RESEARCH PAPER

Functional relationship between mTERF4 and GUN1 in retrograde signaling

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Abstract

Plastid-to-nucleus retrograde signaling plays an important role in regulating the expression of photosynthesis-associated nuclear genes (PhANGs) in accordance with physiological demands on chloroplast biogenesis and function. Despite its fundamental importance, little is known about the molecular nature of the plastid gene expression (PGE)-dependent type of retrograde signaling. PGE is a multifaceted process, and several factors, including pentatricopeptide repeat (PPR) proteins, are involved in its regulation. The PPR protein GUN1 plays a central role in PGE-dependent retrograde signaling. In this study, we isolated a mutant exhibiting up-regulation of CHLOROPHYLL A/B-BINDING PROTEIN (CAB) under normal growth conditions (named coe1 for CAB overexpression 1). The coe1 mutant has a single-base mutation in the gene for mitochondrial transcription termination factor 4 (mTERF4)/BSM/RUG2, which plays a role in regulating the processing of certain plastid transcripts. Defects in GUN1 or mTERF4 de-repressed the expression of specific plastid mRNAs in the presence of lincomycin (LIN). In wild-type plants, treatment with LIN or spectinomycin (SPE) inhibited processing of plastid transcripts. Comparative analysis revealed that in gun1 and coe1/mterf4, but not in wild-type, gun4, or gun5 plants, the processing of plastid transcripts and expression levels of Lhcb1 mRNA were affected in opposite ways when plants were grown in the presence of LIN or SPE. In addition, the coe1 mutation affected the intracellular accumulation and distribution of GUN1, as well as its plastid signaling activity. Taken together, these results suggest that GUN1 and COE1 cooperate in PGE and retrograde signaling.

Key words: Chloroplast, GUN1, mTERF4/COE1, PGE, retrograde signaling.

Introduction

Chloroplasts evolved from a free-living cyanobacterium, following its endosymbiotic integration into a non-photosynthetic eukaryotic host (Douglas and Raven, 2003). However, a large amount of genetic information has been transferred to the nuclear genome during the evolution of chloroplasts (Abdallah et al., 2000; Rujan and Martin, 2001). At present, the plastid genome of higher plants contains only about 100 genes, which encode proteins for plastid gene expression (PGE) and photosynthesis (Abdallah et al., 2000). In contrast, more than 95% of chloroplastic proteins are now encoded in the nucleus, translated in the cytoplasm, and then imported into the organelle (Barbrook et al., 2006). Having been imported into the chloroplast, some nucleus-encoded plastid proteins interact with chloroplast-encoded proteins.
to form multi-subunit complexes involved in, for instance, the replication and expression of plastid-encoded genes, or in photosynthesis. To ensure correct stoichiometric assembly of these multi-subunit complexes, and enable their reorganization in response to developmental or environmental cues, the activities of the nuclear and chloroplast genomes must be coordinated through an intracellular signaling network (Kleine et al., 2009; Grimm et al., 2014). This network includes signaling pathways that originate in the nucleus (anterograde signaling) and the plastids (retrograde signaling) (Grimm et al., 2014). Anterograde signaling is involved mainly in the regulation of PGE (Jiao et al., 2007). By contrast, retrograde signaling conveys information about the developmental and metabolic state of the chloroplast to the nucleus, modifying nuclear gene expression in accordance with the current status of the organelle (Nott et al., 2006; Kleine et al., 2009; Pfannschmidt, 2010; Chi, et al., 2013). Plastid signals are classified into five distinct groups, depending on their source: (i) PGE; (ii) tetrapyrrole biosynthesis; (iii) reactive oxygen species generation; (iv) plastid redox state; and (v) metabolites, such as 3-phosphoadenosine 5’-phosphate, methylethritol cyclodiphosphate, and β-cyclocitrinal (Gray et al., 2003; Baier and Dietz, 2005; Pesaresi et al., 2007; Mochizuki et al., 2008; Moulin et al., 2008; von Gromoff et al., 2008; Pfannschmidt et al., 2009; Bräutigam et al., 2009; Voigt et al., 2010; Galvez-Valdivieso and Mullineaux, 2010; Estavillo et al., 2011; Woodson et al., 2011; Ramel et al., 2012; Xiao et al., 2012; Kim and Apel, 2013; Terry and Smith, 2013).

Tetrapyrrole biosynthesis and PGE-dependent signaling are the best characterized types of plastid signaling (Nott et al., 2006). Much information on their operation has been obtained from studies on gun (genomes uncoupled) mutants of Arabidopsis thaliana (Nott et al., 2006). Six independent gun mutants have been identified. Five of them (gun2–gun6) are defective in different steps in the tetrapyrrole biosynthetic pathway (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Strand et al., 2003; Woodson et al., 2011). GUN2 (heme oxygenase), GUN3 (phytochromobilin synthase), and GUN6 (FC1) operate in the heme branch of tetrapyrrole biosynthesis pathway (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Strand et al., 2003; Woodson et al., 2011). GUN2 has a subtle effect on PhANG expression and possibly even in the absence of overt inhibition of PGE, implying that GUN1 is either not directly involved in the regulation of PGE or that its function in PGE becomes manifest only under certain conditions. PGE signaling normally represses nuclear Lhcb expression in response to perturbations in chloroplast protein production. However, in gun plants, expression of Lhcb is slightly higher than in the wild type (WT), even in the absence of overt inhibition of PGE, implying that GUN1 has a subtle effect on PhANG expression and possibly also PGE under normal growth conditions (Sun et al., 2011).

