CpLEPA Is Critical for Chloroplast Protein Synthesis Under Suboptimal Conditions in Arabidopsis thaliana

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Abstract

LEPA is one of the most conserved translation factors and is found from bacteria to higher plants. However, the physiological function of the chloroplast LEPA homolog in higher plants remains unknown. Herein, we demonstrate the physiological role of cpLEPA in enabling efficient photosynthesis in higher plants. The cplepa-1 mutant displays slightly high chlorophyll fluorescence and pale green phenotypes under normal growth conditions. The growth of the cplepa-1 mutant is reduced when grown on soil, and greater reduction is observed under intense light illumination. Photosynthetic activity is impaired in the cplepa-1 mutants, which is reflected in the decreased steady-state levels of chloroplast proteins. In vivo protein labeling experiments explained the decrease in the steady-state levels of chloroplast proteins. An abnormal association of the chloroplast-encoded mRNAs with ribosomes suggests that the protein synthesis deficiencies in cplepa-1 are due to defects in translation initiation in the chloroplasts. The cpLEPA protein appears to be an essential translation factor that promotes the efficiency of chloroplast protein synthesis.

Introduction

LEPA is one of the most conserved proteins, and it has the unexpected ability to back-translocate tRNAs on the ribosome [1]. LEPA homologs are highly conserved in terms of both their structure and their amino acid sequence, and they are found in bacteria, mitochondria and chloroplasts, but not in archaea or in the cytoplasm of eukaryotes [1]. Based on the domain definition of EF-G, LEPA can be divided into five domains, four out of the five EF-G domains—I, II, III, and V—are present in LEPA. Domain IV and the G’ subdomain of domain I of EF-G are absent. LEPA has a special C-terminal domain called CTD with an unusual fold which might interact with tRNA or 23S rRNA [2].

Although the overall structure of LEPA has been described in great detail, the physiological functions involved in translation have not yet been resolved. In E. coli, LEPA is located upstream of the LEP gene, which encodes nonspecific signal peptidase I [3]. Deletion of LEPA does not cause any apparent phenotype under optimal growth conditions [4,5]. These observations are difficult to reconcile with the ubiquity of LEPA and its essential conservation. Other results have demonstrated that, although E. coli LEPA-defective cells grown in rich medium have no phenotype [4], under several stress conditions, including high salt, low pH, and low temperature, the LEPA mutant is overgrown by wild-type bacterial cells [6]. In bacteria, LEPA strains have been shown to be hypersensitive to potassium tellurite and penicillin [7] and to enhance the production of the calcium-dependent antibiotic in Streptomyces bacteria [8]. Recent studies suggested that LEPA may react with both the PRE and POST ribosome complexes, leading to the formation of an intermediate complex that effectively sequesters a catalytically active ribosome, resulting in a transient inhibition of elongation that provides a mechanism for the optimization of functional protein synthesis [9,10].

The physiological function of the chloroplast homologs of LEPA (cpLEPA) in vivo has not been characterized. In this study, we report the identification of an Arabidopsis ΔLEPA mutant, which was termed cplepa-1. A slightly high chlorophyll fluorescence and pale green phenotype are detected in the cplepa-1 mutant when grown under normal growth conditions. Physiological and biochemical analyses of the mutant revealed that cpLEPA has an important function in chloroplast biogenesis and plays an essential role in chloroplast translation.

Results

Chloroplast LEPA in Arabidopsis is a Highly Conserved Homolog of EF-G

Database searches and protein sequence alignments revealed that cpLEPA shares significant sequence identity with its homologs, from bacteria to eukaryotes (64%–87%) (Figure 1). CpLEPA encodes a 601-amino acid protein with a calculated molecular mass of 75 kD. This protein was predicted to be localized to the chloroplast, and the N-terminal 51 amino acids were predicted to be a chloroplast transit peptide by the programs TargetP 1.1 and ChloroP 1.1 (Figure 1). Analysis by the TMHMM program suggests that cpLEPA does not contain a transmembrane domain (data not shown). Four out of the five CpLEPA domains share strong similarity to the counterpart of EF-G, except for domain IV, whereas the CTD is unique to cpLEPA (Figure 1).
Figure 1. CpLEPA Protein Sequence Alignment. The amino acid sequence of CpLEPA was compared with the sequences of homologous proteins from mitochondria in Arabidopsis, Oryza sativa, Glycine max, Physcomitrella patens, Hordeum vulgare, Micromonas pusilla, Synechococcus, Microcystis aeruginosa, and Bacillus cereus. The black boxes indicate strictly conserved amino acids, and the gray boxes indicate closely related residues. The predicted chloroplast transmembrane peptides are underlined in green, The LEPA domains are underlined in red, and the LEPA-II domain is underlined in blue. LEPA-C is underlined in purple, and the CTD is underlined in yellow.

