Short title: CGL20 is a plastid 50S ribosome biogenesis factor

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The Arabidopsis Protein CGL20 is Required for Plastid 50S Ribosome Biogenesis

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One-sentence summary: A green lineage-specific protein is involved in late stages of plastid ribosome biogenesis.

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AUTHOR CONTRIBUTIONS
B.R., E.V., D.L., and T.R. designed research. B.R., E.V., G.M., T.K., C.B., P.J., and T.R. performed research. B.R., E.V., G.M., T.K., P.J., C.B., D.L., and T.R. analyzed data. The manuscript was written by B.R., D.L., and T.R., with contributions from all other authors.

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ABSTRACT

Biogenesis of plastid ribosomes is facilitated by auxiliary factors that process and modify ribosomal RNAs (rRNA) or are involved in ribosome assembly. In comparison to their bacterial and mitochondrial counterparts, the biogenesis of plastid ribosomes is less well understood and few auxiliary factors have been described so far. In this study, we report the functional characterization of CONSERVED ONLY IN THE GREEN LINEAGE20 (CGL20) in Arabidopsis thaliana (AtCGL20), which is a proline-rich, ~10 kD protein that is targeted to mitochondria and chloroplasts. In Arabidopsis, CGL20 is encoded by segmentally duplicated genes of high sequence similarity (AtCGL20A and AtCGL20B). Inactivation of these genes in the atcgl20ab mutant led to a visible virescent phenotype and growth arrest at low temperature. The chloroplast proteome, pigment composition, and photosynthetic performance were significantly affected in atcgl20ab mutants. Loss of AtCGL20 impaired plastid translation, perturbing the formation of a hidden break in the 23S rRNA and causing abnormal accumulation of 50S ribosomal subunits in the high-molecular-mass fraction of chloroplast stromal extracts. Moreover, AtCGL20A-eGFP fusion proteins co-migrated with 50S ribosomal subunits in sucrose density gradients, even after RNase treatment of stromal extracts. Therefore, we propose that AtCGL20 participates in the late stages of the biogenesis of 50S ribosomal subunits in plastids – a role which presumably evolved in the green lineage as a consequence of structural divergence of plastid ribosomes.
INTRODUCTION

The vast majority of the ~3000 chloroplast proteins are encoded in the nuclear genome and only ~120 genes have been retained in a small, ~150-kb plastid genome of cyanobacterial origin (Leister, 2003). Plastid genes are either transcribed by a plastid-encoded, bacterial-type or a nucleus-encoded, phage-type RNA polymerase (reviewed in Pfannschmidt et al., 2015). Several plastid genes are organized in operons, and polycistronic transcripts undergo a variety of processing steps, including splicing, editing and endonucleolytic cleavage (reviewed in Germain et al., 2013). Despite the complexity of post-transcriptional RNA metabolism in the organelle, plastid gene expression is considered to be controlled mainly at the translational level (Sun and Zerges, 2015; Zoschke and Bock, 2018) and therefore depends on the activity of plastid ribosomes. Like those of their bacterial ancestors, plastid ribosomes are made up of a small 30S (SSU) and a large 50S (LSU) subunit, and contain catalytic ribosomal RNAs (rRNA) as well as at least 50 ribosomal proteins (RPs). The overall length of the unprocessed chloroplast rRNA corresponds approximately to that of rRNAs found in bacteria; however, the 3’ end of the 23S plastid rRNA is further processed to yield a 4.5S fragment after LSU maturation (Keus et al., 1984; Leal-Klevezas et al., 2000). In higher plants, the 23S rRNA is additionally subjected to post-maturation processing resulting in three fragments, separated by so-called hidden breaks (Kössel et al., 1985). In comparison to bacterial ribosomes, chloroplast ribosomes show differences in RP composition, and some harbour additional extensions, leading to an overall increase in molecular mass of about 170 kDa (Yamaguchi et al., 2000; Yamaguchi and Subramanian, 2000; Yamaguchi and Subramanian, 2003). No homologs of the bacterial subunits bl25 and ul30 have been identified in chloroplasts, but five plastid-specific ribosomal proteins (PSRPs) are associated with chloroplast ribosomes in stoichiometric amounts (Yamaguchi et al., 2000; Yamaguchi and Subramanian, 2000; Yamaguchi and Subramanian, 2003; Sharma et al., 2010).

Recently, structures of chloroplast ribosomes from spinach (Spinacia oleracea) have been determined at high resolution (Ahmed et al., 2016; Bieri et al., 2017; Graf et al., 2017; Perez Boerema et al., 2018), revealing the exact positions and binding partners of PSRPs. PSRPs 2, 3, and 4 form part of the SSU, whereas PSRPs 5 and 6 associate with the LSU. Moreover, only the loss of PSRP3, PSRP4, or PSRP5 significantly disrupts ribosome biogenesis and translation (Tiller et al., 2012). Hence, as in bacteria, not all RPs are essential for chloroplast ribosome function or biogenesis, whereas some non-essential proteins in bacteria have been shown to be indispensable for plants (reviewed in Tiller and Bock, 2014).
Biogenesis of bacterial ribosomes has been studied extensively (reviewed in Shajani et al., 2011; Davis and Williamson, 2017) but the corresponding process in chloroplasts is less well understood. The rRNAs are encoded in a single plastid operon, whereas the majority of RPs are encoded in the nucleus and imported post-translationally into the chloroplast. As expected from the bacterial model, chloroplast ribosome biogenesis is initiated by rRNA transcription and co-transcriptional binding of RPs (Miller et al., 1970). Subsequent rRNA processing and RP-assisted folding result in the independent assembly of the SSU and LSU (Davis and Williamson, 2017). In bacteria, approximately 100 auxiliary factors that are involved in rRNA processing and the integration of RPs have been identified (Shajani et al., 2011). Some of these have been shown to mediate similar functions in chloroplasts (reviewed in Bohne, 2014; Liu et al., 2015; Jeon et al., 2017; Janowski et al., 2018). Despite the dual genetic origin of plastid RPs – which necessitates spatiotemporal orchestration of RP assembly – and significant structural differences with respect to bacterial ribosomes, few chloroplast-specific ribosome biogenesis factors have been identified to date (Bohne, 2014; Wang et al., 2016; Meurer et al., 2017; Paieri et al., 2017; Pulido et al., 2018).

Here, we report the functional characterization of CONSERVED ONLY IN THE GREEN LINEAGE 20 (CGL20) in Arabidopsis thaliana (AtCGL20). AtCGL20 are small, proline-rich proteins that are conserved in photosynthetic eukaryotes. Their loss is associated with a general reduction in chloroplast protein content, which leads to a virescent growth phenotype. Our analyses of mutant lines strongly support the notion that AtCGL20 proteins are required for efficient ribosomal biogenesis in plastids.
RESULTS

The GreenCut protein CGL20 is a small proline- and glutamic acid-rich protein

In a search for previously uncharacterized factors required for chloroplast biogenesis, proteins were considered as candidates if they (i) are shared by photosynthetic eukaryotes from the green lineage but are not found in non-photosynthetic eukaryotes (the so-called “GreenCut” proteins; Merchant et al., 2007; Grossman et al., 2010) and (ii) were identified as recently segmentally duplicated genes in Arabidopsis thaliana (Bolle et al., 2013). One of these gene pairs comprises At2G17240 and At3G24506 (the Arabidopsis equivalents of the Chlamydomonas (Chlamydomonas reinhardtii) gene CGL20, Karpowicz et al., 2011); hence, At2G17240 and At3G24506 are designated hereafter as AtCGL20A and AtCGL20B, respectively. These genes code for proteins of 140 (AtCGL20A) and 149 (AtCGL20B) amino acids, (Fig. 1) and each contain a predicted N-terminal chloroplast transit peptide, such that the mature proteins have a calculated molecular mass of ~10 kD and a calculated isoelectric point of 4.3. All of the CGL20 proteins examined in the green lineage contained proline-rich N-terminal sequences, whereas the C-terminal regions were characterized by a high proportion of acidic amino acids (Fig. 1, Supplemental Table 1).

