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Review

Conditional repression of essential chloroplast genes: Evidence for new plastid signaling pathways☆

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ABSTRACT

The development of a repressible chloroplast gene expression system in *Chlamydomonas reinhardtii* has opened the door for studying the role of essential chloroplast genes. This approach has been used to analyze three chloroplast genes of this sort coding for the α subunit of RNA polymerase (*rpoA*), a ribosomal protein (*rps12*) and the catalytic subunit of the ATP-dependent ClpP protease (clpP1). Depletion of the three corresponding proteins leads to growth arrest and cell death. Shutdown of chloroplast transcription and translation increases the abundance of a set of plastid transcripts that includes mainly those involved in transcription, translation and proteolysis and reveals multiple regulatory feedback loops in the chloroplast gene circuitry. Depletion of ClpP profoundly affects plastid protein homeostasis and elicits an autophagy-like response with extensive cytoplasmic vacuolization of cells. It also triggers changes in chloroplast and nuclear gene expression resulting in increased abundance of chaperones, proteases, ubiquitin-related proteins and proteins involved in lipid trafficking and thylakoid biogenesis. These features are hallmarks of an unfolded protein response in the chloroplast and raise new questions on plastid protein homeostasis and plastid signaling.
This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Introduction

It is well established that the origin of chloroplasts can be traced to an endosymbiotic event in which a cyanobacterium was engulfed by a eukaryotic host. During evolution most of the cyanobacterial genes were relocated to the host nucleus and only a relatively small fraction of these genes (between 100 and 200 in extant plant and algal species) was retained in the chloroplast. Many of these genes encode components of the photosynthetic apparatus and of the chloroplast gene expression system. In addition, a small set of chloroplast genes codes for proteins involved in various metabolic pathways including heme assembly, lipid synthesis and proteolysis. Finally several conserved hypothetical chloroplast open reading frames (usually annotated as *ycf* genes) are present in plastid genomes whose function is unknown in most cases. The remaining chloroplast proteins, in total ca. 3000, are encoded by nuclear genes, translated on cytosolic ribosomes and imported into chloroplasts [1].

Chloroplast transformation has been extensively used to elucidate the role of chloroplast genes through reverse genetics in the green unicellular alga *Chlamydomonas reinhardtii* and in tobacco. This is mainly due to the fact that, in these organisms, homologous DNA recombination occurs very effectively in chloroplasts [2,3] and a powerful selectable marker, *aadA*, is available to confer resistance to spectinomycin and streptomycin on the chloroplast transformants [4,5]. Thus it is possible to perform specific plastid gene disruptions and site directed mutagenesis [6].

All chloroplasts exhibit genome polyploidy with 80 and 100 copies per chloroplast in *C. reinhardtii* and tobacco, respectively. As an example, mesophyll cells have around 100 chloroplasts per cell, thus they contain 10,000 copies of the chloroplast genome. For functional plastid gene analysis, it is essential to inactivate all of the copies following chloroplast transformation. In this respect *C. reinhardtii* offers specific advantages because of its fast replication cycle (ca 6–8 h) and a single chloroplast per cell. In this organism, complete disruption of a plastid gene can be readily achieved by restreaking transformants on agar plates with continued selection for antibiotic resistance. Moreover, *C. reinhardtii* can grow in the absence of photosynthetic activity in acetate-containing medium [4,7,8]. Thus, this alga has been very useful for elucidating the function of chloroplast genes involved in photosynthesis.

In contrast, it is not possible to apply this strategy for genes that are required for cell survival. To overcome this limitation, a genetic system was developed in which any chloroplast gene of interest can be conditionally and reversibly silenced upon addition of thiamine and vitamin

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B12 in the growth media [9]. Under these conditions the corresponding protein product is gradually depleted. Although the cells will die in the long term if the protein is essential, it is possible to analyze the cellular processes that occur before cell death and to obtain important information on the role of these genes by examining the changes in transcript and protein abundance and how chloroplast gene expression is integrated within cellular metabolism and signaling. Because no chloroplast inducible/repressible promoter is available, an indirect method had to be used that takes advantage of some properties of nucleus-encoded chloroplast proteins involved in specific post-transcriptional steps of plastid gene expression in *Chlamydomonas*. The protein chosen for this purpose is *Nac2* which specifically interacts with the *psbD* 5'UTR and is required for the accumulation of the *psbD* mRNA encoding the D2 reaction center protein [10,11]. Initially, the *Nac2* coding sequence was fused to the Cyc_6 promoter of the cytochrome c_6 gene which is only expressed when cells are grown in copper-deficient medium and repressed by copper [12]. Thus with this system, it was possible to specifically block D2 expression by the addition of copper to the growth medium [13].