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CpLEPA is Associated with the Thylakoid Membrane

To investigate the localization of cpLEPA, intact chloroplasts were isolated and fractionated, and the proteins were subjected to immunoblot analysis with a specific cpLEPA antibody. Under normal growth conditions (120 μmol m⁻² s⁻¹), most of the CpLEPA protein was detected in the thylakoid fractions (Figure 2A), and the ratio of cpLEPA in the stroma to cpLEPA in the thylakoid membrane was approximately 0.25. These results indicate that CpLEPA is a membrane-associated protein. To further investigate the degree of membrane association of cpLEPA, we treated the thylakoid membrane with salts and chaotropic agents. Washing the membrane with 0.25 M NaCl did not release the cpLEPA from the membrane, but cpLEPA was barely detectable after washing the membrane with 0.2 M Na₂CO₃, 1 M CaCl₂, or 6 M urea. As a control, the barely detectable after washing the membrane with such treatments, RBcL, which is located in the stroma and thylakoid membrane, yielded results similar to those of cpLEPA (Figure 2B).

CpLEPA is widely expressed in most Arabidopsis green tissues, including the seedlings, leaves, stems, siliques, flowers and cauli tissue (not in the roots), but the expression levels of cpLEPA in seedlings and cauli tissue are reduced compared with those in leaves, as revealed by RT-PCR analysis (Figure 2C). The expression of cpLEPA appeared to be independent of the age and developmental stage of the Arabidopsis leaves (Figure 2C). To examine the effects of light intensity on the expression of cpLEPA in Arabidopsis, the level of cpLEPA transcripts in leaves grown under normal light was compared with the level in plants grown under high light and low light. Relative to normal growth conditions, the cpLEPA transcript level was decreased under low light and increased under high light conditions (Figure 2C).

Knock-out of CpLEPA Leads to Growth Retardation and Impaired Chloroplast Development

To examine the function of cpLEPA in plants, we obtained two cplepa mutants from ABRC. The mutants contain T-DNA insertions within the fifth intron and the eleventh exon of the cpLEPA gene and are termed cplepa-1 and cplepa-2, respectively (Figure 3A). The T-DNA insertions were confirmed by PCR and subsequent sequencing of the amplified products. RT-PCR analysis revealed that expression of the cpLEPA gene was undetectable in the cplepa-1 and cplepa-2 mutants (Figure 3B). Immunoblot analysis showed that the cpLEPA protein was undetectable in the cplepa mutants, and the protein levels of cpLEPA in the complemented mutant plants were comparable to those of wild-type plants (Figure 2A).

The cplepa-1 and cplepa-2 mutants showed no growth differences compared with wild-type plants when grown on solid MS medium supplied with 2% sucrose at 120 μmol m⁻² s⁻¹ light illumination. When the sucrose was decreased to 1% or without sucrose, the growth of cplepa-1 was greatly retarded (Figure 3C). When grown on soil, the cplepa-1 and cplepa-2 mutant plants displayed slightly high chlorophyll fluorescence and a pale green phenotype (Figure 3D). Chlorophyll fluorescence analysis of dark-adapted leaves of wild-type and mutant plants showed that the Fv/Fm ratio was slightly lower (0.76 ± 0.01) in the cplepa-1 plants than in the wild-type plants (0.81 ± 0.01). As shown in Figure 3E, the leaf area of the mutant was approximately 70% that of the wild-type plants 28 d after germination. The chlorophyll content in cplepa-1 was reduced to approximately 88% of the wild-type level, and chlorophyll a/b was decreased to approximately 2.6, compared with 3.0 in the wild-type (Table 1).

To examine the effects of cpLEPA deletion on the chloroplast ultrastructure, we analyzed electron micrographs of ultrathin sections from 3-week-old leaves of wild-type and mutant plants. In total, 100 chloroplasts were examined, and the micrographs showed that the wild-type chloroplasts have well-structured thylakoid membrane systems, whereas the cplepa-1 chloroplasts are smaller (7.0 ± 0.6 and 6.3 ± 0.3 μm, respectively). In addition, cplepa-1 has fewer discs per grana stack (12 ± 1 and 7 ± 2, respectively) and exhibits disrupted thylakoid membrane organization, which further suggests that the cpLEPA mutation affects chloroplast development (Figure S1).