Lack of AtCGL20 affects plant growth, pigment composition, and photosynthesis

Arabidopsis T-DNA insertion lines for AtCGL20A and AtCGL20B were identified (atcgl20a and atcgl20b) and crossed to generate the double mutant line atcgl20ab (Fig. 2). To analyze transcript abundance in atcgl20 mutants, reverse transcription quantitative PCR (RT-qPCR) analyses were carried out (Fig. 2B). In the wild-type (WT) Col-0 ecotype, AtCGL20A transcripts were found to be four-fold more abundant than their AtCGL20B counterparts. As expected, AtCGL20A and AtCGL20B transcripts failed to accumulate in the respective single-mutant lines and in the double mutant atcgl20ab. No obvious growth phenotype was observed for atcgl20b, but atcgl20a had slightly paler leaves than WT (Fig. 2C). However, growth rate and leaf pigmentation of the double mutant atcgl20ab differed clearly from those of WT plants (Fig. 2C and 2D, Supplemental Table 2), producing a virescent phenotype. Growth rates of atcgl20 mutants were also investigated at low temperature (Supplemental Fig. 1A). All mutants germinated at 4°C, but atcgl20ab stopped growing during the seedling stage and failed to assemble functional PSII complexes, as indicated by the lack of any detectable Fv/Fm signal.
To test whether the disruption of AtCGL20A and AtCGL20B affects photosynthetic performance, Chl $a$ fluorescence and $P_{700}$ parameters were determined (Table 1) using the Dual-PAM-100 system (Walz®). The double mutant exhibited only a moderate decrease in PSII functionality ($F_{v}/F_{m}$: 0.76 ± 0.01), but showed remarkably lower nonphotochemical quenching (NPQ) values (0.27 ± 0.05) than WT plants (0.90 ± 0.13). Interestingly, a significant but less pronounced decline in NPQ was detected in atcgl20a plants (0.66 ± 0.13). Moreover, non-photochemical quantum yields of PSI Y(ND) were increased in atcgl20ab plants, which reflects a donor-side limitation of PSI in photosynthetic electron transport. We also tested for NAD(P)H dehydrogenase-like (NDH) complex activity in atcgl20 mutant lines as described in Armbruster et al. (2013). The typical fluorescence rise after a light-dark switch, which can be mainly attributed to NDH-dependent cyclic electron transport, was observed in Col-0, atcgl20a, and atcgl20b, but not in atcgl20ab (Supplemental Fig. 1C).

To confirm that the atcgl20ab phenotype resulted from the double knockout, the AtCGL20A or AtCGL20B gene was fused to the eGFP-encoding reporter gene, placed under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter, and transformed into the atcgl20ab background (Supplemental Fig. 1B). Each GFP fusion construct was able to complement the double-mutant phenotype, indicating that AtCGL20A and AtCGL20B have redundant functions.

Figure 1. Sequence alignment of Arabidopsis CGL20A (AtCGL20A), CGL20B (AtCGL20B) and homologs from other species in the green lineage. Predicted chloroplast transit peptides are not included in the alignment. Stretches of sequence similarity/identity conserved in the proline-rich N-terminal region in at least 80% of the proteins are highlighted by grey/black shading. At the C-terminal end, acidic (E and D) and basic amino acids (K and R) are marked in red and blue, respectively. Sequence identifiers and further features of these proteins are listed in Supplemental Table 1.
In summary, atcgl20ab has a pgr (proton gradient regulation) phenotype similar to that of the Arabidopsis mutant line pgr1, which is deficient in the Cyt b6f complex (Munekage et al., 2001), and this phenotype is present in a milder form in the single mutant atcgl20a. However, atcgl20ab shows additional defects in photosynthesis, such as a lower level of PSII functionality, lack of detectable NDH activity, and substantially altered chloroplast pigment composition even under normal growth conditions.

**AtCGL20A and AtCGL20B are targeted to chloroplasts and mitochondria**

In previous studies, AtCGL20A, AtCGL20B, and their Chlamydomonas homolog were identified as chloroplast proteins (Bayer et al., 2011; Narsai et al., 2011; Terashima et al., 2011). To confirm protein localization, protoplasts of oeAtCGL20A-eGFP and oeAtCGL20B-
eGFP plants were isolated and examined by confocal laser scanning microscopy (Fig. 3A). In both cases, strong eGFP signals were exclusively detectable in chloroplasts. We also examined the subcellular localization of CGL20 homologs in the green lineage using TargetP (Supplemental Table 1). Chloroplast localization could be predicted for 41 CGL20 homologs, whereas a mitochondrial or an ambiguous localization was predicted for four homologs from Chlorophyta (CGL20 of Chlamydomonas, Volvox carteri, Chlorella variabilis, and Ostreococcus lucimarinus). To identify even small amounts of AtCGL20, which might be targeted to mitochondria and are below the eGFP detection threshold of fluorescence microscopy, we performed cell fractionation and immunodetection assays (Fig. 3B). To this end, an antibody was raised against a synthetic peptide found in both AtCGL20 proteins (see Figure 3).
Materials and Methods for details). We were able to detect AtCGL20A- and AtCGL20B-eGFP fusion proteins in whole-leaf extracts of overexpression plants. Thus, chloroplasts and mitochondria were isolated from oeAtCGL20A-eGFP and oeAtCGL20B-eGFP plants, and subjected to SDS-PAGE and immunodetection assays of marker proteins (Fig. 3B). Both fusion proteins could be detected in mitochondria and were present in both the soluble and insoluble chloroplast fractions.

Since both eGFP fusion proteins showed the same pattern of localization, and atcgl20ab plants overexpressing either AtCGL20A-eGFP or AtCGL20B-eGFP complemented the wild-type phenotype (Supplemental Fig. 1B), subsequent biochemical experiments were carried out exclusively with atcgl20ab plants overexpressing AtCGL20A-eGFP. Next, the association of AtCGL20A-eGFP with high-molecular-mass (HMM) complexes in the chloroplast stroma was analysed (Fig. 3C). Most of AtCGL20A-eGFP was found in the free protein fraction in sucrose step-gradient experiments. However, a significant portion was located in the HMM fraction, together with the ribosomal subunits Rpl2 and Rps1. The fact that neither RuBisCO nor the stromal chaperonin Cpn60 were significantly enriched in the HMM fraction indicated that AtCGL20A-eGFP associates with a stromal complex that is larger than the Cpn60 complex (~800 kD). Thus, AtCGL20 proteins are found in mitochondria, but predominantly in chloroplasts, where they are part of a stromal complex with a molecular weight > 800 kD.

Loss of AtCGL20 function alters the composition of the chloroplast proteome and thylakoid membrane complexes

The dual localization of AtCGL20A and AtCGL20B and multiple alterations in chloroplast function prompted us to investigate changes at the transcriptomic and proteomic level in atcgl20ab (Fig. 4, Supplemental Table 3–7). RNA sequencing analysis revealed that 1254 genes were significantly differentially expressed (adjusted p-value (p-adj) < 0.05) in atcgl20ab relative to WT. Of these, 880 gene transcripts were down- (log2 FC < -0.59) and 374 upregulated (log2 FC > 0.59) (Supplemental Table 3). In addition, the relative abundance of 1920 protein groups was quantified in atcgl20ab with respect to the WT control. Of these, 208 and 216 proteins showed a log2 FC < -0.59 (p-adj < 0.05) and a log2 FC > 0.59 (p-adj < 0.05), respectively (Supplemental Table 4). We further addressed the question of whether any particular cellular compartment is specifically affected in atcgl20ab by examining the subcellular targeting of proteins with altered gene expression or abundance (Fig. 4B). Indeed,
plastid components were overrepresented among those affected at the transcript and protein level. For instance, 27% and 6% of the up- and downregulated transcripts encode plastid proteins, respectively. At the protein level, the effect was even more obvious; 30% proteins with significantly higher and 72% with significantly lower abundances in \textit{atcgl20ab} are localized to the plastid. By contrast, only a small number of mitochondrion-targeted proteins showed significant alterations at the transcript or protein level in \textit{atcgl20ab}.

In light of the pronounced photosynthetic defects in \textit{atcgl20ab} (Table 1 and Supplemental Table 2), we investigated transcript and subunit levels of thylakoid protein complexes in more detail (Fig. 4C, Supplemental Table 5). Overall, only minor changes in the amounts of transcripts coding for thylakoid complex subunits were detected in \textit{atcgl20ab}. By contrast, PSII and PSI subunit levels were clearly reduced in \textit{atcgl20ab}. Remarkably, NDH and Cyt b:f

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\textbf{Figure 4.} Comparative transcriptomic and proteomic analyses of Col-0 and \textit{atcgl20ab}. A, Venn diagram of up- (log, FC > 0.59) or down-regulated (log, FC < −0.59 transcripts or proteins. B, Subcellular distribution of the proteins that are up- (log, FC > 0.59) or down-regulated (log, FC < −0.59) in \textit{atcgl20ab} at the transcriptional (mRNA) or protein level (protein). C, Impact of ATCGL20 disruption on the transcriptional and protein-level expression of thylakoid complex subunits. Asterisks indicate the following \(p\)-values: * < 0.05, ** < 0.01 and *** < 0.001. Colors, which represent down- or up-regulated components in \textit{atcgl20ab}, range from dark blue (log, FC < -2) to dark red (log, FC > 2).
complexes exhibited the most pronounced reductions, together with subunit D1 of PSII (encoded by \textit{psbA}), which is known to exhibit a high turnover rate (Sundby et al., 1993).