More recently, regulatory elements that mediate vitamin-dependent nuclear gene repression were used. They comprise the *MetE* promoter whose activity is repressed in the presence of vitamin B12 [14,15] and the 5'UTR of the *Thi4* gene which contains a riboswitch that undergoes a conformational change upon binding thiamine pyrophosphate (TPP) and ultimately turns off expression of the downstream coding sequence [14]. By fusing these two regulatory sequences to the *Nac2* gene in the nuclear genome of a *Nac2* mutant, a strain was generated in which conditional *Nac2* repression is easily achieved by the addition of vitamins B12 and thiamine to the growth medium [9]. To ensure photosynthetic activity in the presence of vitamins, the dependence of *psbD* expression on *Nac2* was eliminated by replacing its endogenous 5'UTR with another 5'UTR.

1.1. Repression of chloroplast gene expression reveals a regulatory negative feedback system

Earlier genetic studies revealed that amongst the numerous chloroplast mutants of *C. reinhardtii* affected in plastid ribosome assembly and impaired in chloroplast translation, none lacked any ribosomal protein or ribosomal RNA gene or was completely deficient in plastid protein synthesis [16,17]. These observations suggested that chloroplast protein synthesis is essential in this alga. Attempts to fully inactivate the RNA polymerase genes through chloroplast transformation with the *aadA* cassette were unsuccessful and thus in agreement with the idea that plastid protein synthesis is essential [18]. However one problem with these experiments was that expression of the selectable marker is dependent on a functional plastid protein synthesis system. Hence one cannot exclude the possibility that the persistent heteroplasmic state of the chloroplast genome is due to the failure of expressing the selectable marker gene when all copies of the chosen RNA polymerase gene are disrupted.

This issue was clarified by using a vitamin-repressible chloroplast gene expression system. For this purpose the chloroplast 5'UTRs of *rpoA*, encoding the α subunit of the chloroplast RNA polymerase or that of *rps12*, coding for a plastid ribosomal protein that plays a key role in translation, were replaced by the *psbD* 5'UTR in a strain in which the *Nac2* gene was placed under the control of the vitamin-repressible *MetE* promoter and *Thi4* riboswitch [9]. Because the *psbD* 5'UTR confers *Nac2* dependence, upon the addition of vitamins, the *rpoA* or the *rps12* gene was repressed and the cells were gradually depleted of the corresponding gene product. This led in both cases to a growth arrest and cell death indicating that plastid protein synthesis in *C. reinhardtii* is essential for cell growth and survival. It is probable that chloroplast translation is vital in this alga because its plastid genome contains several additional essential genes for cell growth and survival besides those involved in plastid gene expression. They include in particular clpP1 [19] and ORF1995 [20]. Another plausible explanation is that chloroplast gene expression is essential by itself because it is closely connected to the cell cycle in *Chlamydomonas* as suggested by the observation that arresting chloroplast translation compromises nuclear DNA replication [21].

In contrast to the inactivation of the *rps12* gene in *Chlamydomonas*, transplastomic tobacco lines with homoplasmic disruptions of the plastid ribosomal protein genes *rps15* and *rpl36* are viable indicating that these proteins are not essential [22]. However photosynthetic activity and growth were severely impaired upon loss of *rpl36*, and to a lesser extent in the absence of *rps15*, indicating that the translational activity is decreased but not fully blocked in the absence of either of these two proteins.

It should be noted that it is not generally true that plastid translation is essential for the viability of plants. For example, transplastomic lines of tobacco with a homoplasmic disruption of the chloroplast *rpoB* gene are still viable [23]. Similarly, barley mutants fully deficient in plastid protein synthesis are also viable [24]. As an example, the variegated *albostrians* mutant of barley has green and white leaf sectors lacking plastid ribosomes. The latter are deficient in plastid translation. Likewise, plastid growth and division are maintained in maize mutants deficient in plastid ribosomes [25]. In addition, several mutations that compromise splicing of chloroplast tRNA or ribosomal protein mRNA precursors in maize fully inhibit plastid translation without interfering with embryo development [26,27]. In contrast, loss of plastid translation leads to embryo lethality in *Arabidopsis* [27]. These differences between monocotyledonous and dicotyledonous plants could be due to the chloroplast locus *accD* required for fatty acid biosynthesis which is essential in *Arabidopsis* but not in maize and rapeseed in which nuclear genes compensate for the absence of *accD* [26].