Accumulation and Synthesis of Chloroplast Proteins in cplepa-1

The levels of chloroplast proteins were examined in the cplepa-1 mutant using specific antibodies. The levels of PEP-dependent plastid-encoded chloroplast proteins were reduced. Subunits of PSI (PsA/B, PsAN) and PSII (D1, D2 and CP43), Cytf of the cytochrome b6f complex and the β-subunit of the plastid ATP synthase accumulated to approximately 60–70% of their wild-type levels in cplepa-1. In contrast, the accumulation of nuclear-encoded PsbO and LHChII was not affected in the cplepa-1 mutant (Figure 4A).

To investigate the possibility of diminished accumulation of chloroplast proteins, we first studied the synthesis of thylakoid proteins by in vivo pulse-chase labeling experiments. For these experiments, the leaf proteins were pulse labeled with [35S]-Met in the presence of cycloheximide, which blocks the synthesis of nuclear-encoded proteins. As shown in Figure 4B, the rates of synthesis of the PSI reaction center PsA/B; the PSII reaction center D1, D2, CP47 and CP43; and the α- and β-subunits of the plastid ATP synthase (CP1-2) were reduced to 60–70% of wild-type levels (Figure 4B).

Chloroplast mRNAs are Associated with Abnormally Few Ribosomes in cplepa-1

The chloroplast protein synthesis deficiency in the cplepa-1 mutant prompted us to examine the association of chloroplast mRNA with ribosomes. Polysome analysis provides an estimate of the efficiency of translation initiation and elongation [10]. Polysome association was conducted using three-week-old plants grown on soil and MS solid medium. Total leaf lysate was fractionated on a sucrose gradient under conditions that preserve polysome integrity, and mRNAs purified from the gradient fractions were identified by hybridization with specific probes. The PEP-dependent plastid-encoded atpB, psbA, psbB, and psaA/B transcripts showed a small shift toward the top of the gradient in the cplepa-1 mutant grown on soil (Figure 5). However, the distribution of mutant and wild-type plastid 23S rRNA, ndhC, petA, and psaB transcripts were unchanged in the sucrose gradients (Figures 5 and S2). In addition, the distribution of 23S rRNA along the sucrose gradients showed a different sensitivity to EDTA compared to that of rbcL mRNA (Figure S2). When grown on MS, however, no significant differences in sedimentation between wild-type and mutant plants were detected for the atpB, psbA, psbB, psbB, or psaA/B mRNAs (Figure S3). These results indicated that, in the soil-grown cplepa-1 mutant, chloroplast protein translation was impaired.

PEP-dependent Chloroplast-encoded mRNA Transcripts are Dramatically Reduced in cplepa-1

The levels of the plastid-encoded transcripts were also investigated by RNA gel blot hybridization using the same
Figure 2. Immunolocalization and Expression of **cpLEPA**. A: Immunolocalization analysis of cpLEPA. The chloroplast, thylakoid, stroma and envelope fractions were subjected to immunoblot analysis with specific antisera against cpLEPA. Equal amounts of protein (20 μg) were loaded in each lane. The lanes marked *cplepa-1*, *cplepa-2* and *cplepa-1/35S::cpLEPA* were loaded with equal amounts of total protein (20 μg). B: Salt washing of the membranes. The thylakoid membranes were incubated with 250 mM NaCl, 200 mM Na2CO3, 1 M CaCl2 and 6 M urea for 30 min at 4°C. Then, the thylakoid proteins were separated by SDS-PAGE and immunoblotted with anti-LEPA, anti-RbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) and anti-CP47 antibodies. RbcL and CP47 were used as markers. Thylakoid membrane preparations that had not been subjected to treatment were used as controls. C: Expression patterns of cpLEPA. Upper panel: cpLEPA expression levels in different organs of Arabidopsis, as
material as described in the polysome association experiments. Our results showed that the levels of mRNAs encoding the PsaA subunit of PSI (psaA-psaB-rps14) were reduced to 20% of wild-type levels in the mutant (Figure 6). Except for 23s rRNA, an approximately two fold decrease was also observed in the levels of transcripts encoding the following photosynthetic proteins: D1 (psbA), CP47 (psbB-psbH-psbT-petB-petD), D2 (psbD-psbC), atpB (CF1 β), and RBcL (rbcL) (Figure 6). The levels of chloroplast transcripts examined were not affected in the mutant plants when grown on MS (Figure S4).