To verify the outcome of the proteome analysis, we compared membrane complex integrity and subunit abundance in mitochondria (Supplemental Fig. 3A) and thylakoid membranes isolated from WT and \textit{atcgl20ab} plants (Supplemental Fig. 3B, C) by 2D-BN/SDS PAGE analysis. In line with the proteome data (Supplemental Table 4), no obvious differences in respiratory complex abundance could be detected. Moreover, the analysis of thylakoid complexes confirmed the specific impact of \textit{atcgl20ab} mutations on NDH-PSI and Cyt \textit{b}f (see also Supplemental Fig. 3B). Since the NDH-PSI and the Cyt \textit{b}f complexes are less abundant than other thylakoid complexes on Coomassie-stained 2D gels, these reductions were verified by immunodetection assays (Supplemental Fig. 3B, 3C). We also examined the integrity of thylakoid membrane complexes in the single mutants \textit{atcgl20a} and \textit{atcgl20b}. Interestingly, NDH-PSI and Cyt \textit{b}f complex formation was clearly affected in \textit{atcgl20a}, but not in \textit{atcgl20b} (Supplemental Fig. 3B), as was confirmed by immunodetection of marker subunits for the different thylakoid complexes (Supplemental Fig. 3D). Thus, the \textit{pgr} phenotype (Table 1) and the lack of the post-illumination fluorescence rise in \textit{atcgl20ab} plants (Supplemental Fig. 1C) can be attributed to substantially reduced levels of Cyt \textit{b}f and NDH-PSI complexes, respectively. In spite of the fact that AtCGL20A and AtCGL20B are also localized to mitochondria (Fig. 3B), their absence appears to have no measurable effect on protein complex abundances in these organelles (Supplemental Fig. 3A).

\textbf{Loss of AtCGL20 function affects the chloroplast translational machinery}

\textit{AtCGL20A} and \textit{AtCGL20B} are co-expressed with genes involved in plastid gene expression, in particular with genes coding for chloroplast RPs (Supplemental Table 8). Given that \textit{atcgl20ab} shows a virescent phenotype (Fig. 2C), which is often associated with disruptions in chloroplast protein homeostasis (Koussevitzky et al., 2007) and reductions in chloroplast protein level (Fig. 4B) and thylakoid complex amounts (Fig. 4C), we investigated perturbations in chloroplast gene expression in more detail. To this end, we compared plastome-encoded transcript and protein levels of 31 RNA-protein pairs between \textit{atcgl20ab} and the WT control (Fig. 5A). Only moderate changes were observed at the transcript level; 11 transcripts were significantly (p-adj < 0.05) upregulated (log\textsubscript{2} FC > 0.59), but only two were downregulated (log\textsubscript{2} FC < 0.59). Remarkably, at the protein level, the opposite trend was
apparent: the abundances of 10 plastid-encoded proteins derived from different plastid operons were reduced (log2 FC > 0.59), whereas only three proteins were increased in amount (Rpl20, Rpl22, and Ycf3).
To test plastid translation in atcgl20ab, we investigated in vivo incorporation of $^{35}$S-methionine into de novo plastid proteins of WT and atcgl20ab plants grown either under 22°C or shifted to 4°C (Fig. 5B). Mutant plants grown under moderate temperature showed ~50% labeling efficiency, whereas atcgl20ab plants subjected to 4°C incorporated only ~25% of $^{35}$S-Met compared to WT plants. To further determine whether atcgl20ab is defective in chloroplast translation initiation or termination, we examined the association of several plastid-encoded RNAs with chloroplast ribosomes. To this end, polysome-enriched samples were isolated from WT and atcgl20ab leaves under polysome-preserving conditions (Barkan, 1993) and further fractionated by sucrose density-gradient centrifugation (Fig. 5C). Subsequently, total RNAs were isolated from twelve fractions, and subjected to denaturing gel electrophoresis and RNA-gel-blot analyses using selected plastid transcript probes. Transcripts of psaA, psbA, rbcL, ndhH, and petB did not show a shift to lower or higher density fractions, indicating that plastid translation initiation or termination is not perturbed in atcgl20ab. Similarly, the distribution of plastid rRNAs (rrn5, rrn16, and rrn23) was unchanged. However, we detected non-processed 23S rRNAs of the LSU in fractions 4–11, implying that, in the double mutant, both free and actively translating chloroplast ribosomes contain unprocessed 23S rRNAs.

Relative amounts of rRNAs are sensitive indicators of plastid SSU and LSU abundances, since their steady-state levels largely depend on their efficient association with ribosomal subunits. In addition, rRNA quantification allows the assignment of a biogenesis defect either to the SSU or LSU, as the subunits are assembled independently and only form an SSU/LSU complex during translation (Walter et al., 2010; Tiller et al., 2012; Fristedt et al., 2014). Thus, we monitored the accumulation of the SSUs and LSUs of cytosolic and chloroplast ribosomes by quantifying their rRNA species using a microfluidics-based approach (Fig. 5D). The ratios of cytosolic LSU to cytosolic SSU (25S/18S), cytosolic SSU to chloroplast SSU (18S/16S), cytosolic SSU to chloroplast LSU (18S/23SHB1), and chloroplast SSU to chloroplast LSU (16S/23SHB1) were determined from WT and atcgl20ab plants (Fig. 5D). In contrast to the 25S/18S and 18S/16S ratios, which were only slightly altered in atcgl20ab, the 18S/23SHB1 and 16S/23SHB1 ratios were two-fold higher in atcgl20ab plants than in WT.

From these results, we deduced that lack of AtCGL20A and AtCGL20B alters chloroplast LSU biogenesis. However, the defect seems not to interfere with translation initiation or termination (Fig. 5C), but affects the elongation step (Fig. 5B), leading to lower accumulation of plastome-encoded proteins in atcgl20ab (Fig. 5A). The fact that the ratio of free to RNA-
associated ribosomes is unchanged in atcg120ab is also consistent with previous studies, which revealed that defects in plastid ribosome biogenesis do not necessarily lead to significant changes in polysome loading patterns (Pesaresi et al., 2001; Nishimura et al., 2010; Chi et al., 2012).

**AtCGL20 is required for LSU biogenesis in chloroplasts**

To investigate the influence of AtCGL20 on chloroplast rRNA maturation, RNA gel-blot experiments were carried out with probes specific for 16S, 4.5S, 5S, and 23S rRNAs, which were hybridized to size-fractionated RNA samples isolated from WT, atcg120a, atcg120b, and atcg120ab plants (Fig. 6A). In contrast to the WT-like levels of 16S, 4.5S, and 5S rRNAs, hybridization experiments with probes specific for 23S rRNAs detected clear changes in the single mutant atcg120a, which were even more pronounced in the double mutant atcg120ab. Unprocessed precursors of 2.9 and 2.4 kb accumulated to significant levels, whereas processed products of the 23S rRNA with lower molecular masses (1.8, 1.3, and 1.1 kb) were markedly reduced (Fig. 6B, Supplemental Table 9). Notably, levels of the smallest hidden-break product (0.5 kb) of the mature 23S rRNA were not affected in atcg120ab.

In light of our observations that a portion of AtCGL20A-eGFP co-migrates with ribosomal subunits in the HMM fraction (> 800 kD) in sucrose step-gradient experiments (Fig. 3C) and that chloroplast LSU biogenesis is impaired in atcg120ab (Fig. 5C, Fig. 5D and Fig. 6A), we performed a comparative proteomic study of the HMM fractions isolated from WT and atcg120ab stromal extracts (Fig 6C, Supplemental Table 10). A MapMan analysis of the data (Supplemental Table 11) showed that the levels of chloroplast LSU proteins (Bin 29.2.1.1.1.2) were significantly changed in atcg120ab (p-value of 2.8 10^-4). Thus, an in-depth comparison of SSU and LSU protein amounts between WT and atcg120ab HMM stromal fractions was carried out (Fig. 6C). No significant reduction in the amounts of any chloroplast RP could be identified in atcg120ab. Although several chloroplast SSU proteins were slightly more abundant in atcg120ab than in the wild-type control, the most pronounced increases (log2 FC > 2) in the double mutant were observed for the LSU subunits Rpl32, Rpl4, Rpl28, and Rpl3 (Fig. 6C).

Taken together, these data confirm that absence of AtCGL20 has a specific impact on chloroplast LSU biogenesis, whereas chloroplast SSU biogenesis is unaffected. This conclusion is corroborated by the finding that only 23S rRNA maturation is clearly altered in...
atcgl20ab (Fig. 6A and B). In addition, several chloroplast LSU proteins are more prominently represented in the stromal HMM fraction (> 800 kD) from atcgl20ab than are constituents of the SSU (Fig. 6C).

**AtCGL20A-eGFP co-migrates with chloroplast ribosomes**

Several factors involved in rRNA processing have been shown to be physically associated with ribosomes or ribosomal precursor complexes (Chi et al., 2012; Meurer et al., 2017; Paieri...
et al., 2017). Since a portion of AtCGL20A-eGFP had been found in the HMM fraction in sucrose step-gradient experiments (Fig. 3C), we extended our co-migration analysis by performing sucrose density-gradient centrifugation of stromal fractions isolated under conditions in which SSU and LSU were partially dissociated from each other (Fig. 7A). Rps1, a marker subunit for the chloroplast SSU, was immunodetected in fractions 22–35, with two distinct peaks in fractions 26/27 and 31/32 (Fig. 7A). By contrast, the marker subunit Rpl2 for the LSU was detected in fractions 27–38 and predominantly in fractions 29–33. These results indicated that the SSU could be partially dissociated from the LSU and was enriched in fractions 26–27, whereas a mixture of dissociated LSU and fully assembled ribosomes was found in fractions 28–35. The fusion protein AtCGL20A-eGFP was found in fractions 22–25,
29–36 and fraction 38, but did not appear in fractions 26–28. Consequently, AtCGL20A-eGFP does not co-migrate with the dissociated SSU, but together with either the detached chloroplast LSU or as part of fully assembled ribosomes (Fig. 7A).