Previous characterization of *Chlamydomonas* nuclear mutants with impaired chloroplast translational activity revealed that ribosomal proteins are preferentially translated as compared to proteins of the photosynthetic apparatus [28]. Thus, in the case of direct silencing of the *rps12* gene, one would expect that this response is even more pronounced. Indeed, a comparative transcriptomic analysis between wild type and a strain in which expression of the *rps12* gene is repressed indicated that the transcript abundance of all plastid ribosomal genes is increased [9]. Because one third of the chloroplast ribosomal proteins are synthesized on plastid ribosomes, this response may be viewed as a compensatory response to attenuate the permanent loss of chloroplast ribosomes. Besides the enhanced expression of the chloroplast ribosomal protein genes, shut-down of chloroplast translation caused an increase in abundance of several other plastid genes including those of subunits of the plastid RNA polymerase, some tRNAs, the elongation factor TufA and ClpP1. A similar increase also occurred for the transcripts of several plastid genes of unknown function. A particularly striking result of this analysis was the inverse correlation between the progressive decrease of ClpP1 protein and the increase of its mRNA suggesting the existence of a negative feedback control exerted by ClpP1 itself [9]. This finding is in agreement with the observation that ClpP1 mRNA increases in a mutant in which its translation is specifically attenuated [29].

A similar change in abundance of transcripts of plastid genes involved in chloroplast gene expression was observed upon depletion of the *rpoA* subunit of the chloroplast RNA polymerase [9]. All together, these data suggest the existence of negative feedback loops for a large set of chloroplast genes that counteract the decrease in their transcription or translation with an increase in their stability or a change in RNA processing. It is possible that plastid ribonucleases associated with the ribosomes degrade the mRNA upon translation but not when translation or transcription is inhibited. Indeed increased mRNA abundance has been detected in mutants of *C. reinhardtii* affected in the translation of *psbA* [30], *atpA* [31] and *psaB* mRNA [32]. Similar regulatory mechanisms involving feedback loops have been found in bacteria for ribosomal operons [33]. Thus it is possible that they have been conserved during the evolution of chloroplasts from cyanobacteria.

The effect of repression of *rps12* and *rpoA* is not only limited to chloroplast transcripts but also affects the abundance of several mRNAs of nucleus-encoded proteins involved in photosynthesis. This process thus resembles the retrograde signaling pathway triggered by inhibition of plastid protein synthesis in land plants [34]. Several mutants deficient in this signaling pathway have been characterized including the constitutively photomorphogenetic *cop1-4* and *gun1* mutants from *Arabidopsis* and *lip1* from pea [35–37]. Whereas the levels of most nucleus-encoded proteins involved in photosynthesis are decreased upon repression of chloroplast gene expression in *C. reinhardtii*, the abundance of several plastid proteins involved in proteolysis and membrane biogenesis is increased. This set of proteins includes two essential proteins such as Alb3.2, implicated in the assembly of membrane photosynthetic proteins [38], and Vipp1 required for thylakoid membrane biogenesis, photosystem assembly and most likely plastid lipid trafficking [39]. Possibly, under conditions of limited plastid protein synthesis, Alb3.2 and Vipp1 are involved in the remodeling of the thylakoid membrane with the participation of the chloroplast chaperones Hsp70B and Hsp90C and the cochaperone Cdj1 [40].

Taken together these data indicate the existence of an elaborate signaling system acting both in the chloroplast and nucleo-cytosol that is able to perceive perturbations in the plastid gene expression apparatus. One feature of this system is a negative feedback loop circuitry, especially for genes involved in plastid gene expression (Fig. 1). The underlying molecular mechanisms are however still largely unknown, and they clearly differ from those of CES (for Control of Epistasy of Synthesis), an assembly-dependent chloroplast feedback process in which specific unassembled subunits of photosynthetic complexes inhibit directly or indirectly their own translation and thereby allow for a coordinate assembly of these complexes (reviewed in [41]).