In order to identify novel components of retrograde signaling, we developed an ethyl methanesulfonate (EMS) screen for mutants that displayed enhanced activity of the promoter of the Lhcb1 gene for chlorophyll a:b-binding protein (CAB) under normal growth conditions. A series of coe (CAB over-expression) mutants was isolated, and the causative mutation in one of them (coe1) was localized to position 844 of the AT4G02990 gene, thus demonstrating that COE1 codes for BSM/mTERF4. Like gun1, coe1 showed increased Lhcb1 mRNA expression under normal growth conditions and displayed a weak gun phenotype in the presence of the herbicide norflurazon (NF), which inhibits carotenoid synthesis and causes photo-oxidative damage. Defects in GUN1 or mTERF4 decreased the expression of certain plastid mRNAs in the presence of the antibiotic lincomycin (LIN) which, like spectinomycin (SPE), inhibits protein synthesis in the chloroplast. Comparative analysis revealed that in gun1 and coe1 mterf4, but not in WT, gun4, or gun5 plants, the processing of plastid mRNAs and expression levels of Lhcb1 were affected in opposite ways when plants were grown in the presence of LIN or SPE. In addition, COE1 has an impact on the intracellular accumulation and distribution of GUN1, as well as on its plastid signaling activity.
Materials and methods

Plant materials and growth conditions

The following *A. thaliana* mutants in the Columbia (Col-1) ecotype were obtained from the Arabidopsis Biological Resource Center: gun1 (SAIL_742_A11, a T-DNA insertion mutant; Sun et al., 2011) and gun4 (SALK_011461, a T-DNA insertion mutant). Homozygous lines were identified by PCR using gene-specific and T-DNA-specific primers (Supplementary Table S1 at *JXB* online). Mutants were backcrossed to WT plants three times before generating double mutants to segregate out additional mutations. To generate oeCOE1-loc1 and oeGUN1-GFP-loc1 strains, pK7FWG2-GOE1 and pB7FWG2-GUN1 (both driven by the cauliflower mosaic virus 35S promoter), respectively, were introduced into lines homozygous for coe1, and gun1 was crossed with coe1 to obtain the double mutant coe1 gun1.

All mutant and WT plants were grown in climate chambers at 22°C and 120 µmol photons m⁻² s⁻¹ on a 12 h light/12 h dark regime. For the NF, L1NE and SPE treatments, surface-sterilized mutant and WT seeds were plated on 1/2 Murashige and Skoog (1/2 MS; Murashige and Skoog, 1962) medium (PhytoTechnology Laboratories, LLC, USA) containing 1% sucrose and 0.8% agar supplemented with either 5 µM NF (Sandoz Pharmaceuticals, Vienna, Austria), 220 µg ml⁻¹ of LIN (Sigma, St Louis, MO, USA), or 500 µg ml⁻¹ of spectinomycin (Sigma).

RNA extraction, Northern blotting, and quantitative real-time reverse transcription PCR (qRT-PCR)

RNA was extracted with a Maxwell® 16 LEV simplyRNA Purification Kit (Promega, WI, USA). Northern blot analysis was performed under stringent conditions, according to Sambrook and Russell (2001). Probes complementary to nuclear or chloroplast genes were used for the hybridization experiments. Primers used to amplify the probes are listed in Supplementary Table S1. All probes were labeled with 32P. Signals were detected with a phosphorimager (Typhoon; GE Healthcare) and quantified using the program ImageJ. For qRT-PCR, 1 µl aliquots of total RNA, treated with DNase I (Roche Applied Science) for at least 30 min, were utilized for first-strand cDNA synthesis using iScript reverse transcriptase (Bio-Rad) according to the supplier’s instructions. The qRT-PCR profiling was carried out on an iCycler Q5 real-time PCR system (Bio-Rad), using the oligonucleotide sequences listed in Supplementary Table S1. Actin was used as an internal standard. Data from three biological and three technical replicates were analyzed with Bio-Rad iQ5 software (version2.0).

Polysome analysis

For polysome analysis, polysomes were isolated from 5-d-old seedlings according to Barkan (1993), with certain modifications. Approximately 0.5 g of seedlings were frozen and grind in liquid nitrogen to a fine powder. 1 ml of polysome extraction buffer [0.2 M Tris/ HCl (pH 9.0), 0.2 M KCl, 35 mM MgCl₂, 25 mM EGTA, 0.2 M sucrose, 1/10 Triton X-100, 1/10 polyoxyethylene-10-tridecyle ether, 0.5 mg ml⁻¹ of heparin, 100 mM β-mercaptoethanol, 100 µg ml⁻¹ of chloramphenicol, and 25 µg ml⁻¹ of cycloheximide] was added, and the tissue was ground until thawed. The samples were incubated on ice for 10 min and pelleted by centrifugation for 7 min at 14 000 x g. Sodium deoxycholate was added to the supernatant to a final concentration of 0.5%, after which the samples were kept on ice for 5 min and then centrifuged at 12 000 x g for 15 min. Next, 0.5 ml samples of the supernatant were layered onto 4.4 ml sucrose gradients that were prepared, centrifuged, and fractionated as described previously (Barkan, 1993). The samples were kept at 4°C during preparation. The RNA in each fraction was isolated, separated, and transferred onto nylon membranes (Amersham Pharmacia Biotech), which were probed with 32P-labeled probes. Signals were detected with a phosphorimager (Typhoon; GE Healthcare).

Run-on analysis

Run-on analysis was performed according to Zoschke et al. (2007). Intact chloroplasts from 3 g of leaves were isolated in a 40/70% Percoll step-gradient. Chloroplasts (5 x 10⁴) were used in *in vitro* transcription experiments, performed at 25 °C for 15 min in 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂, 10 mM β-mercaptoethanol, 20 U RNase inhibitor, and 0.2 mM ATP, GTP, and CTP, in the presence of α-32P-UTP (10 µCi µl⁻¹). Newly synthesized, labeled RNA was extracted and hybridized overnight at 42 °C to DNA fragments (1 µg) dot blotted in duplicate onto nylon membranes. The primers used for the generation of DNA probes are listed in Supplementary Table S1. Signals were detected with a phosphorimager (Typhoon; GE Healthcare) and quantified using the program ImageJ.

Constructs for plant transformation and yeast one-hybrid assays

To generate the pK7FWG2-GOE1 and pB7FWG2-GUN1 plasmids (in which green fluorescent protein (GFP) is fused to c-terminus of GUN1), COE1 and GUN1 cDNAs were amplified using the primer pair COE1-GFPs and COE1-GFPa for COE1, and GUN1-GFPs and GUN1-GFPa for GUN1, respectively (Supplementary Table S1). The PCR products were purified, and BP and LR Clonase reactions (GATEWAY Cloning; Invitrogen) were performed according to the manufacturer’s instructions to yield the final constructs pK7FWG2-GOE1 and pB7FWG2-GUN1.

