Conditional Depletion of the \textit{Chlamydomonas} Chloroplast ClpP1 Protease Activates Nuclear Genes Involved in Autophagy and Plastid Protein Quality Control

Silvia Ramundo,a,1 David Casero,b,2 Timo Mühlaus,a Dorothea Hemme,c,3 Frederik Sommer,c,3 Michèle Crèvecoeur,a Michèle Rahire,a Michael Schroda,c,3 Jannette Rusch,d Ursula Goodenough,d Matteo Pellegrini,n Maria Esther Perez-Perez,f,4 José Luis Crespo,f Olivier Schaad,a,h Natacha Civic,g Michèle Crèvecoeur,a Michèle Rahire,a Michael Schroda,c,3 Jannette Rusch,d Ursula Goodenough,d and Jean David Rochaix,a,5

\textsuperscript{a}Departments of Molecular Biology and Plant Biology, University of Geneva, 1211 Geneva, Switzerland
\textsuperscript{b}Institute for Genomics and Proteomics, University of California, Los Angeles, California 90095
\textsuperscript{c}Max Planck Institute of Molecular Plant Physiology, D-14476 Potsdam-Golm Germany
\textsuperscript{d}Department of Biology, Washington University, St. Louis, Missouri 63130
\textsuperscript{e}Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, California 90095
\textsuperscript{f}Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas, Universidad de Sevilla, 41092 Sevilla, Spain
\textsuperscript{g}Genomics Platform, University of Geneva, 1211 Geneva, Switzerland
\textsuperscript{h}Department of Biochemistry, University of Geneva, 1211 Geneva, Switzerland

Plastid protein homeostasis is critical during chloroplast biogenesis and responses to changes in environmental conditions. Proteases and molecular chaperones involved in plastid protein quality control are encoded by the nucleus except for the catalytic subunit of ClpP, an evolutionarily conserved serine protease. Unlike its \textit{Escherichia coli} ortholog, this chloroplast protease is essential for cell viability. To study its function, we used a recently developed system of repressible chloroplast gene expression in the alga \textit{Chlamydomonas reinhardtii}. Using this repressible system, we have shown that a selective gradual depletion of ClpP leads to alteration of chloroplast morphology, causes formation of vesicles, and induces extensive cytoplasmic vacuolation that is reminiscent of autophagy. Analysis of the transcriptome and proteome during ClpP depletion revealed a set of proteins that are more abundant at the protein level, but not at the RNA level. These proteins may comprise some of the ClpP substrates. Moreover, the specific increase in accumulation, both at the RNA and protein level, of small heat shock proteins, chaperones, proteases, and proteins involved in thylakoid maintenance upon perturbation of plastid protein homeostasis suggests the existence of a chloroplast-to-nucleus signaling pathway involved in organelle quality control. We suggest that this represents a chloroplast unfolded protein response that is conceptually similar to that observed in the endoplasmic reticulum and in mitochondria.

**INTRODUCTION**

Protein degradation plays a key role in chloroplast biogenesis and maintenance. Under certain stress conditions, such as high irradiance, nutrient starvation, or elevated temperature, the primary reactions of photosynthesis can lead to the formation of reactive oxygen species (ROS) and can damage proteins by photooxidation. Damaged proteins are then degraded through an efficient proteolytic system in the chloroplast (Adam and Clarke, 2002). During assembly of the photosynthetic apparatus, most protein subunits that are produced in excess are also rapidly degraded. The plastid contains several proteases including stromal ClpP (Olinares et al., 2011), stromal and thylakoid Deg (Schuhmann and Adamska, 2012), thylakoid FtsH (Adam et al., 2001; Sokolenko et al., 2002; Peltier et al., 2004), the intramembrane rhomboid proteases (Adam, 2013), and the thylakoid-bound SppA (Lensch et al., 2001) and Egy1 proteases (Chen et al., 2005). Moreover, several processing proteases exist in these organelles, such as SPP (stromal processing peptidase) and TPP (thylakoid processing protease), which are required for cleaving the transit peptide and the thylakoid targeting domain, respectively (Oelmüller et al., 1996; Richter and Lampka, 1998). Among these proteases, only the ClpP1 subunit is encoded by the chloroplast genome.

The bacterial ClpP holoenzyme complex consists of two main homo-oligomeric parts, a proteolytic chamber formed by two
stacked heptameric rings of the ClpP subunit and an unfolding antechamber constituted by the hexameric ring of an ATP-dependent chaperone, ClpX or ClpA. This AAA* (for ATPase associated with various cellular activities) complex associates coaxially to one or both external sides of the catalytic rings (Banecki et al., 2001; Kim et al., 2001). ClpA and ClpX have different substrate specificities (Bewley et al., 2006) and can be further modulated through the association of ClpS with the hexameric ring of ClpA. This association changes the affinity of the ClpA chaperone for protein aggregates (Weber-Ban et al., 1999) and strongly enhances the recognition of substrates with N-terminal degradation signals known as N-rule substrates (Tobias et al., 1991; Schuenemann et al., 2009).

Although the plastid Clp proteolytic system shares the basic features of its bacterial homolog, it is considerably more complex (Peltier et al., 2004; Adam et al., 2006) as several ClpP isoforms have evolved in photosynthetic organisms that can be incorporated in the holoenzyme. Moreover, some isoforms, namely ClpR, have lost the catalytic residues for peptide bond hydrolysis and are often found in the ClpP heptameric rings. Three genes encoding ClpP (ClpP1, ClpP2, and ClpP5) and five genes encoding ClpR (ClpR1-4 and ClpR6) are present in Chlamydomonas reinhardtii. With the exception of ClpP1, which is encoded by the chloroplast genome, all the other ClpP components are encoded by nuclear genes. In C. reinhardtii, the plastid-encoded ClpP1 protein contains a 30-kD insertion that can be removed by endoproteolytic cleavage to give rise to a short ClpP1 isoform (Wang et al., 1997; Majeran et al., 2005). Both the long and short isoforms are essential and can be detected within the ClpP1 core (Huang et al., 1994; Derrien et al., 2009). Moreover, chaperones that are orthologs of the bacterial AAA+ proteins (ClpC1/C2 and ClpD) and ClpS adaptors (ClpS1 and ClpS1-like) have been identified (Peltier et al., 2004; Derrien et al., 2012). However, the plastid ClpP protease also possesses unique accessory subunits (ClpT proteins) that are not found in Escherichia coli. Recent biochemical studies suggest that the ClpT subunits have evolved in order to regulate the assembly of the catalytic core (Sjögren and Clarke, 2011; Derrien et al., 2012).

Because ClpP peptidase function is dispensable in bacteria, it was possible to identify the targets of this enzyme using a protein trapping system in which the ClpP subunit is inactivated through a mutation in its active site that prevents degradation and release of the substrates. In E. coli, the targets are transcription factors, metabolic enzymes, and proteins involved in starvation and other stress responses (Dougan et al., 2002; Flynn et al., 2003).

By contrast, the plastid ClpP protease appears to have an essential role based on the experimental failure to generate homoplasmic knockout lines of clpP1 in the three photosynthetic organisms: Nicotiana tabacum (Shikanai et al., 2001; Kuroda and Maliga, 2003), Synechococcus elongatus pcc 7942 (Schelin et al., 2002; Barker-Aström et al., 2005), and C. reinhardtii (Huang et al., 1994). In this alga, attenuated expression of ClpP1 was achieved by replacing the AUG start codon of ClpP1 with AUU, a less efficient initiation codon (Majeran et al., 2000). Although the expression of ClpP1 protein was decreased by 70%, no striking phenotype was observed under normal growth conditions. However, this decrease in protein expression led to delayed degradation of the Cytb6 complex under nitrogen starvation and was also seen in mutants deficient in the Rieske iron-sulfur protein. Partial or complete loss of expression of several ClpP subunits in Arabidopsis thaliana revealed that the ClpP/R protease core is essential and that its subunits exhibit little functional redundancy, with the exception of ClpR1, whose loss can be overcome by ClpR3 (Rudella et al., 2006; Sjögren et al., 2006; Koussevitzky et al., 2007; Zybailov et al., 2009). Also, loss of individual Clp chaperones led to reduced plant growth and chloroplast development (Sjögren et al., 2006; Zybailov et al., 2009).

The identification of ClpP substrates in the chloroplast remains a challenging task because this protease is essential and there are multiple nucleus-encoded ClpP/R catalytic isoforms that cannot be easily inactivated. Quantitative proteomic approaches have been employed for large-scale detection and quantification of differences in protein abundance among wild-type and Clp mutants (Peltier et al., 2004; Sjögren et al., 2006; Kim et al., 2009; Zybailov et al., 2009). A consistent increase in abundance was observed for proteins involved in many different and unrelated pathways: plastid enzymes and transporters involved in basic metabolic pathways; proteins of plastoglobules; proteins involved in protein import, folding, and maturation; proteins involved in RNA metabolism; and various stromal proteases. However, it is not clear to what extent the increased abundance of these proteins is directly or indirectly linked to the decreased activity of ClpP.