A specific characteristic of rRNAs in ribosomal pre-complexes is their susceptibility to RNase treatments, whereas rRNAs in fully assembled ribosomes are tightly packed with RPs and are protected from degradation (Williams and Barkan, 2003; Meurer et al., 2017). As a consequence, biogenesis factors that interact with ribosomal pre-complexes tend to lose their association with rRNAs when treated with RNase (Meurer et al., 2017; Paieri et al., 2017). To investigate an association of AtCGL20A-eGFP with ribosomal pre-complexes, isolated stromal extracts of oeAtCGL20A-eGFP overexpression lines were either treated with RNase A or an RNase inhibitor (control) and subsequently subjected to two-dimensional BN/SDS-PAGE analysis (Fig. 7B). After protein transfer, PVDF membranes were probed with AtCGL20- and Rpl2-specific antibodies. Although most of the AtCGL20A-eGFP fusion and Rpl2 were detected in the free protein fraction, a significant portion of both proteins co-migrated with the HMM fraction (> 800 kD) in the control, which was previously shown to contain ribosomal subunits (Bučinská et al., 2018). Strikingly, Rpl2 and AtCGL20A-eGFP signals increased in the HMM fraction after RNase treatment. Therefore, we conclude that AtCGL20 is involved in late steps in the biogenesis of the chloroplast LSU, since AtCGL20A-eGFP co-migrates with LSU complexes (Fig. 7A) that are resistant to RNase treatment (Fig. 7B).
DISCUSSION

CGL20 function emerged in the green lineage

Although the core structures and functions of bacterial and chloroplast ribosomes are conserved, considerable changes have occurred over the course of chloroplast ribosome evolution. These include differences in rRNA processing and the addition of five new subunits (PSRP2–6) as well as several structural features unique to chloroplast ribosomes (Bieri et al., 2017), which might have evolved as a consequence of the need to translate a substantially reduced number of chloroplast-encoded proteins (Tiller and Bock, 2014; Sun and Zerges, 2015; Graf et al., 2017). Furthermore, the massive transfer of genes to the nuclear genome has increased the complexity of organellar ribosome biogenesis, as both auxiliary factors and RPs have to be imported into chloroplasts and correctly assembled with plastome-encoded rRNAs and RPs. It is therefore evident that, owing to the relocation of genetic information to the nucleus, additional auxiliary factors became necessary in order to maintain efficient ribosome biogenesis. Indeed, several factors required for chloroplast LSU biogenesis, such as DCL, RH39, RH22, or RH50, have no orthologues in bacteria (Bellaoui et al., 2003; Nishimura et al., 2010; Chi et al., 2012; Paieri et al., 2017) and, like CGL20, first emerged in the green lineage.

Plants that lack AtCGL20 show a characteristic perturbation in chloroplast biogenesis

A large number of virescent Arabidopsis mutants with impaired chloroplast biogenesis have been described in the literature (Pesaresi et al., 2001; Sugimoto et al., 2004; Koussevitzky et al., 2007; Tillich et al., 2009; Chi et al., 2012; Janowski et al., 2018). Their defects are often associated with perturbations in chloroplast protein homeostasis (Sugimoto et al., 2004; Koussevitzky et al., 2007; Yu et al., 2008; Chi et al. 2012). Since pronounced pleiotropic effects usually accompany their disruption, the precise molecular functions of chloroplast biogenesis factors are often difficult to derive from analyses of mutant lines. Moreover, the present study was complicated by the low abundance of AtCGL20 proteins, and the absence of a clear domain prediction (Fig. 1). For these reasons, we turned to large-scale transcriptome and proteome analyses to compare atcgl20ab plants with their WT counterpart (Fig. 4). In line with observations made with other virescent Arabidopsis mutants (Kim et al., 2009; Janowski et al., 2018), atcgl20ab exhibited a general reduction in chloroplast protein content (Fig. 4B), as well as diminished abundance of thylakoid membrane complexes (Fig. 4).
Remarkably, Cyt \textit{b6f} and NDH complexes were those most affected in \textit{atcgl20ab} (Fig. 4C, Supplemental Fig. 3B), which were reduced to \textsim30\% and \textsim10\% of WT levels, respectively. This finding effectively accounts for the combined \textit{pgr} and chlororespiratory reduction (\textit{crr}) phenotype of \textit{atcgl20ab} (Table 1, Supplemental Fig. 2). A similar photosynthetic phenotype has been reported for the Arabidopsis mutant \textit{pgr3-1}, which is disrupted in a chloroplast pentatricopeptide-repeptide-repeat protein required for post-transcriptional steps in organellar gene expression (Yamazaki et al., 2004). However, selective disruption of Cyt \textit{b6f} and NDH complexes in \textit{pgr3-1} mutants did not result in any significant changes on PSII and PSI levels or photosynthetic growth rates under moderate light intensities. Accordingly, we can exclude a specific role for \textit{AtCGL20} in Cyt \textit{b6f} or NDH complex biogenesis, and we therefore interpret their low levels as an indirect consequence of impaired chloroplast translation in the double mutant (Fig. 5).

Despite the dual localization (Fig. 3), transcript and protein levels of mitochondrial components (Fig. 4B) and respiratory complexes (Supplemental Fig. 3) were essentially unaffected in \textit{atcgl20ab}. Notably, about 500 proteins with ambiguous transit peptides are predicted in Arabidopsis to be dual-targeted to mitochondria and chloroplasts (Mitschke et al., 2009). However, a function in both organelles could only be attributed to some of them (e.g. Kmiec et al., 2014; Mazzoleni et al., 2015) and seems unlikely for others (Rödiger et al., 2011; Baudisch et al., 2014). It is therefore assumed that a certain degree of mistargeting is tolerated and might be the consequence of the evolution of the plastid import machinery (Sharma et al., 2018). On the other hand, a large group of proteins that were experimentally shown to be dual targeted are involved in organellar gene expression and in particular in translation (reviewed in Carrie and Small, 2013). We therefore conclude that \textit{AtCGL20} is critical for chloroplast processes, although we cannot exclude the possibility that \textit{AtCGL20} might mediate mitochondrial functions in other developmental stages, plant organs, or specific growth conditions.

\textbf{\textit{AtCGL20} functions in chloroplast LSU biogenesis}

Our current knowledge of chloroplast ribosome biogenesis is scant and mostly inferred from bacterial ribosome biogenesis, in which the two subunits are assembled independently in a dynamic process through coordinated folding of rRNAs and association of sets of RPs (Davis and Williamson, 2017). The order of bacterial RP binding is highly flexible and several
parallel assembly pathways have been identified (Mulder et al., 2010; Davis et al., 2016). However, LSU assembly is more complex, owing to a more intricate rRNA folding pathway, a larger number of RPs, and the need to form a functional peptidyl transfer centre as well as the polypeptide tunnel exit. Chloroplast SSUs and LSUs are assembled independently, as has been demonstrated in studies of mutants altered in chloroplast LSU and SSU structure (Nishimura et al., 2010; Chi et al., 2012; Tiller et al., 2012; Janowski et al., 2018). In addition, several auxiliary chloroplast ribosome biogenesis factors have been identified, a significant number of which are DEAD-box RNA helicases, GTPases, or rRNA methylases (reviewed in Bohne, 2014). Recent proteome (Majeran et al., 2012) and several GFP-fusion-based localization studies have shown that many of these factors are enriched in plastid nucleoids, which supports the assumption that ribosome assembly takes place in this intra-organellar sub-compartment (Bohne, 2014). By contrast, protoplast studies (Fig. 3) showed that AtCGL20A- and AtCGL20B-eGFP did not display typical nucleoid localizations (Meurer et al., 2017). The uniform distribution of eGFP signals in the chloroplast might be explained by over-accumulation of fusion proteins in the free stromal fraction (Fig. 3C, Fig. 7B), masking nucleoid-associated AtCGL20A- and AtCGL20B-eGFP signals. Furthermore, it cannot be excluded that the large eGFP tag might interfere with nucleoid association.

Our study provides four lines of evidence that AtCGL20 is involved in late steps in the assembly of the chloroplast LSU: i) 23S rRNA maturation is specifically impaired in atcgl20ab mutants, whereas 16S rRNA processing is not affected (Fig. 6); ii) several of the protein constituents of the chloroplast LSU over-accumulate in the stromal HMM fraction of atcgl20ab; iii) AtCGL20A-eGFP co-migrates with LSU complexes (Fig. 7A); and iv) a sub-fraction of AtCGL20A-eGFP associates with an RNase-insensitive stromal HMM complex (Fig. 7B). Specific impairment of the LSU was reported also by Chi et al. (2012) and other defects in plastid protein homeostasis or chloroplast gene expression can lead to disturbances in 23S rRNA processing (Koussevitzky et al., 2007; Yu et al., 2008). However, the lack of AtCGL20 resulted in a distinct alteration in the 23S rRNA processing pattern (Fig. 6A, B), which has also been observed in nara1-2 and del-1 mutants (Bellaoui et al., 2003; Nishimura et al., 2010). The absence of RH39 in nara1-2 mutants led to a specific disruption in the processing of the second hidden-break site in 23S rRNAs. As a consequence, nara1-2 plants showed normal levels of the 0.5-kb hidden-break product, whereas 1.1- and 1.3-kb fragments were markedly reduced (see also Fig. 6B). RH39 was proposed to be directly involved in introducing the hidden break by binding to the 23S rRNA close to an exposed stem-loop structure. This suggestion was further supported by structural analysis, which confirmed the
accessibility of the putative RH39 binding site (Bieri et al., 2017). However, a function as an RNA chaperone or a direct involvement of AtCGL20 in hidden-break processing is unlikely, since the protein does not contain a canonical RNA-binding domain (Fig. 1). Intriguingly, the mutant line dcl-1, which is disrupted in the chloroplast ribosome biogenesis factor DCL, displayed an altered 23S rRNA processing pattern similar to that seen in atcgl20ab (Bellaoui et al., 2003). DCL does not possess obvious RNA-binding capacity either, but was suggested to enable 4.5S cleavage after binding to the assembled LSU. Likewise, AtCGL20 might be involved in late LSU assembly steps and promote efficient RH39-mediated 23S processing.