Increased Sensitivity of the cplepa Mutants to High Light

When wild-type and cplepa-1 mutant plants that were initially grown at 120 μmol m⁻² s⁻¹ were transferred to low-light and high-light growth conditions for another two weeks, the growth of the mutants was greatly inhibited under high light. The mutants did not differ from the wild-type plants under low light (Figure 7A). To further determine whether the cplepa-1 mutant is sensitive to high light, Fv/Fm was measured in the wild-type and cplepa-1 plants under high-light illumination of 1,000 μmol m⁻² s⁻¹. In the absence of lincomycin, within 2 h of illumination at a light intensity of 1,000 μmol m⁻² s⁻¹, Fv/Fm declined in the wild-type and mutant leaves to approximately 73% and 55% of the dark-adapted values, respectively. After 4 h of illumination, Fv/Fm declined in the wild-type and mutant leaves to approximately 60% and 40% of the dark-adapted values, respectively (Figure 7B). These results clearly demonstrated the increased photosensitivity of the mutants. In the presence of lincomycin, the decrease in Fv/Fm was more rapid and continued until the Fv/Fm values approached approximately 10% of the dark-adapted values in wild-type leaves (Figure 7C). In the presence of lincomycin, the decline in Fv/Fm in the cplepa mutants was similar to that observed in the wild-type leaves during the same photoinhibitory light treatment (Figure 7C). Because lincomycin blocks the repair of PSII by inhibiting de novo chloroplast protein synthesis, these results

Figure 3. Identification and Phenotyping of the cplepa Mutants. A: T-DNA insertion sites in the cplepa gene. Exons are indicated by black boxes, introns by lines, and the T-DNA insertions by vertical arrows. The horizontal arrows illustrate the primers used for T-DNA insertion verification and RT-PCR. The scale bar indicates 500 bp. B: RT-PCR analysis. RT-PCR was performed using specific primers for cplepa or ACTIN. C: Two-week-old WT and cplepa-1 mutants grown on MS medium supplied with 0, 1% sucrose and 2% sucrose. D: Complementation of the cplepa-1 mutant. The cDNA of the cplepa gene was cloned into a binary plant transformation vector and used for complementation of the cplepa-1 mutant (cplepa-1/35S::cplepa). Four-week-old WT, cplepa-1, cplepa-2 and cplepa-1/35S::cplepa plants were grown on soil. Fluorescence was measured with a CF Imager and visualized using a pseudocolor index, as indicated at the bottom, Fm and Fv/Fm value were presented. E: Growth of wild-type and cplepa-1 mutant plants on soil at 120 μmol m⁻² s⁻¹. The values shown are averages ± s.e. (n=6).