To construct the luciferase (LUC) reporter, the promoter region of *Lhcb1.1* (1-976 to -30 nt) was amplified by PCR using gene-specific primers (Supplementary Table S1). The resulting DNA fragments were digested with HindIII and BamHI, and inserted into the corresponding sites in the 35S:LUC vector (Hellens et al., 2000), replacing the 35S promoter to produce P-35S: LUC. The vector was subsequently digested with HindIII and SacI and inserted into the cognate sites of the binary vector pCAMBIA1301, thus generating the pCAMBIA1301-35S: LUC.

To generate pGBK7-GUN1 and pGADT7-COE1, the GUN1 and COE1 cDNAs were PCR amplified from Arabidopsis cDNA using PrimeSTAR® HS DNA polymerase and then inserted into the EcoRI and BamHI sites of the pGBK7 and pGADT7 vectors, respectively.

Plant transformation

The pK7FWG2-GOE1 and pB7FWG2-GUN1 constructs were transformed into *Agrobacterium tumefaciens* strain GV3105 via electroporation, and the resulting strains bearing the pK7FWG2-GOE1 (expressing Kan resistance in planta) and pB7FWG2-GUN1 (Basta resistance) constructs were introduced into coe1. T1 transgenic plants were selected by screening on Basta for *GUN1* and on kanamycin for *COE1*. Homozygous transgenic plants were used in all experiments.

Yeast two-hybrid assay

The plasmids pGBK7-GUN1 and pGADT7-COE1 were cotransformed into the yeast strain AH109 using standard techniques. Growth of diploid yeast colonies on SD–His–Leu–Trp–Ade plates supplemented with 40 µg ml⁻¹ of X-a-Gal would reveal a GUN1–COE1 interaction.

Mutagenesis and mutant isolation

The plasmids pGBK7-GUN1 and pGADT7-COE1 were mutagenized with EMS (Redei and Koncz, 1992). F2 seeds were sterilized and planted individually in 100 x 10 mm plates (150 to 200 seeds per plate) containing 1/2 MS, 1% sucrose, and 0.8% agar (pH 5.7). Five-day-old seedlings grown under light were sprayed with luciferin and immediately placed in dark regime. For selection of F2 transgenic plants, 150 to 200 seeds per plate were grown on SD–His–Leu–Trp–Ade plates supplemented with 40 µg ml⁻¹ of X-a-Gal.
the dark (see below) to remove the chlorophyll fluorescence, which was monitored with a CCD camera. After 5 min in the dark, an LUC image was acquired with a 5 min exposure to identify coe mutants. Putative coe mutants were also transferred to soil. To eliminate false positives, putative mutants were rescreened.

**LUC analysis by CCD imaging**

Imaging of the activity of the firefly LUC reporter requires application of the exogenous substrate luciferin. Luciferin (Promega) was dissolved in sterile water and stored frozen in small aliquots as a 100 mM stock solution. A working solution of 1 mM luciferin in 0.01% Triton X-100 was applied uniformly to seedlings by spraying five times. For LUC imaging, the seedlings were kept for 5 min in the dark after application of luciferin. The imaging system consisted of a high-performance CCD camera mounted in a dark chamber, a camera controller, and a computer. Image acquisition and processing were performed with the WinView software. Exposure time was 5 min, unless stated otherwise.

**Positional cloning**

To generate the mapping population for COE1, coe1 mutant plants were crossed to WT Arabidopsis plants of the Landsberg erecta (Ler) ecotype. A total of 1600 coe1 mutant plants were selected from the segregating F2 population based on high luminescence (expression of P<sub>Lhcb1.1</sub>:LUC) and a pale green phenotype. Genomic DNA from these plants was extracted and analyzed for co-segregation with respect to simple sequence length polymorphism (SSLP) markers. These markers were developed according to Lukowitz et al. (2000). Primer pairs for fine mapping of COE1 are listed in Supplementary Table S1. In addition, nucleotide differences between Ler and Col ecotypes were identified by direct sequencing of the ORF of T4F9.

**Protein extraction and immunoblot analysis**

Five-day-old seedlings were harvested from 1/2 MS plates, and total proteins were prepared according to Sun et al. (2011). For immunoblot analyses, the proteins were fractionated by SDS-PAGE (15% acrylamide) (Schägger and von Jagow, 1987). Subsequently, proteins were transferred to polyvinylidene difluoride membranes (Ihnatowicz et al., 2004) and probed with appropriate antibodies. Signals were detected by enhanced chemiluminescence (GE Healthcare).

**Thylakoid membrane preparation and blue native polyacrylamide gel electrophoresis (BN-PAGE)**

Thylakoid membranes were prepared as described by Zhang et al. (1999). Arabidopsis leaves were ground in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM HEPES/KOH (pH 7.8), 10 mM NaCl, and 2 mM MgCl₂, filtered through two layers of cheesecloth, and centrifuged at 5000 g for 10 min. The thylakoid pellets were washed with isolation buffer, recentrifuged, and finally suspended in isolation buffer. The chlorophyll content was determined spectrophotometrically according to the method described by Porra et al. (1989). BN-PAGE was carried out as described previously (Schägger and Cramer, 1994). The thylakoid membranes were solubilized with 1% (w/v) dodecyl-β-maltoside in 20% glycerol, 25 mM BisTris/HCl (pH 7.0), at 0.5 mg chlorophyll m⁻¹ for 10 min at 4 °C, and unsolubilized material was removed by centrifugation at 12 000 g for 10 min. The supernatant was combined with 0.1 vols of 5% Serva blue G in 100 mM BisTris/HCl (pH 7.0), 0.5 M 6-amino-n-caproic acid, 30% (w/v) glycerol, and loaded onto 6–12% acrylamide gradient BN gels.