Notably, AtCGL20 function seems to be essential for the formation of translationally active chloroplast ribosomes after germination under cold stress, since atcgl20ab plants failed to synthesize chlorophyll or PSII complexes at 4°C and soon stopped growing (Supplemental Fig. 1A). In addition, chloroplast translation is more severely affected in mutant plants shifted to low temperature (Fig. 5B). Pronounced effects at low temperature have also been observed in several other Arabidopsis mutants that are impaired in chloroplast protein synthesis (Sugimoto et al., 2004; Paieri et al., 2017; Pulido et al, 2018), as well as in bacterial strains with defects in ribosome biogenesis (Davis and Williamson, 2017). Thus, alternative LSU assembly pathways might exist which bypass AtCGL20 function. However, its presence becomes crucial under cold stress and LSU intermediates might be blocked in kinetic traps leading to the growth arrest of atcgl20ab plants.

Besides specific alterations in 23S rRNA processing, a significant accumulation of several LSU subunits could be observed in the stromal HMM fraction (> 800 kD) of atcgl20ab plants which might be due to the accumulation of chloroplast LSU pre-complexes (Fig. 6C). On the other hand, no detailed RP compositions of chloroplast ribosome assembly intermediates have yet been described. Reasons for this are technical difficulties in resolving assembly intermediates over their entire molecular mass range and in detecting low-abundant, short-living ribosomal pre-complexes. Moreover, in analogy to the bacterial assembly process, several chloroplast ribosome assembly pathways might exist, which run in parallel or are dynamically regulated during different plant developmental stages. Future work using a combination of sucrose gradient experiments, mass spectrometry, and cryo-EM based technologies (Davis et al., 2016) may provide a deeper understanding about the structure of chloroplast ribosome intermediates and the precise function of AtCGL20 in LSU assembly.
MATERIALS AND METHODS

Bioinformatics Sources

Protein and gene sequences were downloaded from the Arabidopsis Information Resource server (TAIR; http://www.arabidopsis.org), Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and the National Center for Biotechnology Information server (NCBI; http://www.ncbi.nlm.nih.gov/). Protein sequence alignments were performed using the Vector NTI software (Invitrogen). Chloroplast transit peptides were predicted by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/). Information about sequences used in the alignment (Fig. 1) is listed in Supplemental Table 1. Alignments were formatted with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Co-expression analysis was carried out with the ATTED-II webs server (http://atted.jp/; Obajashi et al., 2018). Enrichment analyses of differentially expressed genes and of proteins with altered abundance were carried out with MapMan (Thimm et al., 2004).

Plant Material and Growth Conditions

T-DNA insertion lines for AT2G17240 (atcgl20a, SALK_133989) and AT3G24506 (atcgl20b, SAIL_71_A01) were obtained from the SALK (Alonso et al., 2003) and SAIL collections (Sessions et al., 2002), respectively. The double mutant atcgl20ab was generated by crossing the single-mutant lines atcgl20a and atcgl20b. Plants were grown on potting soil (A210, Stender) under controlled greenhouse conditions (70–90 µE m⁻² s⁻¹, 16/8-h light/dark cycles), or on a 12-h light/12-h dark cycle in climate chambers for biochemical and physiological analyses. Fertilizer was added to plants grown under greenhouse conditions in accordance with the supplier’s recommendations (Osmocote Plus; 15% nitrogen [w/v], 11% [w/v] P₂O₅, 13% [w/v] K₂O, and 2% [w/v] MgO; Scotts Deutschland). Growth kinetics were analyzed on the basis of leaf area, which was determined from photographs taken at different times after germination (7–27 days) and quantified using the ImageJ software (Schneider et al., 2012).

For cold-stress treatment, surface-sterilized mutant and wild-type seeds were plated on Murashige and Skoog (1962) medium (pH 5.8) supplemented with 0.7% [w/v] plant agar (Duchefa). Seeds were allowed to germinate at 4°C under long-day conditions (16-h light/8-h dark) in low light (30 µE m⁻² s⁻¹) for six weeks. Control plates were grown at 22°C under moderate light levels (100 µE m⁻² s⁻¹).
To restore the wild-type phenotype, *AtCGL20A* and *AtCGL20B* coding regions were cloned into the binary Gateway vector pB7FWG2.0 (Karimi et al., 2002), placing the genes under the control of the 35S promoter and fused to the 5′ end of the *EGFP* gene to generate *AtCGL20A*-eGFP and *AtCGL20B*-eGFP fusions. Both constructs were first transformed into *Agrobacterium tumefaciens* strain GV3101 and then into *atcgl20ab* plants by the floral-dip method (Clough and Bent, 1998). T1 plants were selected using a stereo microscope (Lumar.V12, Zeiss) based on a wild type-like growth phenotype and high eGFP signals. After selection of homozygous progenies (annotated as oeAtCGL20A-eGFP and oeAtCGL20B-eGFP), complementation of the *atcgl20ab* mutant phenotype was verified by Dual-PAM analyses.

**Leaf Pigment Analysis**

Leaves were harvested from five-week-old plants grown in climate chambers at 4 h after onset of the light period and homogenized in liquid nitrogen. The samples were extracted with 100% [v/v] acetone and centrifuged (16,000 g) for 20 min at 4°C. Pigment compositions in the supernatants were analysed as described in Färber et al. (1997).

**Chl a Fluorescence and P700 Measurements**

Chlorophyll *a* fluorescence and *P700* measurements were performed using the DUAL-PAM-100 system (Walz) in the dual-channel measuring mode. After five-week-old plants had been dark adapted for 20 min, single leaves were exposed to probe light to measure minimal chlorophyll fluorescence (*F0*) and then to a saturating light pulse (10,000 µE m⁻² s⁻¹, 800 ms) for determination of maximal chlorophyll fluorescence (*Fm*). After steady-state fluorescence yields (*Fₚ*) had been measured during a 10-min exposure to actinic red light (126 µE m⁻² s⁻¹), a saturating light pulse (10,000 µE m⁻² s⁻¹, 800 ms) was applied to determine the maximal fluorescence yield in the light (*Fₘ*'). *Fₘ''* and *F₀''* were measured by applying a saturating light pulse (10,000 µE m⁻² s⁻¹, 800 ms) after a dark relaxation phase of 10 min. Parameters were calculated as described in Rühle et al. (2014). *P700* measurements were carried out using the default settings of the DUAL-PAM software (version 1.19). The parameters Y(I), Y(ND), and Y(NA) were calculated as described in the DUAL-PAM manual.
In vivo Chl a fluorescence of whole plants was measured using an imaging Chl fluorometer (Imaging PAM, Walz). Plants were dark-adapted for 20 min and exposed to a pulsed, blue probe beam and a saturating light flash to calculate $F_v/F_m$. The post-illumination rise in fluorescence was determined as previously described in Shikanai et al. (1998). A saturating light pulse was applied to determine $F_m$, which was followed by a 5-min exposure to actinic light (80 μE m$^{-2}$ s$^{-1}$) and a dark incubation for 5 min to analyze the post-illumination Chl a fluorescence rise.

Confocal Laser Scanning Microscopy and Organelle Isolation

Protoplasts were isolated as described in Rühle et al. (2014) from 20-day-old oeAtCGL20A-eGFP and oeAtCGL20B-eGFP plants. Mitochondria were stained with MitoTracker Red (Thermo Scientific) according to supplier’s instructions. Enhanced GFP signals, MitoTracker and chlorophyll autofluorescence were detected simultaneously with a Leica SP8 confocal microscope. Maximum projections of Z-stacks were processed using the Lightning adaptive deconvolution approach. Chloroplast isolation was performed according to Kunst (1998). Intact chloroplasts were ruptured in a buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5 (KOH) and 10 mM EDTA for 30 min on ice. Soluble proteins were separated from the insoluble fraction by centrifugation (35,000 g) for 30 min at 4°C. Mitochondria were isolated according to Escobar et al. (2006).

AtCGL20 Antibody Generation

Rabbit antibodies were raised against a synthesized peptide sequence (73-PLDFPIEWERPKPG-86) that is conserved in both AtCGL20 proteins. Peptide synthesis, immunization, and affinity purification were performed by BioGenes GmbH (Berlin, Germany). Antiserum was employed in dilutions of 1:200 to 1:1000.

