) transcription factors with chloroplast promoters *in vivo* by using ChIP assays (32, 33). However, *in vivo* dynamics of chloroplast RNA polymerases and/or their associated proteins have not been directly characterized experimentally. Unlike cyanobacteria, the chloroplasts of higher plants have two types of RNA polymerase, PEP and NEP. Transcriptome analyses of PEP- or NEP-deficient mutants and *in vitro* transcription analyses using isolated PEP and NEP have shown that PEP and NEP preferentially initiate transcription from bacterial-type and phage-type promoters, respectively. Here, we provide direct evidence that PEP exclusively associates *in vivo* with PEP promoters of photosynthesis genes and the rRNA operon, but not with NEP promoters by using cpChIP assay (Fig. 3), suggesting selective and accurate recognition of PEP promoters by PEP.

It has been shown that several PEP promoters including *psbA*, *psbD* LRP, *rbcL*, *psaA*, and *rrn* P1 are regulated by unique *cis*-elements, termed as the TATA-box, PGT-box, and AAG-box, CDF1-binding site, region U, and RUA, respectively, which are located upstream of or within the core promoter (28, 34–37) and recognized by promoter-specific transcription factors. However, sequence specific DNA-binding proteins have not been identified and characterized in chloroplasts of higher plants. Chloroplast protein pTAC3 contains a putative DNA-binding SAP domain. Here we show that pTAC3 is localized in chloroplast nucleoids and is essential for PEP-dependent transcription, suggesting that pTAC3 may be a chloroplast transcription factor that regulates PEP-dependent transcription. Thus, we further searched for pTAC3-binding regions on plastid DNA by using cpChIP assays. We found that pTAC3 binds to the transcribed regions of all PEP-dependent genes examined (*psbA*, *psaA*, *psbD*, *rbcL*, and *rrn* promoters), but not to a specific *cis*-element in a particular promoter, suggesting that pTAC3 is a PEP-associated general, rather than sequence-specific transcription factor. In contrast to PEP-dependent transcribed loci, pTAC3 associates weakly with NEP-dependent transcribed loci, suggesting that pTAC3 does not associate with the NEP transcription complex. However, chloroplasts exhibit very low NEP-dependent transcription activity. NEP proteins are thought to be present at very low levels in leaf chloroplasts, because they were not detected in the proteomic analysis of whole chloroplast proteins and pTAC fractions. Thus, there remains a possibility that the cpChIP assay could not detect the association of pTAC3 with NEP-dependent transcribed loci, due to the low levels of accumulation of NEP in chloroplasts.

In bacteria, it is proposed that RNAP accessory proteins modulate the promoter accessibility to RNAP by altering the 3D structure of the nucleoid. The nucleoid proteins factor for inversion stimulation (FIS) and histone-like nucleoid structuring protein (H-NS) are associated with RNAP and bind chromosomal DNA at many regions (38). Furthermore, FIS and H-NS were copurified with RNAP subunits and ribosomes in *Escherichia coli* (39). Similarly, cpChIP assays demonstrated that pTAC3 associates with PEP-dependent transcribed regions of photosynthesis and rRNA genes together with PEP α -subunit. Density gradient and immunoprecipitation analyses of PEP demonstrate that pTAC3 is an essential component of PEP in chloroplasts. Furthermore, pTAC3 is crucial for PEP activity and its expression increases dramatically during chloroplast development, as PEP activity increases. Taken together, these results suggest that pTAC3 is an essential component of the PEP complex and critically involved in chloroplast differentiation from immature plastids. Considering the eukaryotic origin of the SAP domain as a DNA-binding motif, our findings suggest that pTAC3 was

acquired from host cells during plant evolution to enhance and/or maintain transcription of photosynthesis genes in higher plants.