Here, we took advantage of a recently developed system of repressible chloroplast gene expression (Ramundo et al., 2013) for investigating the role of ClpP1. It is based on a vitamin-repressible riboswitch from C. reinhardtii (Croft et al., 2007). We expected that conditional repression of ClpP1 would gradually decrease the ClpP level, making it possible to follow the early and late cellular events caused by its depletion. We performed electron microscopic, proteomic, and transcriptomic analyses to obtain time-course profiles of cell morphology and levels of proteins and transcripts during depletion of ClpP1. Using this approach, we identified a small group of proteins that could be substrates of ClpP1. Moreover, we discovered a compensatory nuclear gene expression program that may allow photosynthetic organisms to sense and respond to perturbations of chloroplast protein homeostasis. This chloroplast-to-nucleus retrograde signaling pathway could involve a chloroplast unfolded protein response.

RESULTS

Generation of a Strain in Which ClpP1 Expression Can Be Conditionally Repressed

We previously generated the A31 strain in which expression of Nac2, a nucleus-encoded chloroplast protein that is required for the stability of the chloroplast psbD RNA, is driven by the MetE upstream sequence and the TPP (thiamine pyrophosphate) riboswitch that can be repressed by addition of the vitamins B12 and thiamine to the medium (Ramundo et al., 2013). In this strain, the chloroplast 5′ untranslated region of psbD (psbD 5′ UTR), which is the target of Nac2, has been replaced by the psaA 5′ UTR. The expression of psbD, which encodes the photosystem II D2
reaction center protein in this strain, is therefore no longer dependent on Nac2; thus, the strain grows photoautotrophically in the presence of vitamins when Nac2 is repressed. A ClpP1-repressible strain was produced through chloroplast transformation by replacing the endogenous ClpP1 5′ UTR of the A31 strain with the psbD 5′ UTR, thus making clpP1 expression dependent on Nac2. The homoplasmic state of this strain was tested by PCR (Supplemental Figure 1).

Whereas the parental A31 strain was not affected by the presence of vitamins in the medium, the chloroplast transformants bearing the psbD5′-clpP1 transgene (DCH strains) displayed a pale-green phenotype and died upon transfer from medium lacking vitamins into medium supplemented with vitamins (Figure 1A). This was expected due to the downregulation of a gene essential for cell survival. This vitamin-mediated phenotype could not be rescued by growing the DCH strains in the dark in the presence of a reduced carbon source (acetate and Tris-acetate phosphate [TAP] medium), indicating that the primary cause of the lethal phenotype in the presence of vitamins is not caused by either photooxidative damage or impairment of photosynthesis.

To test the photosynthetic activity and growth of the DCH transformants containing the vitamin-repressible ClpP1 gene, the transformants were first grown in acetate-containing medium in the light and then vitamins were added. The results obtained with one of these transformants, DCH16, are shown in Figure 1B. During the first 24 h of vitamin treatment, we observed no significant change in growth rate or in photosynthetic activity (as measured by Fv/Fm ratios that reflect the maximal quantum efficiency of photosystem II [PSII]) (Figure 1C). After 48 h, cell growth ceased and photosynthetic activity declined. The physiological state of the DCH16 strain changed drastically 72 and 96 h following vitamin treatment; the photosynthetic activity decreased further and the cells became pale green.

Kinetics of ClpP1 Repression in the DCH16 Strain

To determine the time required for the down-regulation of ClpP1, a liquid culture of DCH16 was treated with vitamins and the levels of ClpP1 mRNA and protein were determined over a period of 72 h. RNA gel blot analysis revealed a significant decrease of ClpP1 mRNA after 12 h, and this mRNA was nearly undetectable after 36 h (Figure 1D). By contrast, in the control strain A31, the ClpP1 mRNA level was unchanged. Because clpP1 is under the control of the strong psbD promoter and 5′ UTR, the amount of ClpP1 mRNA in the absence of vitamins was higher in DCH16 than in A31, which contains the authentic clpP1 gene (Figure 1D). It is noteworthy that the levels of several chloroplast mRNAs, in particular those of ORF1995, rpoA, and rps12 were strongly increased whereas those of others such as tufA, atpB, and psaB did not change markedly.

Transcripts of nuclear genes that were strongly upregulated upon ClpP1 depletion included those of Hsp70B, Hsp22A, and Hsp22F (Figure 1E; Supplemental Figure 2). A similar pattern was also observed for transcripts of the nuclear genes Atg8 and Atg7 involved in autophagy, Vipp1 and Alb3.2 implicated in thylakoid biogenesis, and ubiquitin (Figure 1E; Supplemental Figure 2).

Immunoblot analysis showed that the steady state levels of both the long and short forms of ClpP1 diminished gradually over 48 h of vitamin treatment in the DCH16 strain. By contrast, they remained constant in the A31 strain (ClpP1-H and ClpP1-L, respectively, in Figure 2A). Although the amount of clpP1 mRNA was considerably higher in DCH16 than in A31 in the absence of vitamins (Figures 1D and 1E), ClpP1 accumulated to the same level in both strains, indicating that the regulation of its accumulation occurs at the translational or posttranslational level. The fact that the amount of ClpP1 was reduced to 30% in the DCH16 strain 24 h after addition of vitamins and that no significant change in its growth rate and photosynthetic activity occurred at this time point together suggest that ClpP1 is produced in excess of what is needed.

The levels of several chloroplast-encoded proteins were monitored in DCH16 cells over a 72-h period following vitamin treatment. Levels of the majority of these proteins (Rps12, Rps21, RpoA, and Cytf) remained stable during depletion of ClpP1. The only remarkable exception is ORF1995, a large chloroplast open reading frame encoding a potential protein of unknown function of more than 200 kD. Intriguingly, an antibody raised against the middle region of this protein failed to detect the full-length protein. Rather, it highlighted the two smaller polypeptides, ORF1995-L (70 kD), which increased, and ORF1995-S (35 kD), which decreased, upon ClpP1 depletion (Figure 2A). This result suggests that processing of ORF1995 might occur either at the RNA or protein level and that ClpP1 is potentially involved in this activity. A slightly decreased content was observed for some plastid nucleus-encoded proteins (ClpC) and cytosolic ribosomal proteins (Rpl37), whereas the level of mitochondrial AOX protein did not change significantly (Figure 3A). By contrast, the levels of the plastid nucleus-encoded proteins Alb3.2, Vipp1, Vipp2, Hsp70B, Cdj1, and FtsH1 increased upon ClpP1 depletion (Figures 2B and 3). Both Vipp1 and Alb3.2 are involved in thylakoid biogenesis in cyanobacteria, algae, and plants (Kroll et al., 2001; Göhre et al., 2006). The expression and localization of these two proteins is regulated in an interdependent way; Alb3.2 is associated with Vipp1 and Vipp1 expression is considerably enhanced when the expression of Alb3.2 is downregulated (Göhre et al., 2006). Moreover, Vipp1 expression is influenced by the light conditions (Liu et al., 2005; Nordhues et al., 2012).

As these experiments were performed with cells grown in dim light (10 μmol m−2 s−1), it is rather unlikely that the effect of ClpP1 depletion is indirect and results from photooxidative damage. However, in order to eliminate this possibility, proteins were also analyzed from dark-grown cells (Figure 2B). In the dark, these proteins also increased in abundance, albeit to a lower extent and with slower kinetics compared with the light conditions, indicating that this process is light independent. Whereas a significant portion of Vipp1 and Cdj1 were in the soluble fraction under normal conditions, they were partly relocalized to the insoluble fraction upon depletion of ClpP1 (Figure 3B). Moreover, the immunoblot analysis of Vipp1 revealed a smear in the large molecular weight region after prolonged vitamin treatment (Figure 3C). This smear was detected in light-grown but not in dark-grown cells, suggesting that it arises through photooxidative damage and could represent protein aggregates. Indeed, treatment of the extract with 6 M urea significantly decreased the high molecular weight proteins.
Figure 1. Characterization of the *C. reinhardtii* DCH16 Strain upon ClpP1 Depletion.

(A) Growth patterns of the Rep112, A31, and DCH16 strains. Cells were spotted on HSM (minimal medium) and TAP in the absence or presence (+vit) of vitamins B12 (20 μg/L) and thiamine (20 μM). Irradiance was 10 μmol m⁻² s⁻¹.

(B) Growth curves of the A31 and DCH16 strains after addition of vitamins. To maintain cells in exponential growth during the entire time course, they were diluted to 0.5 × 10⁶ cells/mL when they reached a concentration between 2 and 4 × 10⁶ cells/mL. The experiment was repeated three times with similar results.

(C) Decrease of PSII activity in DCH16 after addition of vitamins. Fv/Fm was measured for the indicated strains at different times after addition of vitamins. The experiment was repeated three times with similar results.