Sucrose Step-Gradient Centrifugation

Sucrose step-gradient centrifugation was performed according to Rivera et al. (2015). Crude stroma was prepared from leaves of 20-day-old seedlings grown under climate-controlled chamber conditions. Plant material was first homogenized in chloroplast isolation buffer (330
mM D-sorbitol, 20 mM Tricine/NaOH pH 7.6, 10 mM NaHCO₃, 0.1% [w/v] BSA, 0.03% [w/v] ascorbate). The homogenate was then filtered through two layers of Miracloth (Calbiochem) and centrifuged (2000 g) for 5 min at 4°C. The pellet was resuspended in chloroplast lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgOAc, 50 mM KCl, 6 mM β-mercaptoethanol). After centrifugation (35,000 g) for 30 min at 4°C, 500 µL of the supernatant (~1.5 mg total protein content) was layered onto 3 mL of a high-salt sucrose solution (10 mM Tris-HCl, pH 7.5, 10 mM MgOAc, 150 mM KCl, 6 mM β-mercaptoethanol, 1 M sucrose). Samples were centrifuged for 12 h (55,000 g) in a TL100.3 fixed-angle rotor. Seven fractions were recovered from the top, and the pellet was resuspended in 100 µL of the residual volume. Samples (20 µL) of each fraction were fractionated by Tricine-SDS-PAGE (Schägger, 2006) containing 10% [w/v] acrylamide, blotted, and probed with antibodies against AtCGL20, Rps1, Rpl2, and Cpn60α as described below (see Supplemental Table 13 for information on the latter antibodies).

Transcriptome Analysis

Total RNA was extracted from four-week-old wild-type and five-week-old atcgl20ab plants, grown under climate-controlled chamber conditions, using the RNasy Mini Kit (Qiagen). RNA quality was tested by agarose gel electrophoresis. Additional quality checks, RNA-Seq library preparation, and long non-coding RNA sequencing (lncRNA-Seq) were performed at Novogene Biotech (Beijing, China) using standard Illumina protocols. The RNA-Seq libraries were sequenced on an Illumina HiSeq 2500 system using the paired-end mode. Three biological replicates were used for each analysis. RNA-Seq reads were analyzed on the Galaxy platform (https://usegalaxy.org/). After grooming FASTQ files, adaptors were removed with Trimmomatic (Bolger et al., 2014), and sequencing quality was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped to the Arabidopsis genome (TAIR10) with the gapped-read mapper TopHat 2.1.1 (Kim et al., 2013) set for Forward Read (FR) unstranded libraries and adjusting the maximum intron length to 5,000 bp. Reads were counted with featureCounts (Liao et al., 2014) with the help of the gene annotation in Araport11 (www.araport.org/data/araport11). Differentially expressed genes were obtained with DESeq2 (Love et al., 2014) running with the fit type set to “parametric”, and applying a 1.5-fold change cut-off and an adjusted p-value of <0.05. Sequencing data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE134415.
Proteome Analysis

Label-free shotgun proteomics was performed with the same plant material as was used for transcriptome analysis. Leaf samples (100 mg, five biological replicates per genotype) were snap-frozen in liquid nitrogen and ground into fine powder. The powder was resuspended in 1 mL of extraction buffer (100 mM HEPES pH 7.5, 150 mM NaCl, 10 mM DTT, 1% [w/v] SDS, 1X Roche cOmplete™ Protease Inhibitor Cocktail) and samples were disrupted by sonication (three 10-sec ON/OFF cycles) with a Branson Sonifier B-12 (Branson Ultrasonics). After removing cell debris by centrifugation at 10,000 g for 30 min, proteins were precipitated in chloroform-methanol (Wessel and Flügge, 1984) and solubilized in 6 M guanidine hydrochloride. Protein concentration was determined by the bicinchoninic assay (BCA, Thermo Fisher Scientific, Waltham, USA). Proteome aliquots (100 μg) were reduced in 10 mM DTT for 30 min at 37°C and alkylated with 50 mM iodoacetamide for 30 min at room temperature in the dark. After purification by chloroform-methanol precipitation, samples were digested with trypsin (proteome/enzyme ratio 100:1 [w/w]) at 37°C overnight. Peptides were desalted with home-made C18 stage tips (Rappsilber et al., 2003), vacuum dried until nearly dry, and stored at -80°C.

The peptide mixture was fractionated on a nano-LC system (Ultimate 3000 RSLC, Thermo Fisher Scientific) equipped with an Acclaim Pepmap nano-trap column (C18, 100 Å, 100 μm × 2 cm, Thermo Fisher Scientific), and an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 μm × 50 cm, Thermo Fisher Scientific). Chromatographic separation was carried out using a 150-min linear gradient of 5–45% acetonitrile at a flow rate of 250 nL per min. The column temperature was set to 50°C. An Impact II high-resolution Q-TOF (Bruker Daltonics, Billerica, USA) was directly coupled to the LC using a CaptiveSpray nano-ESI source (Bruker Daltonics). MS1 spectra were acquired at 3 Hz with a mass range from m/z 200–2000, with the 18 most intense peaks being selected for MS/MS analysis using an intensity-dependent spectrum acquisition time of between 4 and 16 Hz. The dynamic exclusion duration was set to 0.5 min.

The MaxQuant software (version 1.6.1.0 (Cox and Mann, 2008)) was used to process the raw MS files. The built-in Andromeda search engine (Cox et al., 2011) was employed to search MS/MS spectra against the Arabidopsis thaliana UniProt database (version February 2017). Enzyme specificity was set to trypsin, allowing up to two missed cleavages. Cysteine
carbamidomethylation was set as ‘static modification’, and N-terminal acetylation and methionine oxidation as ‘variable modifications’. During the search, sequences of 248 common contaminant proteins and decoy sequences were automatically added. A false discovery rate (FDR) of 1% was applied at the peptide and protein levels. Proteins were quantified across samples using the label-free quantification (LFQ) algorithm (Cox et al., 2014) with default settings. Downstream bioinformatics and statistical analyses were performed using Perseus (version 1.6.1.1 (Tyanova et al., 2016)) and R (version 3.5.0, (Team, 2018)). Potential contaminants, reverse hits, and proteins identified only by site modification were excluded from further analysis. Protein groups were retained if they had been quantified in at least 3 of the 5 total replicates in at least one genotype. Protein LFQ intensities were log2 transformed and missing values were imputed from a normal distribution within Perseus. The resulting matrix was exported, and data were quantile normalized using the R/Bioconductor-package Process (Bolstad, 2018). Protein groups with statistically significant differential abundances were determined employing the R/Bioconductor-package limma (Ritchie et al., 2015), with p-values that were adjusted for multiple comparisons according to the Benjamini-Hochberg approach (Benjamini and Hochberg, 1995). Proteins with a |log2(ratio)| relative to the wild-type (Col-0) larger than 0.59 and with FDR-adjusted p-values of less than 0.05 were considered to be significantly changed. The mass spectrometry proteomics data have been deposited with the ProteomeXchange consortium via the PRIDE partner repository (Vizcaíno et al., 2014) with the dataset identifier PXD014514, and can be accessed during reviewing at www.ebi.ac.uk/pride/ with the following username and password: reviewer25980@ebi.ac.uk, kWOerjc7.

**Nucleic Acid Analysis**

Arabidopsis DNA was isolated from three-week-old leaves as described in Ihnatowicz et al. (2004). The T-DNA insertion sites in *atcgl20a* and *atcgl20b* were determined by PCR using combinations of insertion- and gene-specific primers (see Supplemental Table 12), which were also employed for screening of homozygous double mutants.

Total RNA was extracted from snap-frozen leaves with the TRIzol reagent (Invitrogen) according to the supplier’s instructions. RNA for reverse transcription quantitative PCR (RT-qPCR) analysis was isolated using the RNeasy Mini Kit (Qiagen). Arabidopsis cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). The reaction
mixtures contained iQ™ SYBR Green Supermix (Bio-Rad), cDNA as template, and gene-
specific primers. Levels of the ACT8 actin gene (AT1G49240) transcript served as a reference.
DNA amplification was monitored on the basis of the SYBR-Green fluorescence signal in an
IQ™5 Multicolor Real time PCR Detection system (Bio-Rad). Levels of gene expression
were calculated using the standard-curve method implemented in the IQ™5 Optical System
software.

RNA gel-blot hybridizations were performed under stringent conditions according to standard
protocols (Sambrook and Russell, 2001). Samples equivalent to 1–5 µg of total RNA were
fractionated by electrophoresis in formaldehyde-containing agarose gels (1.2% [w/v]), blotted
onto nylon membranes (Hybond-N+, Amersham Bioscience), and fixed by UV irradiation
(Stratalinker® UV Crosslinker 1800). To control for equal loading, abundant RNAs on nylon
membranes were stained with methylene blue solution (0.02% [w/v] methylene blue, 0.3 M
sodium acetate pH 5.5). To detect gene- and rRNA-specific transcripts, amplified DNA
fragments from cDNA templates were labeled with radioactive [α-32P]dCTP and subsequently
used as probes in hybridization experiments (see Supplemental Table 12 for primer
information). Signals were detected with the Typhoon Phosphor Imager System (GE
Healthcare, Chicago, USA). Polysome-associated mRNAs were isolated as described before
(Barkan, 1993).