This signaling system also acts in the nucleus where expression of a specific set of nuclear genes is altered in response to the inhibition of

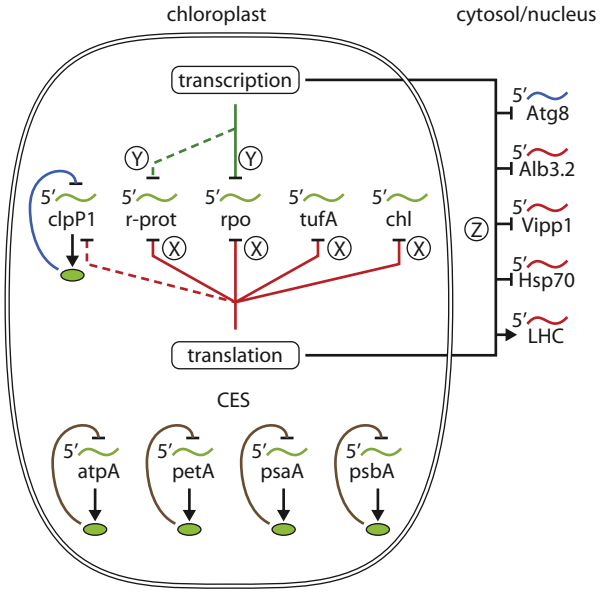

Fig. 1. Negative regulatory feedback loops in the chloroplast gene circuitry. The chloroplast compartment and nucleo-cytosol are represented on the left and right, respectively. A selected set of chloroplast mRNAs is shown comprising those of ClpP1, chloroplast ribosomal proteins (r-prot), subunits of chloroplast RNA polymerase (rpo), elongation factor TufA (tufA), and subunits of the light-independent protochlorophyllide reductase (chl). Nucleus-encoded mRNA genes coding for chloroplast and cytoplasmic proteins are shown in red and blue, respectively, on the right. Negative regulatory feedback loops are revealed through repression of transcription (green lines) or translation (red lines). Factors involved are still unknown (X, Y, and Z) except for the ClpP1 protein, which represses accumulation of its own mRNA directly or indirectly (green circular line). Reproduced from [9] with permission.

plastid gene expression. Genes involved in DNA replication and cell cycle are down-regulated whereas genes implicated in thylakoid biogenesis and various stress responses are up-regulated. While the components of this signaling chain are still unknown in *Chlamydomonas*, in land plants a few components have been identified. They include Gun1, a TPR protein associated with the plastid nucleoid which acts as an integrator of at least three different stress responses in the chloroplast [42], namely, first, inhibition of plastid translation [43], second, interference with the tetrapyrrole biosynthesis pathway [35,44,45] and third, perturbation of the redox state of the photosynthetic electron transport chain [46]. Another important component of this retrograde signaling chain is PTM, a transcription factor bound to the chloroplast envelope which is released from the chloroplast envelope through proteolytic cleavage in stressed plastids [47] and targeted to the nucleus where it activates ABI4 which in turn represses Lhcb genes [42].

1.2. Depletion of the chloroplast ClpP protease induces nuclear genes involved in autophagy and protein quality control

Photosynthetic organisms are often subjected to drastic changes in environmental conditions such as high light, nutrient starvation or elevated temperature. These changes can profoundly affect the primary reactions of photosynthesis and lead to the formation of reactive oxygen species that in turn damage plastid proteins [48]. These damaged proteins are degraded and recycled through a chloroplast proteolytic system consisting of several proteases that include stromal ClpP [49,50], stromal and thylakoid Deg [51] and thylakoid Ftsh [52]. These proteases together with the plastid chaperones Cpn60/Cpn10, Hsp70/DnaJ, Hsp90 and ClpB3 belong to the plastid protein homeostasis network [53].

The plastid ClpP protease is similar to its bacterial counterpart. It contains a proteolytic chamber made of two stacked heptameric rings of the ClpP subunit and an antechamber consisting of a hexameric ring of the ATP-dependent chaperone ClpP or ClpA. In addition, ClpC1/C2 and ClpD chaperones that are orthologous to the bacterial AAA+ proteins and ClpS adaptors have been identified [49,54]. However, the ClpP proteolytic system is more complex in plastids than in bacteria [49] as it contains several ClpP isoforms in the holoenzyme, some of which have lost the catalytic residues. In the case of *C. reinhardtii*, there are three genes encoding catalytic subunits (ClpP1, ClpP2, ClpP3) and five genes encoding non-catalytic subunits (ClpR1–4, ClpR6). In *Arabidopsis* the number of ClpP components is even higher with 9 Hsp100 chaperones (ClpB1–B2, ClpPC1–C2, ClpD, ClpX1–X3), 5 paralogues of ClpP (ClpP1,ClpP3–P6), 4 ClpP-like variants (ClpR1–R4), one adapter protein ClpS and ClpT1, T2, that are absent in bacteria and that may be involved in the regulation of the assembly of the catalytic core [54–56]. All of these Clp components are encoded by the nuclear genome with the exception of ClpP1 which is encoded by a chloroplast gene.