(D) RNA gel blot analysis of chloroplast genes in DCH16, A31, and ClpP1-AUU in the presence of vitamins or rapamycin. RNA was extracted from the strains at different time points after addition of vitamins and hybridized with the indicated probes. ClpP1-AUU is the strain in which the AUG initiation codon of ClpP1 was replaced by AUU. Arrows point to the different transcripts of ORF1995, a chloroplast gene of unknown function.

(E) RNA gel blot analysis of nuclear genes. Strains and conditions were as in (D). Rapamycin treated cells were transferred from 10 to 60 μmol photons m⁻² s⁻¹ upon addition of the drug, vit, vitamins; Rap, rapamycin.
fraction of Vipp1 (Figure 3C). By contrast, upon ClpP1 depletion, the levels of proteins from photosynthetic complexes such as Cytf, D2, and RbcL were decreased under standard light conditions (60 μmol m⁻² s⁻¹), but maintained in the dark or under low-light conditions (10 μmol m⁻² s⁻¹), indicating that they are rather stable but sensitive to photooxidative damage (Figure 2B).

Cellular Characterization of the ClpP1 Repressible Strain

In order to analyze the morphological changes associated with ClpP1 depletion, samples of DCH16 were collected after 0, 48, 96, and 144 h of vitamin treatment under an irradiance of 60 μmol m⁻² s⁻¹ and examined by light and thin-section electron microscopy (Figure 4). Independently, control and treated (0, 48, and 72 h) samples from cells grown under an irradiance of 30 μmol m⁻² s⁻¹ were processed for electron microscopy using the quick-freeze deep-etch technique that employs no chemical fixation or dehydration (Figure 5). The DCH16 strain grown in the absence of vitamins was used as a control.

Thin-section electron microscopy revealed that, upon ClpP1 depletion, the integrity of the thylakoid membranes was disrupted. The membranes were often unstacked or folded back to form unusual whorls, and enlarged vacuolar structures were present in the cytoplasm (Figure 4A). This vacuolization was often associated with cellular swelling and loss of flagella. Furthermore, the starch granules became very irregular in shape and size and in some cases they appeared to occupy a large portion of the plastid. Although a number of vesicle-like structures could be recognized, it was difficult to determine their origin given the highly disorganized cell structure. These changes in cell morphology were also apparent in cells observed using light microscopy (Figure 4B).

Replicas of quick-frozen cells provided additional information. In log-phase cells, the vacuoles were small and filled with a homogeneous flocculent material (Figures 5B and 5C, V), whereas at 48 h, they were enlarged and filled with damaged membrane and starch (Figure 5F, AV); by 72 h, large water-filled vacuoles were often encountered (Figure 5G, WV). In log-phase cells, the chloroplast envelope was smooth, with few intramembranous particles (Figures 5B and 5C, ce), but at 48 h it carried perforations

**Figure 2.** Immunoblot Analysis of Chloroplast Proteins in DCH16, A31, and ClpP1-AUU in the Presence of Vitamins or Rapamycin.

(A) Immunoblot analysis. Proteins were extracted from the three strains grown under an irradiance of 10 μmol m⁻² s⁻¹ at different time points after addition of vitamins and reacted with antibodies directed against the indicated proteins as shown to the left of the immunoblot.

(B) Analysis of proteins from DCH16 grown in the light or the dark upon ClpP1 repression. Proteins were extracted at different time points and immunoblotted with the antisera as indicated to the left of the immunoblot. Irradiance was 60 μmol m⁻² s⁻¹.
(Figure 5E) and by 72 h it was no longer detectable and degenerating thylakoids (Figures 5D and 5E) filled the cell (Figures 5G and 5H). Therefore, many or most of the vesicles seen in thin sections (Figure 4A) may be derived from thylakoid and chloroplast-envelope membranes, although they could also represent autophagosomes engulfing chloroplast membranes.

When the DCH16 strain was grown in medium with vitamins under lower irradiance (5 μmol m⁻² s⁻¹) or in the dark, similar morphological changes were observed, but the time of their appearance was clearly delayed (Figure 4A). Most likely, two factors concur to cause this delay: the absence of photooxidative stress and the slower rate of ClpP1 depletion in the dark. Importantly, none of these changes was detectable in the A31 strain treated with vitamins, thus confirming that the phenotype of DCH16 is caused by the depletion of ClpP1.

**Autophagy-Like Phenotype upon Depletion of ClpP1**

The progressive vacuolization and swelling observed upon ClpP1 depletion (Figures 4A, 4B, and 5) is reminiscent of autophagy. Bulk delivery of cellular components to the vacuole or to lysosomes in eukaryotic cells by autophagy is an evolutionary-conserved and highly regulated response that functions to degrade damaged proteins and/or organelles and to recycle essential macronutrients under adverse conditions such as nutrient starvation and accumulation of protein aggregates. Moreover, upon prolonged cellular stress, massive autophagic vacuolization has been proposed to represent an attempt of adaptation resulting in cell death (Berry and Baehrecke, 2007; Tasdemir et al., 2008).

One of the most widely used autophagy marker is Atg8, a protein that is usually conjugated to phosphatidylethanolamine and is found on the membrane of the autophagosome (Nakatogawa et al., 2009). In *C. reinhardtii*, Atg8 is conserved, and a recent study demonstrated that induction and lipidation of this protein can be triggered by inhibition of the TOR (target of rapamycin) protein kinase and by oxidative or endoplasmic reticulum (ER) stresses (Pérez-Pérez et al., 2010, 2012).

In light of these findings, we performed RNA gel blot, immunoblot, and immunofluorescence analyses to follow the level of expression of Atg8 during ClpP1 depletion in the DCH16 strain. Moreover, after we induced autophagy in the A31 strain by rapamycin treatment, which inhibits TOR (Crespo et al., 2005), we compared the morphological changes caused by activation...
of autophagy with those due to the depletion of ClpP1 by electron microscopy (Figure 6A).

As shown in Figure 1E, the Atg8 mRNA was expressed at a very low level in the DCH16 and A31 strains in absence of vitamins (time 0). Upon addition of vitamins, no significant change in the abundance of the Atg8 mRNA was observed in the A31 strain. Instead, an inverse correlation between the decreasing level of ClpP1 and the increasing amount of Atg8 protein was apparent in the DCH16 strain (Figures 2A, 2B, and 3). A significant increase in Atg8 occurred after 36 h of vitamin treatment when the level of the ClpP1 protein drops below a threshold level and the Atg8 mRNA rises considerably (Figures 1E and 2). Immuno-fluorescence microscopy of DCH16 treated with vitamins for 48 h revealed that Atg8 is not only strongly upregulated but also frequently gives rise to a few speckles (Figure 6B). These structures may reflect the phagophore assembly structure described in other organisms (Xie and Klionsky, 2007). After 72 h, cup-shaped structures were apparent and their location coincided with that of vacuoles, suggesting the fusion of Atg8 with large vacuoles (Figure 6C). Moreover, lipid bodies accumulated as revealed by Nile red staining (Figure 6D). They were also detectable after treatment of A31 cells with rapamycin (Figure 6D). Only weak signals were observed in untreated cells (Figures 6B and 6D). In the dark, enlargement and vacuolization of the cells still occurred although more slowly (Figures 4A and 4B) and was associated with an increase of Atg8 (Figure 2B), indicating that the observed changes cannot be solely explained by photooxidative damage. By contrast, the increase in Atg8 induced by rapamycin in the light was abolished in the dark (Supplemental Figure 3A). This agrees with earlier findings, indicating that oxidative stress is involved in the regulation of autophagy and that ROS act as potent inducers of this process (Pérez-Pérez et al., 2012). These observations suggest that, although there are similarities between the responses induced by ClpP1 depletion and inhibition of the TOR kinase by rapamycin, these two processes also differ from each other. Evidence for the involvement of a subset of these genes in the autophagocytic response to nitrogen starvation has been presented by Goodenough et al.
(2014), indicating that C. reinhardtii is endowed with a diverse repertoire for coping with stress.

**Chloroplast Transcriptome Analysis upon ClpP1 Repression**

Previous studies in Arabidopsis have suggested that the chloroplast Clp protease might play a role in regulating plastid RNA metabolism (Zybailov et al., 2009). To test this hypothesis, total RNA was isolated from the DCH16 and A31 strains at three time points following vitamin treatment (0, 12, and 48 h) (Supplemental Figure 2). This RNA was subjected to quantitative hybridizations with an array containing 58 chloroplast and 27 nuclear gene probes using Nanostring technology as described (Geiss et al., 2008; Ramundo et al., 2013) (Supplemental Figure 2). Independently, the levels of several transcripts were also determined by RNA gel blot experiments (Figure 1D). This analysis revealed that most transcripts involved in chloroplast gene expression were upregulated after 48 h, as seen in the heat map of the Nanostring data (Supplemental Figure 2A). This increase was particularly pronounced for the rpo genes that encode the subunits of the chloroplast RNA polymerase and for several transcripts of plastid genes of unknown function (ORF2971, ORF1995, ORF712, and ORF59) (Figure 1D; Supplemental Figures 2A and 2B). Whereas a similar increase was also detected for most of the transcripts of the chloroplast-encoded subunits of the photosynthetic complexes, the mRNA levels of nucleus-encoded...
proteins involved in photosynthesis including antenna proteins (CP29, Lhcbm1, Lhcbm5, and Lhca1) as well as ferredoxin, plastocyanin, PetC, Rbcs2, and Prx1 were decreased (Supplemental Figures 2A and 2B).