**Chlorophyll fluorescence analysis**

*In vivo* chlorophyll a fluorescence of whole seedlings was recorded using an imaging chlorophyll fluorimeter (ImagingPAM; Walz, Germany). Dark-adapted plants were exposed to a pulsed, blue measuring beam (1 Hz, intensity 4; F0) and a saturating light flash (intensity 4) to obtain Fv/Fm. A 10 min exposure to actinic light (80 μmol photons m⁻² s⁻¹) was then used to calculate the steady-state magnitudes of the quantum yield of photosystem II (PSII) (Fφ), non-photochemical quenching of chlorophyll fluorescence (NPQ), and the fraction of open PSII centers (qL).

**Accession numbers**

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are AT4G29900 (mTERF4/COE1/BSM/RUG2), AT2G31400 (GUN1), AT3G59400 (GUN4), and AT5G13630 (GUN5).

**Results**

**Identification of the coe1 mutant**

To identify components of plastid signaling under physiological conditions, we transformed Arabidopsis plants with a construct containing the Lhcb1.1 [LIGHT HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN 1.1 or CHLOROPHYLL A/B-BINDING PROTEIN 2 (CA2)] promoter fused to the firefly LUC coding sequence. The resulting P<sub>Lhcb1.1</sub>:LUC plants (and the P<sub>35S</sub>:LUC transgenics used as controls) emitted luminescence under normal growth conditions (Supplementary Fig. S1 at JXB online). In contrast to P<sub>35S</sub>:LUC plants, P<sub>Lhcb1.1</sub>:LUC plants displayed significantly lower luminescence levels when grown in the presence of either LIN or NF (Supplementary Fig. S1), as expected, because both agents are known to activate plastid signaling (Dong et al., 2007; Sun et al., 2011; Kindgren et al., 2012).

P<sub>Lhcb1.1</sub>:LUC plants, designated in the following as WT*, were then mutagenized with EMS, and mutants with increased luminescence under normal growth conditions were identified and classified as putative chlorophyll a/b-binding protein overexpression (coe) mutants (Fig. 1A, B). We identified more than 100 coe mutants, and one of them, designated coe1, was chosen for detailed characterization. Compared with WT*, coe1 mutants showed increased luciferase expression under normal growth conditions (Fig. 1C, D). Interestingly, coe1 leaves were pale yellow in color, and this trait first appeared in young seedlings (Supplementary Fig. S2 at JXB online). As suggested by the pale yellow phenotype, the maximum quantum yield of PSII (Fv/Fm) at very early developmental stages was substantially reduced in the coe1 strain relative to WT* but increased gradually as the seedlings got older (Fig. 2A and Supplementary Fig. S2). The higher level of luciferase expression in coe1 was particularly evident on days 3 and 4 after germination (Fig. 2B). This phenotype suggested that COE1 may play a special role during early seedling development.

The coe1 mutant was also backcrossed to the WT*. The resulting F1 plants all exhibited a WT* phenotype (Table 1). The F2 progeny of the selfed F1 segregated at approximately 3:1 for WT:mutant (Table 1), indicating that coe1 is a recessive mutation in a single nuclear gene.

**PhANG expression in coe1**

To determine whether the coe1 mutation also affected endogenous Lhcb1.1 gene expression, we extracted total RNA from
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coe1 and WT* seedlings grown on standard 1/2 MS plates, and performed Northern blotting and real-time PCR analyses. Fig. 3A shows that the steady-state levels of Lhcb1.1 mRNA were higher in coe1 than in WT* under normal growth conditions. Analysis of the changes in Lhcb1.1 expression during development showed that enhanced Lhcb1.1 transcript accumulation was seen at all time points between days 3 and 7 after germination, although the difference was especially evident on days 3 and 4 (Supplementary Fig. S3 at JXB online). In agreement with results obtained for LUC activity and Fv/Fm values, Lhcb1.1 transcript levels in 7-d-old coe1 were close to those of the WT* (Supplementary Fig. S3). We therefore used 3- to 5-d-old seedlings for all subsequent experiments.

To further examine the effects of the coe1 mutation on PhANG expression, we analyzed the expression of Lhcb1.1 in the presence of LIN, NF, or SPE (Fig. 3B–D). While after LIN treatment the expression of Lhcb1.1 in the gun1 and coe1 mutants was about 750 and 250% of WT* levels, respectively, coe1 plants treated with NF displayed almost gun1-like levels of Lhcb1.1 mRNA (Fig. 3C, E). In addition, the expression of other PhANGs (Lhcb2, Lhcb3, Lhcb4, RbcS1a, and CA1) was also slightly higher in coe1 than in WT* in the presence of NF (Supplementary Fig. S4 at JXB online), suggesting that COE1 may be involved in modulating retrograde signaling. In gun1, Lhcb1.1 levels were significantly higher than in WT* in the presence of LIN, SPE, or NF. Unlike Lhcb1.1, accumulation of the rbcL transcript was strongly inhibited in all genotypes by LIN (Fig. 3F, H). Interestingly, levels of rbcL mRNA were significantly higher in gun4 and gun5 than in the other genotypes in the presence of NF (Fig. 3G, H), suggesting that gun4 and gun5 mitigate the effects of NF on the expression of plastid mRNAs, as has been shown for gun5 by Ankele et al. (2007).