Our ChIP analysis of PEP α -subunit shows that PEP preferentially associates with the promoter region rather than the coding region (Fig. 3B and Fig. S3), suggesting that the PEP complex remains at the promoter-proximal region. *E. coli* ChIP analysis using specific antibodies for RNAP core subunits revealed that there is also preferential association of the RNAP core with the promoter-proximal region of transcribed genes and decreased RNAP-binding density downstream from the promoter (31). The accumulation of RNAP at the promoter-proximal region in *E. coli* is controlled mainly by interactions between the elongating complex of RNAP and elongation regulators, such as NusA, NusG, and Rho. The *Arabidopsis* genome encodes only one putative bacterial-type elongation factor, pTAC13 (At3g09210) containing a KOW domain, which is characteristic for members of the NusG protein family (40). Furthermore, maize ET1, which resembles the eukaryotic elongation factor TFIIS, was identified as a component of the chloroplast transcriptionally active fraction, and plants deficient in ET1 showed an aberrant chloroplast development phenotype and decreased PEP-dependent transcription (21), suggesting that bacterial-type pTAC13 and eukaryotic-type ET1 may be involved in PEP elongation. In this study, we demonstrate that pTAC3 is associated with PEP through transcription initiation and elongation steps, suggesting a possible role for pTAC3 in the regulation of PEP transcription elongation in chloroplasts.

Light-dependent activation of PEP transcription may be controlled by PTK and plastid redox signaling. It has been proposed that phosphorylated PEP and/or σ -like factors tightly bind to promoters to arrest transcription under dark conditions (11). If PEP is trapped at promoter regions in the dark, ChIP signals at PEP promoters would not decrease in dark-adapted leaves. The present data, however, showed that ChIP signals at both promoters and coding regions of PEP-dependent photosynthesis and rRNA genes were reduced in dark-adapted wheat seedlings, suggesting that PEP dissociates from chloroplast genomic DNA in the dark. Thus, it is likely that light regulates the association of the PEP–pTAC3 complex with the promoter region possibly through the light-dependent expression of σ -factors, rather than via regulation of σ -factor phosphorylation by PTK.

In conclusion, here we present a characterization of *in vivo* dynamics of chloroplast RNA polymerase PEP and eukaryotic-type nucleoid protein pTAC3 with chloroplast DNA by using cpChIP analysis. We show that PEP α -subunit preferentially associates with the promoter-proximal regions of PEP-dependent photosynthesis and rRNA genes in a light-dependent manner, but not to NEP promoters, suggesting that light-dependent chloroplast transcription is mediated by accurate recognition of PEP promoters by PEP in response to light. Furthermore, we show that eukaryotic-type nucleoid protein pTAC3 is essential for PEP activity and chloroplast development and associates with the PEP complex not only during transcription initiation, but also during elongation and termination. As pTAC3 has been acquired early during land plant evolution, understanding the molecular function of pTAC3 should not only provide insights into the mechanisms enabling developmental regulation of plastid transcription but also offer a unique way of understanding plant evolution.

Materials and Methods

Plant Material. The *Arabidopsis ptac3-1* (SALK_110045) mutant (Columbia ecotype) was identified from the T-DNA insertion mutant line collection generated at the Salk Institute. For expression analysis, wild-type (Columbia) and *ptac3-1* were grown on half-strength MS medium containing 1% (wt/vol) sucrose under 16-h light and 8-h dark conditions (normal light; 50–60 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Wheat (*Triticum aestivum*) seeds were grown on vermiculite at 25 °C under continuous white light (50 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) for 5–6 d. Details regarding the identification of the mutant allele of *pTAC3* are included in *SI Materials and Methods*.

Chloroplast ChIP Assay. Detailed procedures for cpChIP assays are included in *SI Materials and Methods*.

Plastid Gene Expression Analysis. Details for RNA extraction, RNA gel blotting analysis, and construction and analysis of plastid DNA tiling macroarrays are included in *SI Materials and Methods*.

Localization Analysis by Transient Expression in Protoplasts. Protoplast transient expression assays were performed as described previously (41). Details for construction of pTAC3-GFP expression plasmids are included in *SI Materials and Methods*.

Protein Analysis. Details regarding antibody materials, protein extraction from isolated chloroplasts, protein complex separation via glycerol density gradient centrifugation, immunoprecipitation, and immunoblotting analysis are included in *SI Materials and Methods*.

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