In agreement with our immunoblot analyses and electron microscopic studies, we found that ClpP1 depletion strongly enhances the mRNA abundance of nuclear genes encoding chloroplast chaperones (Hsp70B, Hsp22A, and Hsp22F), autophagy-related proteins (Atg8 and Atg7), and proteins involved in thylakoid biogenesis (Vipp1 and Alb3.2) (Figure 1E; Supplemental Figure 2).

Comparative Nuclear Transcriptome Analysis upon Vitamin-Mediated Depletion of ClpP1 and Induction of Autophagy by Rapamycin

The induction of several nucleus-encoded proteins upon ClpP1 depletion prompted us to perform RNA-Seq analysis on the
DCH16 and A31 strains upon vitamin treatment in order to obtain a global view on the remodeling of the nuclear gene expression program induced by the depletion of ClpP1. Based on the kinetics of induction of the Vipp1 and Atg8 mRNAs previously determined by RNA gel blot analysis (Figure 1E), we first selected three different time points at 0, 31, and 43 h following vitamin treatment. To obtain additional information on early time points, a second RNA-Seq analysis was performed at 0, 12, and 48 h. Because treatment of C. reinhardtii cells with rapamycin induces an autophagy-like phenotype that is in many aspects similar to the one caused by the depletion of the Clp protease, we also performed a transcriptomic analysis of the A31 strain treated with rapamycin for 2 and 8 h. For each time point, three biological replicates were used. Only changes >2-fold and statistically significant were considered (see Methods). The numbers of up- and downregulated transcripts identified in this way for the different comparisons are indicated in Table 1. The gene lists corresponding to the different time points are shown in Supplemental Data Sets 1 to 3. Numerous transcripts could be aligned unambiguously on the C. reinhardtii genome, and 5085 of these are differentially expressed in at least one condition. Supplemental Data Set 4 shows a transcriptomic analysis upon ClpP1 depletion and rapamycin treatment.

In order to group genes with similar expression profiles along the time courses for the DCH16 and A31 strains, we performed a gene clustering analysis. A total of 5085 target genes in the DCH16/A31 time courses (0, 12, 31, 43, and 48 h) were pooled and the analysis was performed with 12 clusters (Supplemental Data Set 5). This number was chosen to provide a satisfactory balance between removing redundancies and gaining specificity. A heat map showing the time course of expression of these target genes grouped by similarity in their expression profile from 0 to 48 h for the A31 and DCH16 strains is shown in Figure 7A. Figure 7B displays the same information as kernel-density line plots for each individual cluster (from 1 to 12). The clusters were examined with the algal pathways functional annotation tool (http://pathways.mcdb.ucla.edu/chlamy/) (Lopez et al., 2011) for obtaining Gene Ontology information. Genes involved in DNA replication and repair and the cell cycle were mostly represented in clusters 1 and 2 and display a marked decrease of their transcripts after 43 and 48 h of vitamin treatment, whereas no major changes in the transcript levels of these genes occurred in A31. A similar trend was also observed for cluster 3, which contains genes involved in photosynthesis. Clusters 6 and 7 include genes implicated in amino acid metabolism whose transcripts were significantly increased after 43 and 48 h. This increase in RNA levels was more pronounced for clusters 11 and 12, which include genes involved in autophagy, programmed cell death, protein folding, and proteolysis as well as genes of proteins involved in sulfate and phosphate transport (Supplemental Data Set 4B and Supplemental Figure 4).

### Table 1. Number of Up- and Downregulated Genes upon Vitamin or Rapamycin Treatment

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<th>Time</th>
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<td>48 h</td>
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Genes differentially expressed upon ClpP1 depletion and rapamycin treatment of A31 (strain 1) and DCH16 (strain 2). Number of genes up- or downregulated for these two strains are indicated in the columns up_1, up_2, down_1, and down_2. Number of genes up- and down-regulated in both strains 1 and 2 are listed in columns up_1&2 and down_1&2, respectively. Union refers to the total number of genes differentially expressed at the time points indicated. Genes listed include those with (1) log2 moderate fold change >1, (2) an adjusted P value < 0.01 (false discovery rate < 0.01). The gene lists with expression values are given in Supplemental Data Sets 1 to 3.
Figure 7. Model-Based Clustering of RNA-Seq Data.

Groups of genes with similar expression profiles across all samples were obtained using a negative binomial model as described (Si et al., 2014). 

(A) Heat map of expression levels for significantly regulated genes (a total of 5085; see main text). The map shows the fold change at 0, 12, 31, 43, and 48 h following vitamin addition for the A31 and DCH16 strains (from bottom to top of the heat map), using the gene’s average expression as a reference. The color scale ranges from dark red (positive fold changes) to illustrate significant overexpression, to light yellow (negative fold changes). The tree shows the result of the hybrid-hierarchical clustering, which groups clusters based on their similarity (as computed from the likelihood of the negative binomial model). The lower level of the tree and labels on the x-axis represent the maximum number of clusters used in this work (12).

(B) Kernel-density line plots for individual clusters (from 1 to 12). Each plot compiles the expression profiles for both strains (A31 and DCH16) across the time course (0, 12, 31, 43, and 48 h). For each cluster, the red line shows the cluster’s trend (average expression profile of all genes in the cluster), and the light-blue density map is a histogram of all the individual gene profiles. Also shown are the most significant functional ontologies as described in the main text.
2-fold changes or higher were considered to have a reproducible and statistically significant change in accumulation during the time course.

A list of 348 proteins was obtained whose change in abundance appears to be specifically linked to the depletion of ClpP1 in the DCH16 strain (Table 2; Supplemental Data Set 6). Of these proteins, 27 and 11% are predicted to be targeted to the chloroplast and mitochondria, respectively, by PredAlgo, an algorithm specifically designed for predicting the cellular localization of *C. reinhardtii* proteins that is based on a neural network trained to recognize transit peptide sequences (Tardif et al., 2012). The number of proteins differentially expressed increased at each time point (Supplemental Figure 5). The Venn diagram comparing these proteins reveals a set of proteins whose level changes transiently at each time point. Some of these proteins might also be differentially expressed at the other points but below the threshold of significance, whereas others might be linked to some short-term time-specific cellular events.

Supplemental Data Set 7 provides, for each time point, the list of genes encoding proteins showing at least a 2-fold difference in protein level upon ClpP1 depletion in the DCH16 strain. See Supplemental Data Set 8 for a proteomic analysis of A31 upon vitamin addition. Sorting of these proteins with different abundance in functional categories in Table 3 reveals that upon ClpP1 repression many different basal metabolic functions are affected such as amino acid synthesis and metabolism, RNA metabolism, fatty acid synthesis, lipid metabolism, and cell cycle. Moreover, changes in the abundance of proteins involved in translation and signaling occur. Many of these changes are also seen at the RNA level (Supplemental Data Set 4B); in some cases, they are likely to represent compensatory responses, e.g., stimulation of amino acid synthesis when proteolysis by ClpP1 is impaired.

It is noteworthy that after 36 h of vitamin treatment, proteins with known or predicted ROS scavenger activity accumulate (such as Gshr1 and Cre16.g676600.t1.2), suggesting that the reduced activity of the Clp protease seriously compromises the functionality of the plastid and leads to a response induced by oxidative damage. Unfortunately, the majority of the proteins differentially regulated at this time point have unknown functions and localizations.

At later time points (72 and 96 h) the number of proteins differentially present became quite large and the occurrence of compensatory responses is well illustrated by the increased abundance of many additional proteins that are known to be involved in protein folding and degradation (Hsp22F, ClpB3, Rpn8, UspA, Cpn11, Deg11, FKB-16.2, and Asp1), vesicular trafficking (Art3 and Art1), autophagy (Atg10), DNA replication and repair (RecA and PolE), and tetrapyrrole metabolism (ChlH1).

### Table 2. Number of Proteins of A31 and DCH16 with >2-Fold Changes upon Vitamin Treatment

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Strain</th>
<th>Genes</th>
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<tr>
<td>24 h/0 h vitamin</td>
<td>A31</td>
<td>5</td>
</tr>
<tr>
<td>48 h/0 h vitamin</td>
<td>A31</td>
<td>54</td>
</tr>
<tr>
<td>12 h/0 h vitamin</td>
<td>DCH16</td>
<td>5</td>
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<td>DCH16</td>
<td>34</td>
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<tr>
<td>36 h/0 h vitamin</td>
<td>DCH16</td>
<td>39</td>
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<tr>
<td>48 h/0 h vitamin</td>
<td>DCH16</td>
<td>97</td>
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<tr>
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</tr>
<tr>
<td>96 h/0 h vitamin</td>
<td>DCH16</td>
<td>272</td>
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The listed proteins are satisfying the following conditions: significant fold change (>2) and significant adjusted P value (<0.05).