Accumulation of photosynthetic proteins is differentially affected in coe1

The pale yellow leaf coloration and the photosynthetic defect (Fig. 1C, Supplementary Fig. S5 at JXB online) suggested that chloroplast development in coe1 is impaired. When grown on soil, the lower photosynthetic efficiency of the coe1 mutant resulted in a decrease in growth rate (Supplementary Fig. S6 at JXB online). The decrease in Fv/Fm levels observed in the coe1 mutant during early development (Fig. 2A, Supplementary Fig. S5) might be a consequence of altered thylakoid protein levels. To address this, the steady-state levels of chloroplast proteins in 5-d-old plants was determined by immunoblot analyses with antibodies raised against representative chloroplast proteins. In fact, semi-quantitative determination of protein levels by applying a dilution series and quantification with ImageJ showed that the levels of the plastid-encoded PSI reaction center protein psaA and subunits of the PSII core subunits D1, D2, CP47, and CP43 were reduced to about 15–25% of WT*
levels in coel (Fig. 4A). Nucleus-encoded subunits of the oxygen-evolving complex (Psbo), light-harvesting complex II (Lhcb1), and ferredoxin:NADP(H) oxidoreductase (FNR) accumulated in coel to ~75%, ~30%, and ~15% of WT* levels, respectively (Fig. 4A). In contrast, the level of chloroplast ATPase protein CFβ was virtually equivalent to that of WT* (Fig. 4A). In some respects, coel behaves like the known gun mutants. Like those of coel, the cotyledons of the gun4 and gun5 mutants were pale yellow in color (Supplementary Fig. S5). Interestingly, the effect of gun4 on the accumulation of chloroplast proteins was very similar to that of coel: levels of D1, D2, CP43, CP47, Psaa, and Lhcb1 were reduced to about 10-30% of WT* levels (Fig. 4A), and amounts of FNR, RbcS1a and RbcL declined to about 10, 15, and 40% of WT* levels, respectively, in gun4 (Fig. 4A). The gun5 mutant showed less pronounced effects on the accumulation of the chloroplast proteins. In gun5, D1, D2, CP43, CP47, Lhcb1, Psbo, FNR, Psaa, RbcS1a, and RbcL accumulated to about 35-95% of WT* levels (Fig. 4A), while amounts of CFβ in gun5 were almost identical to WT* (Fig. 4A). Furthermore, in the gun1 mutant, all investigated photosynthesis proteins accumulated to or nearly to WT* levels (Fig. 4A). The significant decrease of thylakoid membrane proteins suggested that thylakoid membrane complexes are also altered in coel. To investigate this, Blue Native (BN) gel analysis was performed. Indeed, the levels of thylakoid membrane complexes, such as PSI and PSII, were clearly reduced in coel and rug2-1 compared with WT* and gun1 (Fig. 4B, C).

The dramatic reductions in chloroplast proteins observed in coel could be due to decreased transcription of the corresponding genes. To assess this possibility, plastid-encoded transcripts were detected by RNA gel-blot hybridization, and levels of the transcripts were determined semi-quantitatively by applying a dilution series followed by quantification with ImageJ. This confirmed that levels of psbA (encoding the D1 subunit of PSI), psbA (encoding the CP47 subunit of PSI), psbC (encoding the CP43 subunit of PSI), psbD (encoding the D2 subunit of PSI), and rbcL mRNAs in the coel mutant were almost identical to that of WT* (Fig. 5A, B). In contrast, levels of atpB, psaB, and petA transcripts (which encode the β-subunit of ATP synthase, the B subunit of PSI, and cytochrome f, respectively) were increased in coel relative to WT* (Fig. 5A, B). In gun4, psbA, psbB, psbC, psbD, psaB, petA, and rbcL transcripts were under-represented, which was consistent with the changes in protein levels (Fig. 5A, B), whereas amounts of atpB and psaB in gun4 were comparable to WT* (Fig. 5A, B). In gun1 and gun5, levels of psbA, psbB, psbC, psbD, rbcL, and psaB RNAs were slightly decreased (Fig. 5A, B), but atpB and petA transcripts were increased relative to WT* (Fig. 5A, B). Amounts of nucleus-encoded Psbo mRNA in gun1, gun4, gun5, and coel were similar to WT*, while nucleus-encoded RbcS1a mRNA levels were slightly increased in gun5, coel, and rug2-1 (Fig. 5A, B). Taken together, these results suggested that COE1 plays an important role in PGE.

Compared with WT*, the levels of chloroplast psba, petA, and atpB transcripts were elevated (Fig. 5); however, the levels of the encoded proteins were reduced in coel (Fig. 4), suggesting that a defect in translation of chloroplast transcripts might be responsible for reduced accumulation of the corresponding proteins. To test this, the association of psbA, petA, and atpB mRNA with polysomes was analyzed (Supplementary Fig. S7 at JXB online). To this end, plant extracts were fractionated in sucrose gradients under conditions that preserve polysome integrity, and mRNAs were identified by hybridization with specific probes. As shown in Supplementary Fig. S7, the amounts of petA, psaB, and atpB mRNA assembled with

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**Table 1. Analysis of coel (WT*×coel)**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Seedlings tested</th>
<th>Mutant</th>
<th>WT*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>465</td>
<td>113</td>
<td>352</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

* Female×male.
* For 3:1 segregation.
ribosomes (fractions 7–12) was generally similar in WT* and coel, whereas mRNA accumulation of polycistronic versions of these transcripts, especially the transcript of petA, was higher in the non-polysomal fractions (fractions 1–6) in coel. These results suggested that the majority of these mRNAs in coel chloroplasts were not engaged in translation, which accounts for the reduction in synthesis of chloroplast proteins. The distribution of petA, psaB, and atpB mRNAs in non-polysomal and polysomal fractions of gun1 was similar to that of WT* (Supplementary Fig. S7), suggesting that gun1, as expected, does not affect the translation of these proteins.
Positional cloning of COE1

To map coe1 genetically, homozygous coel mutant plants (Col ecotype) were crossed with WT plants of the Ler ecotype. The resulting F1 plants were selfed and homozygous coe1 mutant plants were selected from the segregating F2 population based on their pale yellow phenotype. A survey of representative molecular markers from each of the five Arabidopsis chromosomes localized COE1 to chromosome IV (Fig. 6). Further analysis showed that COE1 is closely linked with the SSLP marker nga8. Several new SSLP markers were selected from the Arabidopsis Mapping Platform (http://amp.genomics.org.cn/) and TAIR (http://www.arabidopsis.org/) between the markers cih6 and nga8. Fine-scale mapping using these new markers delimited COE1 to the BAC clone T4I9 (Fig. 6).