Microfluidics-based quantification of rRNA species was carried out in initial quality-control
experiments for RNA sequencing at Novogene Biotech (Beijing, China) using an Agilent
2100 Bioanalyzer (Agilent) system. Ratios of rRNA species were calculated from three
biological replicates of Col-0 and atcgl20ab as described previously (Walter et al., 2010;
Tiller et al., 2012).

**In Vivo Translation Assay**

In vivo incorporation of [35S]-methionine was performed according to Meurer et al. (2017).
Col-0 and atcgl20ab were either grown under climate-controlled chamber conditions for 20
days or shifted to 4°C conditions for 7 days prior to the experiment. Plant material (200 mg)
was incubated for 30 min in labeling buffer (1 mM KH₂PO₄/K₂HPO₄ pH 6.3; 20 µg mL⁻¹
cycloheximide; 0.1% [w/v] tween 20). [35S]-Met was added and plants were vacuum-
infiltrated. Samples were exposed to 50 µmol photons m⁻² s⁻¹ for 40 min at room temperature.
Plants were washed three times in labeling buffer and snap-frozen. Whole-protein extract was
isolated from ground tissue by resuspension in loading buffer (see below). Samples were
adjusted according to equal fresh weight and subjected to Tricine-SDS-PAGE. Gels were stained with Coomassie brilliant blue G-250 and signals were detected with the Typhoon Phosphor Imager System (GE Healthcare).

**Protein Analysis**

Leaves from five-week-old plants grown under climate-controlled chamber conditions were harvested 4 h after the onset of the light period and directly homogenized in loading buffer (100 mM Tris-HCl, pH 6.8, 50 mM DTT, 8% [w/v] SDS, 24% [w/v] glycerol, and 0.02% [w/v] bromophenol blue). Denaturation for 5 min at 70°C and protein fractionation on Tricine-SDS-PAGE gels (10% gels) was carried out according to Schägger (2006).

Sample preparation for BN-PAGE was performed as described in Peng et al. (2008) using freshly prepared thylakoids. BN-PAGE gels (4–12% gradient) were prepared as described in Schägger et al. (1994). Solubilized samples corresponding to 80 µg Chl were loaded per lane, and gels were run at 4°C overnight. To separate complexes into their subunits, strips of the BN-PA gel were treated with denaturing buffer (0.2 M Na2CO3, 5% [w/v] SDS, 50 mM DTT) for 30 min at room temperature and layered on 10% Tricine-SDS-PAGE gels. In the case of stromal extract analyses, chloroplast-enriched pellets were resuspended directly in BN washing buffer (25 mM Bis-Tris-HCl, pH 7, 20% [w/v] glycerol) and passed 20 times through a 0.45-mm syringe to mechanically disrupt intact chloroplasts. The supernatant was separated from the membranous pellet by centrifugation at 35,000 g. Protein concentration was determined using the Bradford Protein Assay (Bio-Rad). RNase A (Qiagen) was added (1/10 of total protein content) to isolated stroma and incubated for 15 min at room temperature. BN sample buffer (1/10 sample volume, 100 mM Bis-Tris-HCl, pH 7, 750 mM aminocaproic acid, 5% [w/v] Coomassie-G 250) was added to the supernatant and samples (150 µg total protein content) were separated on 4–12% BN gradient gels (Schägger, 1994). Isolated mitochondria equivalent to 150 µg total protein content were solubilized in BN washing buffer containing 1.5% [w/v] n-dodecyl-β-maltoside. After centrifugation (35,000 g for 30 min at 4°C), BN sample buffer (1/10 sample volume) was added and mitochondrial complexes were separated on 5–12% BN gradient gels. Second-dimension electrophoresis was performed as described earlier.

Proteins fractionated by gel electrophoresis were transferred to polyvinylidene difluoride membranes (PVDF) (Immobilon®-P, Millipore) using a semi-dry blottting system (Biorad) as
described in the supplier’s instructions. After blocking with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% [v/v] Tween-20) supplemented with 3% [w/v] skim-milk powder, the membranes were first incubated with primary antibodies at 4°C overnight and then with secondary antibodies for 2–3 h. Signals were detected by enhanced chemiluminescence (Pierce, Thermo Scientific) using an ECL reader system (Fusion FX7, PeqLab). Antibodies used in this study are listed in Supplemental Table 13.

Proteome Analysis of the High-Molecular-Mass Fraction in the Chloroplast Stroma

Stroma fractions of three biological replicates of Col-0 and atcgl20ab plants were isolated from 20-day-old plants as described earlier and subjected to sucrose step-gradient centrifugation with 50 mM KCl (instead of 150 mM) in the cushion. The protein concentration of the last fraction was appropriately adjusted with chloroplast lysis buffer. Aliquots (30 µg) of protein were reduced, alkylated and digested with trypsin (0.4 µg) as described above. Peptides were desalted with home-made C18 stage tips, vacuum dried to near dryness and stored at -80°C. LC-MS/MS and data analysis were performed as described above.

Sucrose Density-Gradient Centrifugation

Crude stromal extracts were prepared as described above. Chloroplast-enriched pellets were resuspended in chloroplast lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM MgOAc, 6 mM β-mercaptoethanol). After centrifugation (35,000 g) for 30 min at 4°C, 500 µL of the supernatant (~2 mg total protein content) was layered onto a continuous (10–40%) sucrose gradient (2 mL) prepared in lysis buffer. The gradients were centrifuged at 45,000 rpm (~273,000 g) in a SW60 Ti rotor (Beckmann Coulter) for 3 h at 4°C. Samples were separated into 38 fractions, subjected to 10% Tricine SDS-PAGE, blotted and probed with antibodies against AtCGL20, Rps1, Rpl2 and GFP.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S1.
SUPPLEMENTAL DATA

Supplemental Figure S1. Cold treatment, complementation, and NDH activity analyses of atcgl20 mutants.

Supplemental Figure S2. Graphical illustration of MapMan enrichment analyses.

Supplemental Figure S3. BN/SDS-PAGE analysis of mitochondrial and thylakoid complexes.

Supplemental Table S1. CGL20 homologs in the green lineage.

Supplemental Table S2. Pigment analysis.

Supplemental Table S3. Transcriptomic analysis (RNAseq) of WT (Col-0) and atcgl20ab plants.

Supplemental Table S4. Shotgun proteome analysis of WT (Col-0) and atcgl20ab plants.

Supplemental Table S5. Combined proteome and transcriptome data.

Supplemental Table S6. MapMan pathway analysis of the atcgl20ab transcriptome.

Supplemental Table S7. MapMan pathway analysis of the atcgl20ab proteome.

Supplemental Table S8. AtCGL20A co-expression analysis.

Supplemental Table S9. Quantification of processed 23S rRNA products in WT (Col-0) and atcgl20ab.

Supplemental Table S10. Proteome analysis of the high-molecular-mass fraction in the chloroplast stroma of WT (Col-0) and atcgl20ab.

Supplemental Table S11. MapMan pathway analysis of the high-molecular-mass proteome in the chloroplast stroma of WT (Col-0) and atcgl20ab.

Supplemental Table S12. Primers used in this study.

Supplemental Table S13. Antibodies used in this study.

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Table 1. Chl a fluorescence and P700 parameters of WT (Col-0), atcgl20a, atcgl20b, atcgl20ab, oeAtCGL20A-eGFP, and oeAtCGL20B-eGFP plants.

Leaves were exposed to 130 µE m⁻² s⁻¹ for 15 min. The dark-relaxation phase lasted 10 min. Mean values (n=5) ± SD are provided. \( F_{v}/F_{m} \), maximal quantum yield of PSII; \( \Phi_{II} \), effective quantum yield of PSII at 100 µE m⁻² s⁻¹; 1-qP, excitation pressure; NPQ, nonphotochemical Chl fluorescence quenching; qE, energy-dependent quenching of Chl fluorescence; qI, photoinhibitory quenching; Y(I), photochemical quantum yield of PSI; Y(ND), nonphotochemical quantum yield of PSI, donor-side limited; Y(NA) nonphotochemical quantum yield of PSI, acceptor-side limited.