Comparison between Transcriptomic and Proteomic Data: Potential ClpP1 Substrates

Having identified many mRNAs and proteins that change significantly during the time course of ClpP1 depletion, we next determined how the transcriptomic and proteomic data compare with each other. Among the 348 identified proteins, we tested to what extent the changes in protein and corresponding mRNA levels correlate with each other during ClpP1 depletion. Of these, 148 and 53 are increased and decreased, respectively, both at the protein and mRNA level, whereas in 33 cases, the proteins decrease and their mRNAs increase and, for 78 cases, the opposite pattern occurs. Comparison of the RNA and protein changes for individual genes at 48 h (Supplemental Data Set 6 and Supplemental Figure 6) revealed that they differ widely. Because in the absence of ClpP1, the levels of its chloroplast protein substrates would be expected to increase presumably with only modest changes in the corresponding RNA levels, we examined the proteins that follow such a pattern. We chose cutoffs of <2-fold for the RNA changes and >2-fold for protein changes due to ClpP1 depletion. In this way, 78 genes were identified, of which 16 were predicted to encode chloroplast proteins that are involved in a variety of cellular processes (Supplemental Data Set 6C and Supplemental Figure 6). This includes several unknown proteins with possible functions in lipid and pyrimidine metabolism and signaling, proteins involved in amino acid metabolism, RecA, which is involved in chloroplast DNA recombination, pyruvate dehydrogenase, a putative aspartate kinase, ClpP4 (part of the ClpP protein complex), the chaperonin Cpn11, the plastid ribosomal protein Psrp6, and Tic110, a component of the chloroplast inner membrane translocon. These proteins could be potential substrates of ClpP1.

Specificity of the Response Induced through ClpP1 Depletion

The transcriptomic and proteomic data clearly indicate specific changes in both RNA and protein accumulation upon depletion of the ClpP1 protease that differ at least to some extent from those induced by other stress conditions. To further study the specificity of this response, we first compared the transcriptomic responses induced by ClpP1 depletion and rapamycin treatment. Table 1 shows that among the up- and downregulated genes of DCH16 upon ClpP1 depletion, significant fractions, 58 and 64%, respectively, are similarly affected in the A31 strain treated with rapamycin. It should be noted, however, that the irradiance was changed from 10 to 60 μmol m⁻² s⁻¹ when rapamycin was added because induction of autophagy by rapamycin requires this light intensity (Pérez-Pérez et al., 2010). We noticed that, in some cases, this increased irradiance alone caused a change in transcript level that was larger than that induced by rapamycin.
that it is not caused by phototoxic damage alone. However, contrary to the Vipp2-gLUX reporter, the Deg11-gLUX reporter was also activated by tunicamycin.

**DISCUSSION**

The major goal of this study was to investigate the role of the chloroplast ATP-dependent ClpP protease in cellular metabolism and growth. Because it is an essential protein, we took advantage of our recently developed repressible chloroplast gene expression system that is based on the vitamin-sensitive Thi4 riboswitch (Ramundo et al., 2013). This allowed us to examine the fate of *C. reinhardtii* in which ClpP1 is progressively depleted. Our results demonstrate that ClpP1 is essential for cell survival and show that ClpP1 depletion leads to the arrest of cell growth and protein aggregation associated with a massive upregulation of chaperones and proteases, a hallmark of an unfolded protein response, and metabolic adaptation. Moreover, the cellular membrane system is considerably perturbed with a striking increase in the number of vacuoles in the cytosol and vesicles in the chloroplast which presumably are derived from the thylakoid membrane (Figures 4 and 5). Our comparative study of the stress response induced by ClpP1 depletion and of rapamycin reveals that these processes share common features but also differ in many respects from one other.

It is particularly striking that many of the proteins with increased abundance are involved in sulfur transport and recycling (Sul2, Slt3, Met16, and Aot4), iron-sulfur cluster biogenesis (SufD), and in trans-sulfuration pathways (DhpS). Because glutathione is a key factor for buffering the cellular redox state, the induction of sulfur metabolism upon ClpP1 depletion may be linked to an increased demand of cysteine, the limiting reagent for glutathione production. Alternatively, it is possible that depletion of ClpP protease impairs a key component of the sulfur metabolic pathway, thus activating a sulfur starvation response.
Interestingly, among the proteins with increased accumulation involved in protein homeostasis, some are known to have isoforms with different subcellular localizations. Examples are the heat shock proteins Hsp22 and Hsp70, the Deg proteases, the ClpB protein disaggregases, and the GroES-type chaperonins. In our proteomic analysis, the isoforms identified are mainly the ones targeted to the chloroplast compartment. Another protein whose level increases is ClpP4, one of the other two paralogous active catalytic subunits of the Clp protease encoded by the nuclear genome. It is possible that the cell senses the impaired stoichiometry during assembly of the Clp catalytic chamber and may attempt to overcome this limitation by overexpressing another catalytic subunit of the complex.

Genes whose transcripts are downregulated upon both ClpP1 depletion and rapamycin treatment are involved in the cell cycle and in DNA replication, repair, and recombination as well as in various signaling pathways and photosynthesis (Supplemental Data Sets 4A and 4B). Genes that are upregulated under both conditions are implicated in amino acid metabolism, vacuolar function, tetrapyrrole metabolism, autophagy, and transport of metabolites. Among the most highly upregulated transcripts were those of several small HSP proteins and chaperones, proteases, proteins involved in autophagy and thylakoid membrane biogenesis, protein kinases and transporters, and ion channels (Supplemental Data Set 4B). Among the latter, one gene of particular interest is Msc1, which encodes a mechanosensitive ion channel suggested to be localized both in the cytosol and chloroplast (Nakayama et al., 2007). It could be involved in sensing osmotic stress, allowing the cell to counteract changes in osmotic pressure during the cellular swelling that accompanies autophagy.

Depletion of ClpP Affects Chloroplast and Cytosolic RNA Metabolism

A striking effect of ClpP depletion is the increased accumulation of many chloroplast mRNAs (Figure 1; Supplemental Figure 2). In this respect, one chloroplast gene of particular interest is ORF1995. It gives rise to a full-length transcript of 6600 nucleotides and to several smaller transcripts, which all increase upon vitamin treatment (indicated by arrows in Figure 1D), suggesting the existence of a plastid negative feedback control. However, it is not clear whether the smaller transcripts represent functional processed transcripts or degradation products of the full-length
transcript. It is particularly noteworthy that in most of the cases, chloroplast and nuclear genes involved in photosynthesis respond in opposite ways with chloroplast transcripts increasing while the cytosolic transcripts decrease.

The transcriptomic and proteomic analyses indicate that prolonged ClpP1 depletion causes a drastic upregulation of several ribonucleases, such as Rbn1 and Rbn2, which act as 3′-5′ exoribonucleases in the exosome complex and actively participate in RNA maturation and quality control (Supplemental Data Set 4), and Xrn1, a putative organelle-localized homolog of the cytosolic 5′-3′ exoribonuclease and a potential ribonuclease Z. Importantly, RNases Z are mainly known for their role in tRNA processing, but recently an additional function in mRNA decay has been suggested, especially in organisms lacking RNase E (Perwez and Kushner, 2006). The upregulation of these genes and the unexpected upregulation of many chloroplast mRNAs may indicate a restructuring of intracellular RNA populations and/or changes in an RNA quality control mechanism. Consistent with this hypothesis, we found that, among the most upregulated transcripts in this functional category, three of them encode uncharacterized proteins (Cre17.g725750.t1.11, Cre17.g726850.t1.12, and Cre10.g447800.t1.2) that show remarkable sequence similarity to the bacterial Rsr and to the human Ro 60-kD autoantigen but are not predicted to be targeted to the chloroplast (Stein et al., 2005) (Supplemental Data Set 4 and Supplemental Figure 4F). Intriguingly, both in bacteria and in humans, these proteins are known to act as cofactors to increase the association of exoribonucleases with misfolded RNA substrates during stress (Chen et al., 2005; Stein et al., 2005; Fuchs et al., 2006; Wolin and Wurtmann, 2006). We found that the Cre17.g725750.t1.1 transcript is also upregulated under high light (Supplemental Figure 4F). These results raise the question whether an unfolded RNA response exists in C. reinhardtii as part of an integrated stress response.