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supplied with 2% sucrose under a light intensity of
mutants and wild-type plants when grown on MS medium
on soil, and the reduction is increased under high light
could be washed out by Na\textsubscript{2}CO\textsubscript{3} and CaCl\textsubscript{2}, indicating that
with the thylakoid membrane. Membrane-associated cpLEPA
peptide (Figure 1). Immunoblot analysis verified that cpLEPA is
cpLEPA was hypothesized to function as a chloroplast signal
stress conditions.
might facilitate the production of functional protein under
association with the membrane is flexible (Figure 2). Pech
suggested that the membrane acts as a storage depot for LEPA and
Discussion
LEPA is an extremely conserved and widely distributed
translation factor [11]. The amino acid sequence of Arabidopsis
cplLEPA shows 64% amino acid identity with that of E. coli LEPA
(Figure 1). This degree of sequence conservation is particularly
high for a comparison between higher plants and bacteria. CplLEPA contains four domains: LEPA, LEPA-II, LEPA-C and a
CTD domain. The LEPA and LEPA-II domains contain the
extremely conserved key amino acids that are important for GTP
binding, which are known as the G1, G2, G3 and G4 sequence
motifs. The G1, G3 and G4 motifs are responsible for binding and hydrolyzing GTP and for interacting with the cofactor Mg\textsuperscript{2+} [12]. The G2 motif undergoes a conformational change that is essential
for GTPase function [13]. LEPA-C was predicted to function in
translation elongation. The structure and sequence similarity of
cpLEPA to E. coli LEPA indicates a role for this protein in the
efficiency of chloroplast protein translation.
LEPA was initially reported as the leader peptide of the lcp
operon and was described as a membrane-associated GTP-
binding protein [4]. The N-terminal 51 amino acids of Arabidopsis
cplLEPA was hypothesized to function as a chloroplast signal
peptide (Figure 1). Immunoblot analysis verified that cpLEPA is
located in the chloroplast and is primarily found in association
with the thylakoid membrane. Membrane-associated cpLEPA
could be washed out by Na\textsubscript{2}CO\textsubscript{3} and CaCl\textsubscript{2} indicating that
cplLEPA is not an integral membrane protein and that the
association with the membrane is flexible (Figure 2). Pech \textit{et al}
suggested that the membrane acts as a storage depot for LEPA and
that LEPA is released into the cytoplasm as needed under specific
stress conditions in \textit{E. coli} [5]. Considering the association of
cplLEPA with the thylakoid membrane, such an arrangement
might facilitate the production of functional protein under
different stress conditions.
We also observed no growth differences between the cplepa-1
mutants and wild-type plants when grown on MS medium
supplied with 2% sucrose under a light intensity of
120 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} (Figure 3C). However, the growth of cplepa-1
was greatly retarded on MS medium supplied with 1% sucrose or
without sucrose under the same light intensity (Figure 3C). Sucrose
is an important nutrient which affects overall plant growth
features. Plant makes and transports sucrose for store or for use
through photosynthesis activity. If photosynthesis was impaired,
sucrose starvation will greatly decrease plant growth [14]. In
addition, the growth of the cplepa-1 mutant is reduced when grown on soil, and the reduction is increased under high light
illumination (Figure 7A). Moreover, the cplepa-1 mutant shows a
slightly pale green phenotype and impaired chloroplast develop-
ment (Figure S1). PSI and PSII activities are also decreased when
grown on soil. These results indicate that, although cplLEPA is not
essential under optimal conditions, it becomes critical under nutrient limitation or light stress conditions. PsiII activity,
indicated by the Fv/Fm value, revealed enhanced sensitivity to
high-light treatment in the cplepa-1 mutant in the absence of
lincomycin compared with the wild-type plants. The rate of PsiII
photoinhibition was similar in the mutant and wild-type plants in
the presence of the protein synthesis inhibitor lincomycin (Figure 7B, C). The adverse effect of high light on the cplepa-1
mutant indicates that the repair of PSI was perturbed. Thus, cplLEPA might be involved in the regulation of the synthesis of
PSII proteins.
The association of the chloroplast-encoded psbA, psbB, psaA/
psb3 and atpB mRNAs with ribosomes in the mutant grown on soil
showed a small shift toward the top of the gradient in the ribosome
loading assay (Figure 5), this indicated that translation initiation
was impaired in these transcripts. However, the distribution of
mutant and wild type plastid 23S rRNA, ndhA, petA and psaJ
transcripts were unchanged in the sucrose gradients (Figure S2B). Further exploration of the distribution of polysome association
revealed that 23S rRNA displayed a different sensitivity to EDTA
compared with \textit{vbcL} mRNA (Figure S2A). It is likely that a
significant proportion of the 23S rRNA is found in ribonucleo-
protein complexes other than polysomes. Alternatively the
ribosomes on which these chloroplast mRNAs are translated
represent only a small part of the total ribosome pool (Figure 5).
The steady-state transcript levels of PEP-dependent genes, including
psbA, psbB, \textit{vbcL}, psaA, atpE and psbD, decreased drastically in cplepa-1 mutants grown on soil (Figure 6). Changes in
chloroplast translation might modulate the stability of a subset
of chloroplast mRNA molecules [11,15]. The inactivation of
\textit{atrpB} affects the polysomal association of the \textit{atpE} transcript and leads to a 50% reduction in the amount of \textit{atpE} transcripts [16]. In
\textit{atpB}-1, the abnormal polysomal association of UAG-containing
transcripts leads to decreased stability of the transcripts [17]. In
\textit{apsB}-1, the abnormal polysomal association of \textit{apsB} transcript
causes the stability of the \textit{apsB} transcript and leads to a significant
reduction in its steady-state level [18]. In addition, decreased
protein levels of RPOA and RPOB (the \textit{a}- and \textit{b}- subunits of PEP)
were observed in the \textit{cplepa} mutant (Figure 4A). Thus, it is likely
that the dramatic loss in chloroplast transcripts observed in the
\textit{cplepa} mutant might be the synergistic effect of decreased
chloroplast translation and decreased PEP transcription.
Photosynthetic activity is somewhat impaired in cplepa-1
mutants, which is reflected in the decreased steady-state level
of chloroplast proteins (Figure 4A). Although a dramatic loss in
chloroplast transcripts and a perturbation in chloroplast polysome
loading were observed in the \textit{cplepa} mutant, only an approximate
20% decrease was observed in the steady-state levels of the
proteins. One possibility is that chloroplast genes are transcribed in
excess [19]. The \textit{psbA} mRNA levels are 30-fold higher than the
\textit{psbB} mRNA levels, but the steady-state protein level of RpoB is
approximately 50% of that of RpoA [20,21]. Similarly, the \textit{psbA}
mRNA levels are fivefold greater than those of the \textit{psaB} transcripts because of the increased turnover rate of \textit{psbA} needed to
maintain normal photosynthetic activity, whereas the protein
levels of these genes remain similar [22,23]. Polysomes analysis
provides an estimate of the efficiency of translation initiation and
elongation [11]. There was a relative increase in nonpolysomal
chloroplast mRNAs in the \textit{cpl2} mutant, but a substantial fraction of
mRNAs still remained associated with multiple ribosomes [11]. In
this mutant, chloroplast protein translation was only very mildly
affected. The effects of the \textit{cplLEPA} mutation on the association of