Candidate ORFs on T4I9 were sequenced in WT* and coe1 mutant plants, revealing a single-nucleotide substitution in AT4G02990 in the coe1 mutant. This mutation was predicted to lead to a change from Arg to Trp at position 282 of the protein (Fig. 6).

To prove that the alteration in AT4G02990 was responsible for the phenotypes observed in the coe1 mutant, the isolated full-length AT4G02990 cDNA was fused to the 35S promoter in the plant transformation vector pK7FWG2. The construct was introduced into coe1 mutant plants via Agrobacterium-mediated transformation. Transformants were selected based on kanamycin resistance. Both kanamycin resistance and pale yellow leaf coloration segregated in the T2 generation. All kanamycin-resistant plants exhibited the WT* phenotype. A deeper analysis of two complemented coe1 mutant plants in the T2 generation, COM.-1 and COM.-2, demonstrated the full restoration of the WT phenotype: Luminescence of \( P_{Lhcb1}LUC \), \( Fv/Fm \), \( \phi_{PSII} \), and NPQ, as well as growth of COM.-1 and COM.-2 were the same as in WT plants (Supplementary Fig. S8 at JXB online).
The coe1 mutation is a new allele of mterf4/bsm/rug2

Arabidopsis AT4G02990 encodes BELAYA SMERT (BSM; Babiychuk et al., 2011)/RUGOSA2 (RUG2; Quesada et al., 2011), a plastid-localized mTERF protein, which has been designated mTERF4 in the systematic nomenclature of Kleine (2012). The mTERF4 protein is essential for normal plant development and for maintenance of adequate levels of transcripts in both mitochondria and chloroplasts (Babiychuk et al., 2011; Quesada et al., 2011). In rug2-1, the conserved proline residue at position 420 is replaced by leucine, and the mutant shows a variegated phenotype similar to var1 and var2 (Quesada et al., 2011). The maize RUG2 ortholog ZmmTERF4 is localized to the chloroplast, and an allelic series of Zmmterf4 mutants showed pale yellow/green and albino phenotypes (Hammani and Barkan, 2014). mTERF4 contains 10 mTERF motifs between aa 98 and 444, and the Arg282 to Trp mutation in COE lies in the fifth of these. In order to compare the effects of coe1 and rug2 on AT4G02290/BSM/RUG2 function, we analyzed growth and photosynthesis in these genotypes (Quesada et al., 2011). In fact, growth rate, cotyledon coloration, and photosynthesis parameters (Fv/Fm, $\phi_{II}$, NPQ, and qL) were very similar in coe1, rug2-1, and rug2-2 (Fig. 7). In addition, as in coe1, the levels of chloroplast proteins D1, CP43, and CP47 in rug2-1 were equivalent to only about 25% of WT amounts, while PsbO in rug2-1 was reduced to about 60% of WT levels, but RbcL protein amounts were not affected in rug2-1 (Fig. 4). Furthermore, analysis of the expression of Lhcb1.1 revealed that Lhcb mRNAs were also slightly up-regulated in rug2-1, both under normal growth conditions and in the presence of NF and LIN (Fig. 3C, E). These results confirmed that coe1 is a new allele of bsm/rug2.

mTERF4 plays an important role in group II intron splicing of certain plastid transcripts, and the null bsm mutant seriously affected the global expression of plastid genes (Figs...
In this respect, our coe1 mutant showed a relatively weak bsm phenotype (Figs 5 and 8A–D) (Babiychuk et al., 2011). This suggested a possible connection between processing of plastid transcripts and retrograde signaling. To address this possibility, the processing of plastid-encoded transcripts was investigated in gun1, gun4, gun5, and coe1 plants. As shown in Fig. 8E–H and Supplementary Fig. S9 at JXB online, under standard growth conditions, the processing of atpF, clpP, rpl2, and rps12 was normal in gun1, gun4, and gun5, but was strongly perturbed in coe1 compared with WT*. Similarly, exposure to LIN, like treatment with SPE (Babiychuk et al., 2011), strongly inhibited the processing of atpF, rpl2, and rps12 in WT* seedlings (Fig. 8E–H and Supplementary Fig. S9). Strikingly, gun5 markedly mitigated the effects of SPE on the processing of atpF and clpP transcripts (Fig. 8E–H and Supplementary Fig. S9). In contrast, gun1 did not alter the inhibitory effect of LIN and SPE on the processing of atpF, clpP, rpl2, and rps12 RNAs (Fig. 8E–H and Supplementary Fig. S9), and further repressed the mRNA levels of atpF, clpP, rpl2, and rps12 in the presence of LIN but not SPE (Fig. 8A–D and Supplementary Fig. S9). In fact, in this context, coe1 behaved like gun1 with respect to atpF and rpl2. In WT*, gun4, and gun5 seedlings grown in the presence of LIN and SPE, levels of unprocessed chloroplast transcripts and of Lhcb mRNA followed opposing trends (Fig. 8I), i.e. the more unprocessed chloroplast transcripts present, the less Lhcb1.1 mRNA was detected. However, in gun1 and coe1 grown with either antibiotic, disruption of plastid RNA processing was accompanied by a rise in levels of Lhcb1.1 mRNA. These results suggested a link between the accumulation of non-processed transcripts and PGE-dependent signaling.

Furthermore, the higher accumulation of atpF, clpP, rpl2, and rps12 in coe1 and also partly in gun1 (Fig. 8) and psaB, atpB, and petA transcripts (Fig. 5) in coe1 could be the result of higher transcription rates of these transcripts. To test this, run-on transcription assays were carried out on isolated chloroplasts of 2-week-old WT*, coe1, and gun1 seedlings. As shown in Supplementary Fig. S10 at JXB online, no signals were detected in the controls with the nucleus-encoded RbcS1a gene and the mitochondrial-encoded atp1 gene, but the transcripts of the chloroplast-encoded genes atpB, clpP, atpF, petA, psbC, psaB, rpl2, and rps12 were detected. In general, in all genotypes, psbC, psaB, and rpl12 showed relatively strong transcription rates, while the other genes were relatively
weakly transcribed (Supplementary Fig. S10). Compared with those of WT*, the transcription rate for \( \text{psaB} \) was 3-fold higher in \( \text{coe1} \), and those for \( \text{atpF} \) and \( \text{clpP} \) were 2-fold higher in \( \text{coe1} \) but only slightly elevated in \( \text{gun1} \) (Supplementary Fig. S10). These results suggested that elevated mRNA levels of some chloroplast-encoded genes in \( \text{coe1} \) might be caused by higher transcription rates.