**Potential of ClpP1**

Our comparative transcriptomic and proteomic analysis of the 348 proteins that are differentially expressed upon ClpP1 depletion revealed that 137 (39%) also display changes in their corresponding mRNAs. For the remaining 211 proteins (61%), no significant changes in transcript accumulation could be detected, indicating that the changes in protein levels are mainly due to translational or posttranslational processes. In the absence of ClpP1, its chloroplast protein substrates would be expected to accumulate, presumably with only small changes in the corresponding RNA levels. At 48 h following vitamin treatment, we identified 15 chloroplast proteins with this property (Supplemental Data Set 6C and Supplemental Figure 8). These proteins are involved in plastid chloroplast metabolism, protein synthesis, turnover, and folding, and DNA recombination. Some of the proteins in this list were also found to increase in the ClpR2 mutant of Arabidopsis (Zybailov et al., 2009). Moreover, RecA has been proposed to be a ClpP substrate in Staphylococcus aureus (Feng et al., 2013). All of these proteins are soluble, with the exception of Tic110, which is located in the inner chloroplast envelope membrane. This broad range of potential substrates is compatible with the fact that ClpP1 is the most abundant protease in the chloroplast stroma. However, we cannot rule out that the increased abundance of these proteins is due to an indirect effect of ClpP1 depletion or is caused by increased translation rather than reduced proteolysis. Alternatively, removal of posttranslational modifications from quantified peptides as a consequence of ClpP1 depletion would also lead to an apparent increase of these peptides as phosphorylated peptides would have escaped detection.

**Loss of ClpP1 Leads to Increased Accumulation of the Thylakoid FtsH Protease**

Chloroplasts are known to contain a number of proteases; some are localized in the stroma, whereas others, such as the FtsH and the Deg proteases, are associated with the thylakoid membranes and involved in the PSII photorepair cycle (Kato and Sakamoto, 2013). Recent results indicate that in the Arabidopsis var2 mutant deficient in FtsH2, ClpP1 is upregulated and a portion of this protease is relocalized from the stroma to the thylakoid membranes, suggesting compensation for the limitation in FtsH (Kato et al., 2012). Similarly, upon exposure to excess light, ClpP1 is also upregulated, implying cooperation between different chloroplast proteases under stress conditions (Kim et al., 2009). In this study, we showed that the reverse is true when ClpP1 is limiting. Upon gradual depletion of ClpP1 induced by vitamin treatment of DCH16, the level of FtsH increased significantly, suggesting a compensatory proteolytic response to mitigate the loss of ClpP1 (Figures 2A and 3B). Importantly, this compensation effect was only observed when ClpP1-depleted cells are grown in the dark or dim light (10 μmol m⁻² s⁻¹), suggesting that this process is sensitive to photooxidative damage (Figure 2B).

**Autophagy-Like Phenotype and Membrane Trafficking upon ClpP1 Depletion**

Direct comparison of the mRNAs differentially expressed as the consequence of ClpP1 depletion or rapamycin treatment revealed that the genes that are upregulated under both conditions comprise 28 and 58% of the total number of the upregulated genes in the vitamin-treated DCH16 and A31 strains, respectively. The corresponding numbers for the downregulated genes are 48 and 64% (Table 1). These results suggest common regulatory mechanisms used by the cells to orchestrate these stress responses. The list of the most upregulated genes (Supplemental Data Set 4B) includes a group of autophagy-related genes including Atg8, Atg3, Atg6, Atg7, and Atg12. The induction of these genes is a typical hallmark of autophagy. Moreover, the set of highly upregulated mRNA encode proteins involved in membrane biogenesis and vesicular trafficking, including two SNARE proteins (Vmp12 and Vamp74) that catalyze the homotypic membrane fusion during the late stages of autophagosome formation both in mammalian cells and yeast (Nair et al., 2011; Renna et al., 2011; Stroupe, 2011). The strong upregulation of the related chloroplast Vipp1 and Vipp2 genes raises the possibility that they are involved in the breakdown/disassembly of the thylakoids and in chloroplast lipid trafficking and membrane remodeling. Whether the released polar lipids serve as a cellular membrane source during autophagy remains an open question. Vipp1 and Vipp2 are partially redundant
and have been proposed to be involved in the assembly and biogenesis of thylakoid core complexes, perhaps by providing structural lipids (Nordhues et al., 2012). The absence of induction of Lhcsr3, Hsp70B, and Hsp90C in Vipp1-RNAi strains upon high-light exposure suggests that Vipp1 might play a role in the high-light retrograde signaling pathway. As the expression of Vipp2 is not markedly affected by changes in the redox state of the photosynthetic electron transport chain, it was suggested that specific photoreceptors may be involved (Im et al., 2006). However, in this work, we show that the amount of Vipp2 protein can also be increased in the dark upon depletion of the chloroplast ClpP1 protease.

Marked changes in protein levels were already detected at early time points. A decrease of 50% in ClpP1 expression after 12 h of vitamin treatment (Figure 1D) was sufficient to trigger significant changes in accumulation of a few proteins (Supplemental Figure 6 and Supplemental Data Set 7). In particular, the levels of two proteins, Vipp2 (Cre11.g468050.t1.1) and a protein encoded by Cre16.g683350.t1.1, were strongly increased. The other protein (Cre16.g683350.t1.1), which is not predicted to be targeted to the chloroplast, is a member of the thioesterase superfamily. These enzymes catalyze the hydrolysis of long-chain fatty acyl-CoA thioesters (acyl-CoAs) to free fatty acids and CoA (CoASH). Importantly, acyl-CoA is not only a building block in lipid metabolism, it can also serve as a signaling molecule in a wide range of biological activities such as development, stress responses, vesicular trafficking, membrane biogenesis, and other signaling pathways (Xiao and Chye, 2011). Therefore, the presence of this protein in the early phase of ClpP1 depletion raises the possibility that retrograde signaling may be mediated in part by lipid molecules.

**Genes Regulated by ClpP1 Depletion but Not by Rapamycin Treatment May Be Involved in a Chloroplast Unfolded Protein Response**

Together with the proteomic analysis, our transcriptomic analysis indicates that depletion of the ClpP1 plastid protease subunit causes induction of genes coding for factors involved in chloroplast and cytosolic protein folding and proteolysis (Supplemental Data Sets 4 and 7). In particular, the chloroplast small heat shock proteins Hsp22E and Hsp22F and the HtrA protease Deg1 are three of the most highly upregulated genes identified in our study. Other upregulated mRNAs of protein quality control factors include the ubiquitin conjugating enzyme E2 (Cre01.g027200.t11.1), the chaperones ClpB3, DnaJ5, and DnaJ34, and a set of poorly characterized proteases. Taken together, our data suggest the existence of an unfolded protein response.

Genes that are downregulated both by ClpP1 depletion and rapamycin treatment include many genes involved in nuclear DNA replication and repair, plastid division, centriole and basal body duplication, and in response to a chloroplast dysfunction (Supplemental Data Set 4 and Supplemental Figure 4B). Under these stress conditions it appears that cells devote their resources to maintenance and survival at the expense of cell growth and division.

Although the stress responses elicited through depletion of the plastid ClpP1 protease and by the inhibition of the TOR kinase by rapamycin show similarities, there are also significant differences as revealed by the global comparative transcriptional analysis (Table 1). The list of the most upregulated genes with an annotated function includes chaperones, proteases, and ubiquitin-related factors in addition to those already identified in the list of the genes commonly regulated by ClpP1 depletion and rapamycin treatment. Intriguingly, some of these transcripts encode different isoforms of proteins belonging to the same family, in particular in the case of the small heat shock proteins. These proteins form dimers with a hydrophobic surface at their N-terminal end for interaction with denatured proteins with which they form a large complex to prevent their irreversible aggregation (Lee et al., 1997; Haslebeck et al., 2004). In C. reinhardtii, this family contains eight members (Schroda and Vallon, 2009). Of these, isoforms A and B are predicted to be in the cytosol and isoforms C, E, and F in the chloroplast, all of which are upregulated upon ClpP1 depletion. Several transcriptomic analyses have revealed that Hsp22C, Hsp22E, and Hsp22F can be induced by a variety of stresses, such as heat shock, oxidative stress, and phosphorus and sulfur starvation (Gonzales-Ballester and Grossman, 2009; Moseley and Grossman, 2009; Schroda and Vallon, 2009). We found that Hsp22B, Hsp22C, and Hsp22F are upregulated by ClpP1 depletion but not by rapamycin treatment under constant dark or light (Supplemental Figures 4D and 8 and Supplemental Data Set 4). Moreover, the loss of the ClpP1 protease caused a specific upregulation of Cgl41, an uncharacterized protein containing an RbcX domain, usually found in an assembly chaperone for hexadecameric Rubisco (Saschenbrecker et al., 2007) (Supplemental Figure 4 and Supplemental Data Set 4). Taken together, these findings suggest that the disruption of protein homeostasis in the chloroplast compartment can be sensed and transduced to the nucleus to induce the expression of a specific set of protein quality control factors. Since in Arabidopsis constitutive reduction of the ClpPR protease also results in upregulation of chloroplast chaperones and protein sorting components, it is possible that this retrograde signaling pathway is evolutionarily conserved (Zybailov et al., 2009).