| Table 1. Chlorophyll Contents in Wild-Type and cplepa-1 Plants. |
|-------------------|-----------------|-----------------|
| chlorophyll | WT | cplepa-1 | cplepa-1/WT(%) |
| Chl a | 792.6±17 | 699.6±12 | 88 |
| Chl b | 261.4±12 | 261.1±11 | 100 |
| Chl a+b | 1083.8±31 | 963.7±14 | 89 |
| Chl a/Chl b | 3.2±0.1 | 2.6±0.1 | 87 |

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Figure 4. Accumulation and Synthesis of Chloroplast Proteins in cplepa-1 Plants. A: Immunoblot analysis of total protein extracts from wild-type and cplepa-1 plants. Wild-type and cplepa-1 plants grown on soil at a photon flux density of 120 μmol m⁻² s⁻¹ were used. For wild-type and cplepa-1 plants, 10 μg of total proteins were loaded. The antibodies used are indicated on the right. Actin served as a control to normalize the protein levels. Similar results were obtained in two additional independent experiments. B: Pulse labeling of thylakoid proteins. Primary leaves of 12-day-old plants were radiolabeled with [³⁵S] S-methionine in the presence of cycloheximide for 20 min. The thylakoid membranes were isolated, separated by SDS-urea-PAGE and visualized autoradiographically, lanes were loaded with equal protein contend. C: A coomassie blue-stained gel is presented to show that equal amounts of proteins were loaded.

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the *psbA*, *psbB*, and *psaA/B* mRNAs with ribosomes were similar to those of *cps2* [11] (Figure 5). In *vivo* protein labeling experiments showed a moderately decreased synthesis rate for the chloroplast-encoded proteins, which may account for the accumulation of photosynthetic proteins (Figure 4B).

Biochemical analysis of LEPA in *E. coli* has demonstrated its function as a translation factor in *vivo*. The elongation cycle of protein synthesis is characterized by tRNA movement between pre-translocation (PRE) and post-translocation (POST) complexes. Under stress conditions, such as high salt concentration or low temperature, translation could be blocked, possibly by perturbation of the ribosome structure [9]. LEPA could effectively compete with EFG for binding to the PRE complex. This binding could lead to the formation of an intermediate complex, I3, which could allow for the correction of an incorrect translocation event by replacing LEPA-GDP with EF-G-GTP (EF-G is present at considerably higher concentrations in bacterial cells compared with LEPA) [10]. A high Mg²⁺ concentration could stabilize the I3 complex by inhibiting the conversion of I3 to a PRE complex, which explains why LEPA accelerates protein synthesis at increased Mg²⁺ concentrations [6,10]. Our study is consistent with the proposed function of LEPA as a translation factor that contributes to the efficiency of protein synthesis.

In summary, we have demonstrated the physiological role of cpLEPA in efficient photosynthesis in higher plants. In addition, we have presented evidence highlighting the importance of this protein for chloroplast translation, which provides further insights into the conserved function of LEPA in chloroplast protein synthesis.