**COE1 genetically interacts with GUN1**

Mutations in mTERF4 and GUN1 have similar effects on \( \text{atpF} \) and \( \text{rpl2} \) RNA processing (Fig. 8). Moreover, both proteins potentially interact with nucleic acids (Koussevitzky et al., 2007; Babiychuk et al., 2011, and this study). We therefore tested whether these two proteins functionally interacted with each other. To this end, we first investigated the genetic relationship between GUN1 and mTERF4 (Fig. 9). In fact, overexpression of \( \text{GUN1 (oe} \text{GUN1-GFP} / \text{coe1)} \) could partially rescue the pale green phenotype of \( \text{coe1} \) under normal growth conditions (Fig. 9B). Levels of luminescence of \( P_{\text{Lhcb1.1}:LUC} \) were also slightly lower in \( \text{oe} \text{-GUN1-GFP} / \text{coe1} \) than in \( \text{coe1} \) (Fig. 9C). In parallel, the value of \( \text{Fv/Fm} \) was slightly higher in \( \text{oe} \text{-GUN1-GFP} / \text{coe1} \) than in \( \text{coe1} \) (Fig. 9D). These results suggested that overexpression of \( \text{GUN1} \) may partially compensate for the defect of \( \text{coe1} \) in the regulation of chloroplast biogenesis with respect to leaf coloration and \( \text{Fv/Fm} \). In addition, we also generated a \( \text{gun1 coe1} \) double mutant. Compared with \( \text{coe1} \), the \( \text{gun1 coe1} \) mutant showed a more severe leaf color phenotype and grew more slowly (Fig. 9B), but levels of \( P_{\text{Lhcb1.1}:LUC} \) luminescence were not affected in the \( \text{gun1 coe1} \) double mutant (Fig. 9C). The value
of Fv/Fm of gun1 coe1 was also lower than that in coe1 (Fig. 9D). These results indicated that GUN1 is required to maintain chloroplast biogenesis and function when COE1 is impaired.

The potential for functional interaction between GUN1 and COE1 prompted us to test whether GUN1 could physically interact with COE1 in a yeast two-hybrid system. The coding sequence of GUN1 was cloned into the yeast bait vector pGBKT7 to generate GUN1-BD and the coding sequence of COE1 was cloned into the prey vector pGADT7 to produce COE1-AD. The resulting co-transformants expressing both GUN1-BD and COE1-AD were analyzed for growth on plates lacking His, Leu, Ade, and Trp (SD–His–Leu–Trp–Ade), and their α-galactosidase activities were assayed. As in the negative control, no interaction between GUN1 and COE1 was detectable, suggesting that the two proteins did not physically interact (Supplementary Fig. S11).

Alternatively, COE1 might affect the function of GUN1 by modulating its distribution in the plant. Analysis of the tissue localization of GUN1 fused to green fluorescent protein (GFP) indicated that the GFP fluorescence was prominent primarily in guard cells and in leaf-vein cells of cotyledons in WT* under normal growth conditions (Fig. 10A).

Interestingly, in the presence of NF, enhanced GFP fluorescence was observed in all epicotyls and hypocotyls of the seedlings (Fig. 10B). In the coe1 genetic background, the GUN1–GFP fluorescence showed a similar trend to that seen in NF-treated WT seedlings (Fig. 10C). These results suggest that coe1 may affect the accumulation or distribution of GUN1. Alternatively, the defects in processing of plastid transcripts in coe1 may cause a stress syndrome similar to that induced by NF, thereby changing the behavior of GUN1–GFP.

**Discussion**

Plastid retrograde signaling is essential for the biogenesis and development of chloroplasts because of its impact on the expression of PhANGs (Mochizuki et al., 1996; Larkin et al., 2003; Koussevitzky et al., 2007; Kleine et al., 2009). However, in spite of its fundamental importance, little is known about the molecular nature of the process. In order to dissect the mechanism of retrograde signaling, we analyzed the expression of plastid and nuclear genes in gun mutants and WT seedlings. Interestingly, under normal growth conditions, levels of nucleus-encoded Lhcb transcripts were slightly
higher in gun mutants than in WT at early stages of seedling development (Fig. 3A, E). Furthermore, gun mutants showed a subtle decrease in the expression of the plastid-encoded \( \text{rbcL} \) mRNA during the same developmental phase (Fig. 3F, G). These results clearly indicated that the activity of PGE is impaired in gun mutants. However, how the PGE-dependent signal is produced, and how it is modulated by GUN1, is unknown. In this study, we isolated a novel mutant, coe1, which shows up-regulation of \( \text{Lhcb1.1} \) expression under normal growth conditions (Figs 1 and 3A, E). The coe1 mutant is also characterized by slightly higher PhA NG expression than WT in the presence of NF and LIN (Fig. 3B, C, E and Supplementary Fig. S4), suggesting that COE1 plays a role in modulating retrograde plastid signaling.