In mammalian cells, such a homeostatic pathway is well known to operate when functional perturbations occur in the ER in which most proteins are folded, modified, and assembled before being secreted or displayed on its surface. The discovery of this signaling network, named eRUPR, arose from a pioneering study in which the pharmacological inhibition of folding led to the transcriptional up-regulation of several key ER chaperones (Cox et al., 1993). More recently, a similar pathway named mitochondrial unfolded protein response (mtUPR) was proposed to counteract the accumulation of unfolded proteins within the mitochondrial matrix through the transcriptional upregulation of nuclear genes encoding mitochondrial stress proteins such as chaperones Hsp60, Hsp70, mtDNAJ, and ClpP1, but not those encoding stress proteins of the ER or the cytosol (Aldridge et al., 2007).

The observed changes in gene expression caused by ClpP1 depletion raise the possibility that a specific chloroplast unfolded protein response exists. It should be noted that all of the upregulated chaperones are not exclusively localized in the chloroplast, although the majority of these proteins are predicted to be in this organelle. Moreover, because the vast majority of the chloroplast proteins are encoded by the nuclear genome, are synthesized in the cytoplasm, and are imported in an unfolded
state into the organelle via specialized translocases, impairment of the stromal protein folding environment is expected to limit the availability of the stromal chaperones and ATP required for protein import and the refolding of the mature proteins upon translocation. In this case, the protein import capacity of the translocon complex is likely to be compromised at the chloroplast envelope, which would impact protein homeostasis in the cytosolic compartment and necessitate the upregulation not only of chloroplast but also of cytosolic chaperones. In this respect, it is interesting to note the appearance of perforations in the chloroplast envelope upon ClpP1 depletion (Figure 5E). They could reflect changes in the envelope structure that may affect protein import.

In the list of the transcripts specifically upregulated by ClpP1, we identified some mRNAs of poorly characterized and potentially organelle-associated proteins that are related to E2 ubiquitin-conjugating enzymes or contain UBX and UFD domains (Meyer et al., 2012) (Cre10.g429001.t1.1, Cre03.g179100.t1.1, and Cre12.g510300.t1.2) and to the mitochondrial Vms1 protein (Cre26.g772100.t1.2) (Supplemental Figure 4E). Others contain the DUB/PPDE domain in Cre16.g662450.t1.1 proposed to act as deubiquitinating and desumoylating peptidase. Importantly, in yeast, Vms1 was recently shown to be induced upon oxidative stress and to interact with Cdc48/p97, a conserved chaperone-like ATPase that forms a homohexameric complex and regulates a large array of cellular functions, including protein degradation, cell division, membrane fusion, autophagosome biogenesis, retrotranslocation of unfolded proteins from the ER to the cytosol, and segregation and delivery of ubiquitylated proteins to the proteasome for degradation (Meyer et al., 2012). Vms1 recruits Cdc48/p97 to the outer mitochondrial membrane and thereby mediates the extraction and ubiquitination and proteasome-mediated degradation of mitochondrial proteins in the cytosol (Heo et al., 2010).

A pathway for protein degradation through the extrusion of chloroplast proteins to the vacuoles was proposed for *C. reinhardtii* (White et al., 1998; Park et al., 1999). Major pulse-labeled polypeptides synthesized on chloroplast ribosomes were recovered in small granules in cytoplasmic vacuoles. Moreover, protrusions of the outer membrane of the chloroplast envelope were detected that enclosed stroma, suggesting that chloroplast proteins from the stromal phase were extruded from the chloroplast in membrane-bound structures to the vacuoles. Similarly, greening of the *C. reinhardtii* yf1 mutant in the light at 38°C revealed the presence of light-harvesting proteins inside the chloroplast near the envelope and also in granules within vacuoles in the cytosol. This suggests that this pathway may operate when excess light-harvesting proteins are synthesized with respect to integration of these proteins into thylakoid membranes (White et al., 1996; Park et al., 1999). The observed upregulation of Vms1 and Cdc48 upon ClpP1 depletion raises the possibility that several chloroplast protein extrusion processes may operate in *C. reinhardtii* under specific stress conditions to degrade excess proteins or to provide free amino acids when amino acid supply is limiting.

The use of the reporter constructs Vipp2-gLUX and Deg11-gLUX provided several new important insights into the regulation of the expression of these genes. First, both Vipp2 and Vipp2-gLUX are specifically induced by the depletion of ClpP1 or by high-light treatment, indicating that upregulation of the Vipp2 gene is mediated by its promoter and/or 5′ UTR (Figure 8). Second, this induction also occurs in the dark and does not require a light signal. Instead, it may be triggered by accumulation of unfolded proteins and/or damaged thylakoid membranes, which is also likely to occur upon photodamage induced under high light (Figure 8). However, VIPP2 is not induced by heat shock, a treatment that is also expected to unfold proteins (Nordhues et al., 2012). Possibly, depletion of ClpP1 may induce a stronger and more chloroplast-specific response to which Vipp2 is reactive. Third, in these experiments performed either under constant light or in the dark, rapamycin did not upregulate Vipp2 RNA in contrast to the experiments profiled by RNA-Seq in which the light irradiance was changed after addition of the drug (for more details, see Supplemental Data Sets 3 and 4 online). Vipp2 therefore appears to be well suited for genetic screens aimed at identifying the components of the retrograde signaling system, which lacks ClpP1 depletion with nuclear gene expression. The Deg11-gLUX reporter responds in a similar manner as Vipp2-gLUX except that it is also induced by tunicamycin, which inhibits glycosylation in the ER. This raises the question of possible interactions between ER and chloroplasts. The observation of contact sites between these two organelles suggests that they interact physically (Andersson et al., 2007). Moreover, it is interesting to note that a few nucleus-encoded plastid protease use the secretory pathway and are N-glycosylated in the ER before they enter the chloroplast (Villarrejo et al., 2005).

In conclusion, we took advantage of the conditional repressible chloroplast gene expression system in *C. reinhardtii* to conduct a detailed study of the role of the essential plastid *clpP1* gene through a comparative cell biology, transcriptomic, and proteomic analysis. This approach has revealed that ClpP1 is involved, directly or indirectly, in a variety of molecular events that underlie protein homeostasis and retrograde signaling. Thus, this work opens new avenues for identifying new molecular components involved in these processes and pathways.

**METHODS**

**Strains, Growth Conditions, and Media**

The *Chlamydomonas reinhardtii* strains generated in this study were maintained on TAP or minimal (HSM) medium plates supplemented with 1.5% Bacto-agar (Gorman and Levine, 1965; Harris, 1989) at 25°C under constant light (60 to 40 μmol m⁻² s⁻¹ dim light (10 μmol m⁻² s⁻¹)) or in the dark. Growth medium containing vitamins was prepared as previously described (Ramundo et al., 2013). See Supplemental Methods for more details on growth conditions.

**Construction of the Strains DCH16, CS174, CS193, and CS365**

Nuclear and chloroplast transformations were performed as previously described (Ramundo et al., 2013). Plasmids used for generating the strains DCH16, CS174, CS193, and CS365 are described in Supplemental Methods.

**Fluorescence Measurements**

Maximum quantum efficiency of photosystem II (Fv/Fm) was measured as described (Ramundo et al., 2013).
Protein Extraction and Immunoblot Analysis
Total protein extraction and immunoblot analysis was performed as described (Ramundo et al., 2013). For details, see Supplemental Methods.

Nucleic Acid Extraction
Isolation of total RNA and DNA from C. reinhardtii strains was performed as described (Ramundo et al., 2013).

RNA Gel Blotting
Hybridizations of RNA gel blots were performed as described (Ramundo et al., 2013). The probe used for ORF1995 corresponds to the region between amino acids 1082 and 1463.

Immunofluorescence
C. reinhardtii cells were fixed and stained for Apg8 immunofluorescence as described (Diaz-Troya et al., 2008). Time and light conditions are indicated in the text.

Nile Red Staining
C. reinhardtii cells were stained with Nile Red and images were acquired through a Leica TCS-SP2 confocal microscope as described (Wang et al., 2009). Time and light conditions are indicated in the text.

Optical and Transmission Electron Microscopy
A total of 4 × 10⁷ cells/mL were pelleted by centrifugation (5 min, 1000g, room temperature), resuspended very gently in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7, and incubated at room temperature for 2 h. The cells were then pelleted by centrifugation (2 to 3 min, 500g), washed in cacodylate, and postfixed in 1% OsO₄ in cacodylate for 1 h at room temperature. They were rinsed in buffer and further fixed for 1 h in 1% aqueous uranyl acetate. Samples were washed in water, dehydrated in a graded ethanol series, and embedded in Epon 812. Semithin sections, 1 μm in thickness, were cut with a histoDiatome diamond knife (Diatome). Ultrathin sections 80-nm thick were also cut with an ultr Diatome knife. The semithin sections were first stained for 20 min at 65°C with a 1:20 dilution of a methylene blue solution (0.15% in 0.1 M phosphate buffer containing glycerol 1% and methanol 1%). They were dried and restained with a 1:20 solution of basic fuscin (0.1% in 50% ethanol) for 10 min at room temperature. The sections were dried, mounted in Eukitt, and observed using a Nikon eclipse 80i microscope. They were photographed with a Nikon Digital Color Camera Sight DS-Fi-1. The ultrathin sections were stained with 2.5% uranyl acetate and subsequently with Reynolds lead citrate. They were viewed in a FEI Tecnai G2 Sphera transmission electron microscope at 120 kV. Surface areas were measured using Leica Twin Image analysis software. For quick-freeze deep-etch electron microscopy, cells were pelleted, placed on a cushioning material, and dropped onto a liquid-He-cooled copper block; the frozen material was transferred to liquid nitrogen and fractured, etched at −80°C for 20 min, and Pt/C rotary replicated as described (Heuser, 2011). Replicas were examined with a JEOL electron microscope, model JEM 1400, equipped with an AMTV601 digital camera. The images are photographic negatives; hence, protuberant elements of the fractured/etched surface are most heavily coated with platinum and appear white.