Materials and Methods

Plant Material and Growth Conditions

The *cplepa-1* (T-DNA insertion line, Salk_140697) and *cplepa-2* (T-DNA insertion line, CS464145) mutants were obtained from ABRC, and the homozygous mutants were verified by PCR using the primer pairs LEPA-LP and LEPA-RP as well as LEPA-GKF+LEPA-GKR (for primer sequences, see Table S1). The T-DNA insertion was confirmed by PCR and sequencing with the primers SALKLB1b and LEPA-LP for the *cplepa-1* mutant and with the primers GABILB and LEPA-GKR for the *cplepa-2* mutant. Wild type and mutant seeds were sterilized with 10% sodium hypochlorite for 15 min, washed five times with distilled water, and placed on solid MS medium [24] supplemented with sucrose as needed. Wild type and mutant seeds were sown and grown on soil according to a standard protocol. To ensure synchronized germination, the seeds were kept in the dark at 4°C for two days. The *Arabidopsis* plants were kept in a growth chamber at 22°C with a 12-h photoperiod at a photon flux density of 120 μmol m⁻² s⁻¹.

Photoinhibitory Treatment

Detached wild type and *cplepa-1* mutant leaves were floated face down on water and illuminated under a photon flux density of 1,000 μmol m⁻² s⁻¹, and the chlorophyll fluorescence was measured at 0.5 h, 1 h, 2 h, 3 h, and 4 h after exposure to high light using a PAM-2000 fluorometer (Walz). The temperature was maintained at 22°C throughout the photoinhibitory treatments. The synthesis of chloroplast-encoded proteins was blocked by incubating detached leaves with 1 mM lincomycin at low light (20 μmol m⁻² s⁻¹) for 3 h before photoinhibition treatment. To investigate the effects of high light on plant growth, we transferred 2-week-old *Arabidopsis* plants grown on soil under normal illumination of 120 μmol m⁻² s⁻¹ to 500 μmol m⁻² s⁻¹ for another 2 weeks.

Complementation

To complement the *cplepa* mutation, a full-length *cplepa* cDNA was amplified using nested antisense primers ([LEPAH-F, LEPAH-R1 and LEPAH-R2] with His tags, and the product was subcloned into the pSN1301 vector under the control of the CAMV 35S promoter. The constructed plasmids were then transferred into *Agrobacterium tumefaciens* strain C58 and introduced into the *cplepa-1* mutant plants by a floral dip method, as described previously [25]. Transgenic plants were selected on MS medium containing 50 μg/mL hygromycin. Complemented plants were selected and transferred to soil to produce seeds. The success of the complementation was confirmed by PCR, immunoblot and chlorophyll fluorescence analysis.

Chloroplast Ultrastructure

Wild type and mutant leaves from 3-week-old plants grown on soil were used for transmission electron microscopy analysis. The leaves were chopped into 1×2 mm pieces and immersed in fixative solution (2.4% glutaraldehyde in phosphate buffer) for 4 h at 4°C. After fixation, the samples were rinsed and postfixed in 1% OsO₄ overnight at 4°C and then dehydrated in an ethanol series, infiltrated with a graded series of epoxy resin in epoxy propane, and embedded in Epon 812 resin. Thin (80–100 nm) sections were obtained using a diamond knife on a Reichert OM2 ultramicrotome. The sections were stained with 2% uranyl acetate, pH 5.0, followed by 10 mM lead citrate, pH 12, and observed with a transmission electron microscope ([Jem-1230; JEOL]).

In vivo Protein Labeling Assays

In *vivo* protein labeling was performed essentially according to Meurer et al [26]. For pulse labeling, primary leaves from 12-d-old plants were labeled with 1 μCi/μL [³⁵S]-Met in the presence of 20 μg/mL cycloheximide for 20 min at 25°C. After labeling, the leaves were washed twice with homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2 mM EDTA) and ground with 300 μL of the same buffer. The thylakoid membranes were isolated and separated by SDS-PAGE, and the labeled proteins were visualized by autoradiography.

Chloroplast and Thylakoid Membrane Preparation

Intact chloroplasts were fractionated into envelope, stromal and thylakoid membrane fractions as described previously [27–29]. The thylakoid membranes were isolated according to Cai et al [30]. The chlorophyll contents were measured as described previously [31].
Figure 6. Northern Blot Analysis for Chloroplast Transcripts in Wild-Type and cplepa-1 Plants. Northern blot analysis of the chloroplast transcripts psbA, psbB, psbD, atpB, psaA, petB, rbcl, rpoA, rpoB and rrn23 in wild-type and cplepa-1 mutant plants. Each lane was loaded with 10 μg of total RNA. The plants were grown on soil for 3 weeks under 120 μmol m$^{-2}$ s$^{-1}$ illumination. Additionally, 25S rRNA stained with EtBr was loaded as a control. The size of the transcript (in kb) is shown.
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Protein Localization Analysis