The coe1 mutant displayed a pale yellow leaf phenotype, suggesting that the biogenesis of chloroplasts is impaired (Supplementary Fig. S2). The maximum quantum efficiency of PSII (Fv/Fm) was indeed substantially reduced in coe1 relative to WT and other gun mutants (Supplementary Figs S2 and S5). The accumulation of chloroplast proteins was also significantly decreased in coe1 and rug2-1 (Fig. 4), and the levels of thylakoid membrane complexes were also clearly decreased in coe1 and rug2-1 but almost unchanged in gun1 compared with WT* (Fig. 4B, C). Furthermore, as a secondary effect of reduced thylakoid complex accumulation in coe1, Lhcb1 protein levels were also decreased (Fig. 4). Like protein levels of Lhcb1, RbcS proteins levels were also reduced in coe1 (Fig. 4), which is likely the consequence of the reduced RbcL levels due to disturbed chloroplast translation in this mutant. Although chloroplast proteins accumulated to lower levels in coe1, the amount of plastid transcripts in coe1 was about the same or even up-regulated compared with WT* (Fig. 5). This can be explained by the fact that the majority of transcripts for \( \text{petA} \), \( \text{psaB} \), and \( \text{atpB} \) might not be engaged in translation (Supplementary Fig. S7). Interestingly, in run-on experiments, levels of \( \text{psaB} \), \( \text{atpF} \), and \( \text{clpP} \) were increased in coe1 and slightly increased in gun1, suggesting that at least COE1 might modulate the transcription activity of plastid genes.
Unlike prosl, which is defective in PGE and associated with a reduction of PhANG expression under normal growth conditions (Pesaresi et al., 2006), transcription of Lhcb1.1 was increased in coel (Figs 2B and 3A), while levels of Lhcb1 protein were significantly lower in coel than in WT under normal growth conditions (Fig. 4).

Molecular cloning of COEI revealed that it codes for mTERF4, and is allelic to BSM/RUG2/ZmmTERF4 (Babiychuk et al., 2011; Quesada et al., 2011; Hammani and Barkan, 2014). The mTERFs form a large and complex protein family in both metazoans and plants (Kleine, 2012; Kleine and Leister, 2015). In stark contrast to the case in mammals, the functions of mTERFs in plants are poorly understood (Kleine, 2012). The mTERF family in plants is considerably larger than in Metazoa; for example, A. thaliana and Oryza sativa Japonica contain at least 35 and 48 genes for mTERF proteins, respectively (Babiychuk et al., 2011; Kleine et al., 2012), all of which are predicted or known to localize to mitochondria or chloroplasts (Babiychuk et al., 2011; Kleine, 2012). So far, only four plastid mTERFs—SOLDAT10, BSM, ZmmTERF4, and TWIR1/mTERF9—have been identified and functionally characterized (Meskauskiene et al., 2009; Babiychuk et al., 2011; Mokry et al., 2011; Quesada et al., 2011). In this study, we described coel as a new allele of bsmrug2/Zmmterf4 that causes similar defects in the accumulation of chloroplast proteins and the biogenesis of chloroplasts (Fig. 4). Expression of PhANGs was slightly up-regulated in coel under normal growth conditions. Furthermore, coel also showed a gun phenotype in the presence of NF (Fig. 3). Genetic analysis revealed that the effects of coel on the expression of Lhcb1 were partially dependent on GUN1 (Fig. 9). Yeast two-hybrid analysis indicated that GUN1 does not interact with COEI (Supplementary Fig. S11), but overexpression or loss of GUN1 in coel can partially rescue or aggravate its defects in the regulation of the biogenesis of chloroplasts. More interestingly, coel can also regulate GUN1 function by affecting its accumulation and distribution. For instance, compared with WT, both the level and the distribution of GUN1–GFP fluorescence are altered in coel under normal growth conditions (Fig. 10). Taken together, these results suggest that GUN1 and COEI do interact at some level in regulating the expression of plastid genes and PhANGs under certain physiological conditions.

In WT*, LIN and SPE treatments lead to the accumulation of non-processed RNA (Fig. 8). The coel mutant accumulates high levels of unprocessed RNAs even under normal growth conditions (Fig. 8). The gun1 mutation did not dramatically alter RNA processing in the presence of LIN but instead seemed to affect the expression of plastid genes (Fig. 8). Alterations in chlorophyll metabolism might affect the processing of transcript stability/mutation, because for example, the chlorophyll-deficient mutants atcsl and atcaf2 (Asakura and Barkan, 2006) and rice white stripe leaf (wst) (Tan et al., 2014) show defects in the processing of plastid transcripts. The gun4 and gun5 mutants, in which chlorophyll metabolism is perturbed, indeed showed subtle alterations in the processing of rps12 and atpF transcripts under normal conditions but not in the presence of LIN and SPE (Fig. 8). Comparative analysis revealed that levels of unprocessed plastid transcripts are negatively correlated with expression levels of Lhcb1.1 in WT plants exposed to LIN and SPE, and in gun4 and gun5 plants, but not in gun1 or coel. These results suggest that the accumulation of unprocessed plastid transcripts might trigger plastid signaling to inhibit gene expression of nuclear photosynthesis genes. In addition, altered mTERF4 levels affected the intracellular accumulation and distribution of GUN1, as well as its plastid signaling activity. Taken together, these results suggest that GUN1 and COEI cooperate in PGE and retrograde signaling (Supplementary Fig. S12).

**Supplementary data**

Supplementary data are available at *JXB* online.

**Table S1.** List of oligonucleotides used in this study.

**Fig. S1.** LUC activity in *P*Lhcb1::LUC plants can be suppressed by treatments with LIN or NF.

**Fig. S2.** The coel phenotype is especially prominent during early development of chloroplasts.

**Fig. S3.** Quantification of steady-state *Lhcb1.1* mRNA levels in coel and WT* plants during early plant development.
Fig. S4. Transcripts of PhANGs are slightly increased in NF-treated coel plants.

Fig. S5. Photosynthetic performance of mutant (gun1, gun4, gun5, coel, rug2-1 and rug2-2) and WT* plants.

Fig. S6. Growth phenotype of gun1, gun4, gun5, coel and WT* on soil. Plants were grown on soil in a climate chamber for 3 weeks, on a 12-h light/12-h dark regime.

Fig. S7. Polysome association analysis for chloroplast transcripts in WT*, coel and gun1 plants.

Fig. S8. Complementation of the coel mutation by AT4G02990.

Fig. S9. Analysis of plastid transcript processing in gun1, gun4, gun5, coel, and WT*.

Fig. S10. Transcription rates of plastid genes in WT*, coel and gun1 seedlings.

Fig. S11. GUN1 does not interact with COE1 in yeast-two-hybrid experiments.

Fig. S12. A model for the functional relationship of mTERF4 and GUN1.

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References