Nanostring nCounter Expression Analysis
RNA was isolated from the A31 and DCH16 strains after 0, 12, and 48 h of vitamin treatment. Three biological replicates were prepared for each sample. Nanostring nCounter Expression analysis was performed as previously described (Ramundo et al., 2013).

RNA-Seq
RNA was isolated from the A31 and DCH16 strains grown under 60 μmol m⁻² s⁻¹ after 0, 31, and 43 h of vitamin treatment and after 2 and 8 h of rapamycin treatment of A31 as described (Ramundo et al., 2013). RNA-Seq libraries were essentially built as previously described (Castruita et al., 2011) and sequenced on a HiSequence 2500 from Illumina. Raw sequence files were obtained using Illumina’s proprietary software and are available at NCBI’s Gene Expression Omnibus (accession number GSE56259).

Nanostring nCounter Expression analysis was performed using STAR v2.3.0 (Dobin et al., 2013). The v4 assembly of the C. reinhardtii genome and the Augustus v10.2 gene models were used as reference for STAR. The counts-per-gene matrix was generated with HTSeq-count v0.5.4p3 (Simon Anders; http://www-huber.embl.de/users/anders/HTSeq) using strict intersection and unique alignments to the genome only. For each gene, expression estimates were obtained after normalizing by sequencing depth and transcript mappable length and reported in units of reads per kilobase of transcript mappable length and millions of mapped reads. Log₂-fold changes relative to the 0 time point were calculated for each strain and experiment using average expression estimates from three biological replicates. For each pairwise comparison, moderate fold changes, P values, and adjusted P values were computed with DESeq v1.12.1 (Anders and Huber, 2010), using a negative binomial model and per-condition dispersion estimates. For each comparison, a gene was deemed differentially expressed if it met the following three criteria: (1) significant fold change (≥2); (2) significant expression value (bigger than the median for the condition with highest expression); and (3) significant adjusted P value (padj<0.01). Altogether, 5038 genes passed these criteria in at least one comparison. Model-based gene clustering (Figure 6) was performed in R using the MBCluster. Seq package (Si et al., 2013). RNA-Seq data quality control was performed with Partek Genomics Suite 6.6 beta.

Proteomics
Quantitative shotgun proteomics using a uniform ¹⁵N-labeled standard was performed as described by Mühlaus et al. (2011) with some modifications. In brief, one culture of the A31 strain and two cultures of the DCH16 strain were grown for ~10 generations in TAP medium containing ¹⁵N in the case of A31 and ¹⁴N and ¹⁵N-ammonium in the case of DCH16. At time point 0 h, vitamins were added to the three cultures that were maintained in exponential phase through daily dilutions. Samples were taken from A31 (¹⁴N) after 12 and 48 h of vitamin treatment and from DCH16 (¹⁴N and ¹⁵N) after 0, 12, 24, 36, 48, 72, and 96 h of vitamin treatment. The seven time point samples from DCH16 grown in ¹⁵N-containing medium were pooled to generate a ¹⁵N -labeled reference standard that included all induced and repressed proteins during ClpP1 depletion. Prior to protein extraction, each ¹⁴N sample from the A31 and DCH16 time course was spiked with the ¹⁵N-labeled reference sample at a ratio of 0.8 ¹⁵N/¹⁴N based on protein content determined by the BCA assay (Thermo Scientific). Mixed cells were ruptured by freeze/thawing cycles and afterwards centrifuged in a tabletop centrifuge at 4°C and 21,500g for 35 min. The supernatant, containing soluble proteins, was recovered. The soluble proteins were precipitated with acetone, digested with trypsin and LysC, and desalted. For each sample, extracted peptides of three biological replicates were subjected in triplicates to reverse-phase separation by nanoACQUITY UPLC (Waters). Separated peptides were directed by ESI to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific), which was operated in a cycle of one full-scan mass spectrum (Orbitrap; 300 to 1800 m/z) at a set resolution of 60,000 at 400 m/z followed by serial data-dependent tandem mass spectrometry scans of the five most intense peaks. Four different search engines were employed to achieve spectra-
to-peptide mappings, and a total of 7342 peptides could be identified. Of these, 5038 were mapped with high confidence, i.e., could be recognized by all four search engines. Protein abundance was quantified using 14N/15N ratios from three biological replicates for a total number of 11,702 protein models. Of these 5035 proteins with values for at least three time points were retained. From these data, the following comparisons were done: (1) A31(12 h)/A31(0 h), (2) A31(48 h)/A31(0 h), (3) DCH16(12 h)/DCH16(0 h), (4) DCH16(24 h)/DCH16(0 h), (5) DCH16(36 h)/DCH16(0 h), (6) DCH16(48 h)/DCH16(0 h), (7) DCH16(72 h)/DCH16(0 h), and (8) DCH16(96 h)/DCH16(0 h). In total, 57 proteins from the A31 comparisons and 348 proteins from the DCH16 comparisons were retained with a fold change >2. Protein data evaluation was performed using Partek Genomics Suite 6.6 beta.

Accession Numbers
Sequence data from this article can be found in at NCBI’s Gene Expression Omnibus under accession number GSE56295. Accession numbers for the psbD genes used in making constructs for this work are EDP09084.1, and Cre11.g46805.t1.1.

Supplemental Data
The following materials are available in the online version of this article.

**Supplemental Figure 1.** Homoplasmicity of the DCH16 Strain.

**Supplemental Figure 2.** Comparative Chloroplast RNA Analysis in A31 (Wild-Type) and DCH16 upon ClpP1 Depletion.

**Supplemental Figure 3.** Stress-Induced Responses.

**Supplemental Figure 4.** Quantitative RT-PCR of Selected Nuclear Transcripts.

**Supplemental Figure 5.** Venn Diagram for Proteins Differentially Expressed in DCH16 upon Vitamin Treatment.

**Supplemental Figure 6.** Comparison of Protein and RNA Accumulation in DCH16 after 48 h of Vitamin Treatment.

**Supplemental Figure 7.** Immunoblot Analysis of CS191, CS174, and DCH16.

**Supplemental Figure 8.** Quantitative RT-PCR of Deg11, Hsp22B, Hsp22F, and Vipp2 in A31.

**Supplemental Figure 9.** Comparison of Changes in Gene Expression between Cells Shifted from Low Light/Dark to Higher Light in the Presence or Absence of Rapamycin.

**Supplemental Table 1.** List of Primers.

**Supplemental Methods.**

**Supplemental References.**

**Supplemental Data Set 1.** List of Genes with Significant Changes in RNA Levels in A31 after 12, 31, 43, and 48 h of Vitamin Treatment.

**Supplemental Data Set 2.** List of Genes with Significant Changes in RNA Levels in DCH16 after 12, 31, 43, and 48 h of Vitamin Treatment.

**Supplemental Data Set 3.** List of Genes with Significant Changes in RNA Levels in A31 after 2 and 8 h of Rapamycin Treatment.

**Supplemental Data Set 4.** Transcriptomic Analysis upon ClpP1 Depletion and Rapamycin Treatment.

**Supplemental Data Set 5.** Clustering of 5085 Genes According to Their Expression Patterns after Vitamin Depletion in DCH16 (0, 12, 31, 43, and 48 h).

**Supplemental Data Set 6.** Global Proteomic Analysis.

**Supplemental Data Set 7.** Proteomic Analysis during ClpP1 Depletion in DCH16.

**Supplemental Data Set 8.** Proteomic Analysis of A31 upon Vitamin Addition.

**REFERENCES**


Conditional Depletion of the *Chlamydomonas* Chloroplast ClpP1 Protease Activates Nuclear Genes Involved in Autophagy and Plastid Protein Quality Control

Silvia Ramundo, David Casero, Timo Mühlihaus, Dorothea Hemme, Frederik Sommer, Michèle Crèvecoeur, Michèle Rahire, Michael Schroda, Jannette Rusch, Ursula Goodenough, Matteo Pellegrini, Maria Esther Perez-Perez, José Luis Crespo, Olivier Schaad, Natacha Civic and Jean David Rochaix

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