The thylakoid membranes from wild type plants were suspended to a final concentration of 0.1 mg chlorophyll/mL in 10 mM HEPES-KOH, Ph 8.0, 10 mM MgCl₂, 330 mM sorbitol, and 1 mM PMSF supplemented with either 250 mM NaCl, 1 M CaCl₂, 200 mM Na₂CO₃ or 6 M urea. The membrane fractions without treatment were used as controls. All of the samples were kept on ice during the experiment. The treated samples were washed with 10 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂, 330 mM sorbitol, and 1 mM PMSF, and the pellets were collected by centrifugation for western blot analysis [32,33].

Immunoblot Analysis

Total protein was extracted from 3-week-old wild-type and mutant plants using E buffer (125 mM Tris-HCl, pH 8.8; 1% (v/v) SDS; 10% (v/v) glycerol; 50 mM Na₂S₂O₅) as described by Martínez-García et al [34]. Protein concentration was determined using the BioRad Dc Protein Assay (BioRad, Hercules, CA, USA) according to the manufacturer’s instructions. Total proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. After incubation with specific primary antibodies, the signals from secondary conjugated antibodies were detected by the enhanced chemiluminescence method. The anti-cpLEPA antibody was raised against the N-terminus of the cpLEPA protein (cpLEPA36-170). The procedures involved in generating an antibody were performed according to Sun et al [35].

RT-PCR, Northern Blot and Polysome Association Analyses

For the RT-PCR analysis, the total RNA was isolated from 3-week-old leaves using the Total RNA Isolation Kit (U-Gene), and RT-PCR was performed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using the primers LEPA RTF and LEPA RTR.

For northern blot analysis, total RNA was extracted from 3-week-old wild type and mutant plants after germination on MS or soil as described above. The northern blot was performed according to Cai et al [36]. The following primer pairs were used to amplify the appropriate probes: psbA, psbB, psbD, atpB, petB, rbcL, petA, psaA, psaB, ndhA, petA and psaJ (Table S1 for primer sequence).

For polysome association analysis, polysomes were isolated from 3-week-old leaves according to Barkan [37], with certain
of MgCl₂ in parallel on a similar gradient containing 1 mM EDTA instead. A polysome sample supplemented with 20 mM EDTA was analyzed. The samples were incubated on ice for 10 min and pelleted by centrifugation for 7 min at 14,000 rpm. Sodium deoxycholate was added to the supernatant to a final concentration of 0.5%, after centrifugation for 7 min at 14,000 rpm. Sodium deoxycholate was g/mL chloramphenicol, and 25 μg/mL cycloheximide was added, and the tissue was ground until thawed. The samples were kept on ice for 5 min and then centrifuged at 12,000 rpm for 15 min. Next, 0.5 mL samples of the supernatant were layered onto 4.4-mL sucrose gradients that were kept on ice for 5 min and then centrifuged.

Supporting Information

Figure S1 Transmission Electron Micrographs of the Chloroplasts. Transmission electron microscopic images of the chloroplast ultrastructure in WT and cplepa-1 leaf sections. Three-week-old plants grown on soil at 120 μmol m⁻² s⁻¹ were used. The scale bar indicates 1 μm. In total, 100 chloroplasts of the WT and cplepa-1 were examined and measured.

Figure S2 Polysome Association Analysis for Chloroplast Transcripts in Wild-Type and cplepa-1 Plants Grown in Soil. A: The association of rrn23 and rbcL transcripts with EDTA treated polysomes. Crude leaf lysates treated with 20 mM EDTA from wild-type were size fractionated on 15% to 55% sucrose gradients containing 1 mM EDTA. B: The association of ndhA, petA, and petJ transcripts with polysomes. Total extracts from wild-type and cplepa-1 leaves grown on soil for 3 weeks at 120 μmol m⁻² s⁻¹ were used. The rRNAs were purified from each fraction were collected from the top to the bottom of the sucrose gradients, and equal proportions of the RNA purified from each fraction were analyzed by northern blot. The RNA was stained with EtBr and the size of the transcript (in kb) is shown.

Table S1 Primer sequences and probes used in this work.

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Author Contributions

Conceived and designed the experiments: D-LJ L-XZ. Performed the experiments: D-LJ HL. Analyzed the data: D-LJ HL WC L-XZ. Contributed reagents/materials/analysis tools: D-LJ HL. Wrote the paper: D-LJ L-XZ.
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