|                   | Col-0     | atcgl20a | atcgl20b | atcgl20ab | oeAtCGL20A-eGFP | oeAtCGL20B-eGFP |
|-------------------|-----------|----------|----------|-----------|-----------------|-----------------|
| \( F_{v}/F_{m} \) | 0.80 ± 0.00 | 0.80 ± 0.01 | 0.81 ± 0.00 | 0.76 ± 0.01 | 0.81 ± 0.00 | 0.81 ± 0.00 |
| \( \Phi_{II} \)   | 0.41 ± 0.04 | 0.43 ± 0.03 | 0.39 ± 0.04 | 0.45 ± 0.04 | 0.40 ± 0.02 | 0.38 ± 0.04 |
| 1-qP              | 0.41 ± 0.06 | 0.39 ± 0.04 | 0.43 ± 0.05 | 0.37 ± 0.05 | 0.42 ± 0.03 | 0.44 ± 0.06 |
| NPQ               | 0.90 ± 0.13 | 0.66 ± 0.13 | 0.92 ± 0.11 | 0.27 ± 0.05 | 0.97 ± 0.07 | 1.00 ± 0.10 |
| qE                | 0.75 ± 0.13 | 0.50 ± 0.11 | 0.76 ± 0.10 | 0.15 ± 0.05 | 0.81 ± 0.07 | 0.84 ± 0.10 |
| qI                | 0.35 ± 0.04 | 0.33 ± 0.05 | 0.39 ± 0.04 | 0.26 ± 0.01 | 0.40 ± 0.02 | 0.41 ± 0.03 |
| Y(I)              | 0.70 ± 0.03 | 0.69 ± 0.03 | 0.71 ± 0.03 | 0.65 ± 0.04 | 0.70 ± 0.02 | 0.69 ± 0.04 |
| Y(ND)             | 0.19 ± 0.04 | 0.21 ± 0.04 | 0.21 ± 0.04 | 0.29 ± 0.03 | 0.21 ± 0.03 | 0.23 ± 0.05 |
| Y(NA)             | 0.10 ± 0.02 | 0.10 ± 0.02 | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.09 ± 0.02 | 0.08 ± 0.01 |

FIGURE LEGENDS

Figure 1. Sequence alignment of Arabidopsis CGL20A (AtCGL20A), CGL20B (AtCGL20B), and homologs from other species in the green lineage. Predicted chloroplast transit peptides are not included in the alignment. Stretches of sequence similarity/identity conserved in the proline-rich N-terminal region in at least 80% of the proteins are highlighted by grey/black shading. At the C-terminal end, acidic (E and D) and basic amino acids (K and R) are marked in red and blue, respectively. Sequence identifiers and further features of these proteins are listed in Supplemental Table 1.

Figure 2. Characterization of Arabidopsis atcgl20ab knockout mutants. A, Structures and T-DNA insertion sites in the AtCGL20A and AtCGL20B genes. Left (LB) and right (RB) T-DNA borders are indicated. Exons are numbered and shown as white rectangles, UTRs as black rectangles. B, Quantification of At2G127240 and At3G24506 transcripts by real-time
PCR analyses using transcripts of the actin-encoding gene ACT8 (At1G49240) as reference. Means ± SD were calculated from three technical replicates. C, Growth phenotypes of the genotypes analyzed. Plants were grown for 5 weeks under a 12/12-h light/dark regime. D, Leaf-area measurements of the genotypes shown in C. Means ± SD were calculated from data for 12 leaf areas per genotype.

**Figure 3.** Subcellular localization of AtCGL20A and AtCGL20B. A, Chl autofluorescence (Auto), MitoTracker™, and eGFP fluorescence emission (eGFP) of protoplasts isolated from oeAtCGL20A-eGFP and oeAtCGL20B-eGFP plants. Protoplasts were analyzed by confocal laser scanning microscopy and overlaid in a single image (Merged). White scale bar indicates 5 µm. B, Immunodetection of AtCGL20A-eGFP and AtCGL20B-eGFP in cell fractionation experiments. Mitochondria (Mito) and chloroplasts (Chloro) were isolated from oeAtCGL20A-eGFP and oeAtCGL20B-eGFP plants and chloroplasts were further separated into an insoluble (Insol) and a soluble (Sol) fraction. The purity of the mitochondrial fraction was examined by immunodetection of CoxII, whereas Lhcb3 and Csp41b served as marker proteins for the insoluble and soluble chloroplast fractions, respectively. To control for loading, PVDF membranes were stained with Coomassie brilliant blue G-250 (C.B.B.). C, Accumulation of AtCGL20A-eGFP in the high-molecular-mass (HMM) fraction isolated by sucrose step-gradient centrifugation from stroma extracts of oeAtCGL20A-eGFP chloroplasts. Fractions were characterized by SDS-PAGE. Cpn60a1, Rps1, and Rpl2 were immunodetected as marker subunits for the stromal protein complex chaperonin 60, and the SSU and LSU of chloroplast ribosomes, respectively. C.B.B. staining is shown as loading control. The position of the large RuBisCO subunit RbcL is highlighted.

**Figure 4.** Comparative transcriptomic and proteomic analyses of Col-0 and atcgl20ab. A, Venn diagram of up- (log_2 FC > 0.59) and downregulated (log_2 FC < -0.59) transcripts and proteins in atcgl20ab compared to wild-type (Col-0). B, Subcellular distribution of the proteins that are up- (log_2 FC > 0.59) or downregulated (log_2 FC < -0.59) in atcgl20ab at the transcriptional (mRNA) or protein (protein) level. C, Impact of AtCGL20 disruption on the transcriptional and protein-level expression of thylakoid complex subunits. Asterisks indicate the following p-values: * < 0.05, ** < 0.01 and *** < 0.001. Colors, which represent down- or up-regulated components in atcgl20ab, range from dark blue (log_2 FC < -2) to dark red (log_2 FC > 2).
Figure 5. Chloroplast gene expression and polysome loading in AtCGL20 loss-of-function mutants. A, Relative abundances of selected plastome-encoded mRNAs and proteins in atcgl20ab compared to wild-type (Col-0). Asterisks indicate following p-values: * < 0.05, ** < 0.01, and *** < 0.001. Colors, which represent down- or upregulated components in atcgl20ab, range from dark blue (log2 FC < -2) to dark red (log2 FC > 2). B, Pulse-labeling analysis of de novo synthesized plastid proteins in 20-day-old seedlings of Col-0 and atcgl20ab grown under 22°C or shifted for seven days to 4°C prior to the experiment. Labeling was performed with [35S]-Met for 40 min under moderate light intensity in the presence of cycloheximide. Whole leaf extracts corresponding to equal amounts of fresh weight were separated by SDS-PAGE, stained with Coomassie brilliant blue (C.B.B.) and subjected to autoradiography. Abundant signals were identified as RbcL, CP43/CP47, and D1/D2 according to Rühle et al. (2014) and Pulido et al. (2018). C, RNA gel-blot hybridization of fractions obtained following sucrose density-gradient centrifugation under polysome-preserving conditions. Free ribosomes (monosomes) are found in fractions 2–6, whereas fractions 7–12 contain mRNA-polysome complexes. Membranes were hybridized with probes specific for psaA, rbcL, psbA, ndhH, petB, rrn5, rrn16, and rrn23. Methylen blue (M.B.) staining is shown as loading control. D, Micro-fluidics-based quantification of rRNA abundance in atcgl20ab compared to the wild-type control (Col-0). Means ± SD were calculated from three biological replicates. Relative amounts of large cytosolic (25S), small cytosolic (18S), small chloroplast (16S), and the 1.3-kb hidden-break product of the 23S species (23S HB1) in Col-0 were compared to the values for atcgl20ab.

Figure 6. RNA gel-blot hybridization of plastid rRNAs and quantification of ribosomal proteins in the HMM fraction of atcgl20ab stroma. A, Total RNA isolated from 20-day-old wild-type (WT), atcgl20a (a), atcgl20b (b) and atcgl20ab (ab) plants was fractionated by denaturing agarose gel electrophoresis and blotted onto nylon membranes. Membranes were hybridized with probes specific for the 16S rRNA (rrn16), hidden-break (HB) fragments of 0.5 kb (rrn23.1), 1.3 kb and 1.1 kb (rrn23.2), and 1.1 kb (rrn23.3) of the 23S rRNA, as well as the 4.5S (rrn4.5) and 5S rRNA (rrn5), respectively. B, Quantification of 23S hidden-break products. RNA gel-blot hybridization signals for atcgl20ab and wild-type (Col-0) samples were quantified (Supplemental Table 9). Means ± SD were calculated from three technical replicates. Probes for rrn23S used in panel A are depicted in gray and subspecies of processed.
23S rRNA are colored according to their abundance in *atcgl20ab* as per a scale extending from dark blue (log<sub>2</sub> FC < -2) to dark red (log<sub>2</sub> FC > 2). C, SSU and LSU protein abundance in the high-molecular-mass fraction isolated from *atcgl20ab* chloroplast stroma (Supplemental Table 10). Asterisks indicate the following p-values: * < 0.05, ** < 0.01 and *** < 0.001.

**Figure 7.** Co-migration studies of AtCGL20A-eGFP. A, Sucrose density-gradient centrifugation of stromal extracts containing AtCGL20A-eGFP. Stromal extracts of AtCGL20A-eGFP plants were prepared with reduced amounts Mg<sup>2+</sup> and subjected to sucrose density-gradient centrifugation. Sucrose fractions were collected and proteins were separated by SDS-PAGE. After protein transfer, PVDF membranes were decorated with antibodies specific for Rps1, Rpl2, AtCGL20 and GFP. Coomassie brilliant blue (C.B.B.) staining is shown as a loading control and the position of the large RuBisCO subunit RbcL is indicated. B, BN/SDS-PAGE analysis of stromal extracts isolated from AtCGL20A-eGFP plants. Stroma was either treated with RNase A or an RNase inhibitor (RNasin, Promega, Mock control) and subjected to BN/SDS-PAGE analyses. After protein transfer, PVDF membranes were probed with antibodies specific for AtCGL20 and Rpl2. C.B.B. staining is shown as loading control. Positions of RuBisCO (~520 kDa) and RNase A are indicated.
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