Whereas ClpP is dispensable in bacteria, it appears to be essential in chloroplasts. This conclusion is based on the failure to produce homoplasmic knockout lines of *clpP* in *C. reinhardtii* [19], *Nicotiana tabacum* [57,58] and *Synechococcus elongatus pcc 7942* [59,60]. In *Chlamydomonas*, expression of ClpP1 could be decreased by 70% through replacement of the AUG start codon of ClpP1 with AUU which is less efficient for initiation of translation [29]. No conspicuous phenotype of this mutant was observed under normal growth conditions although the degradation of the Cytb_6f complex was delayed under nitrogen starvation or in Cytb_6f mutants deficient in the Rieske iron–sulfur protein. In *Arabidopsis* the ClpP/R core is essential as revealed by the failure to isolate mutant plants with a partial or complete loss of expression of any of the nucleus-encoded ClpP or ClpR paralogues indicating that there is little functional redundancy between them [61]. The only exceptions are the *clpR1–1* and *clpR2–1* mutants which are still able to accumulate small amounts of intact Clp proteolytic

core, most likely because of partial compensation by the ClpR3 subunit [62–65].

To study the role of ClpP in cellular metabolism and growth in *Chlamydomonas*, a repressible chloroplast gene expression system was used in which ClpP1 is progressively depleted [66]. As expected, silencing ClpP1 expression leads to a drastic arrest of cell growth and to cell death in the long term, thus confirming that ClpP is essential for cell survival. During ClpP depletion, protein aggregation occurs accompanied with a large increase in the abundance of chaperones and proteases, reminiscent of an unfolded protein response. Also the cellular membrane system is vastly perturbed with a considerable increase in the number of vacuoles in the cytosol and the appearance of vesicles in the chloroplast.

ClpP depletion leads to an autophagy-like response as revealed by light and electron microscopy suggesting common regulatory mechanisms that orchestrate these stress responses. Further support for an autophagy-like response comes from the transcriptomic analysis which showed that many autophagy-related genes (Atg8, Atg3, Atg7 and Atg12) are highly upregulated [66].

Rapamycin, an inhibitor of the TOR protein kinase, has been shown to induce autophagy in *Chlamydomonas* [67,68]. It was therefore possible to compare the responses to ClpP depletion and rapamycin treatment. This comparative analysis revealed the existence of a set of general stress-responsive genes whose expression is altered in response to both conditions. Many of these mRNAs encode proteins involved in membrane biogenesis and vesicular trafficking, in particular those of two SNARE proteins which were recently shown to catalyze membrane fusion during the late stages of autophagosome formation [69–71].

An unexpected result was that the loss of ClpP leads to an increase in abundance of many chloroplast RNAs raising the possibility that ClpP may be involved in the plastid transcriptional regulatory feedback control system mentioned above. In addition, the levels of several ribonucleases both in the cytosol/nucleus and in the chloroplast increase, thus raising the possibility that a drastic perturbation of chloroplast protein homeostasis might trigger the restructuring of sets of intracellular RNAs and/or changes in RNA quality control. In this respect it is particularly interesting that amongst the transcripts with the highest increase in abundance upon ClpP depletion, three encode proteins structurally related to the bacterial Rsr and to the human Ro 60-kD autoantigen [72]. These proteins are known to act as cofactors for promoting the association of exonucleases with misfolded RNA substrates during stress [72–75]. Some of these *Chlamydomonas* transcripts are also increased in abundance under high light and by rapamycin. Hence, this raises the question whether a RNA unfolded response occurs in this alga as part of an integrated stress response. Perturbations of RNA metabolism have also been observed in the *Arabidopsis clpR2-1* mutant in which plastid ribosome assembly is delayed and the abundance of the RH3 DEAD box RNA helicase and the exoribonuclease polynucleotide phosphorylase are greatly increased [65].

An extensive time-course proteomic analysis during ClpP depletion revealed an increased abundance of proteins involved in protein folding, proteolysis and more generally in protein quality control [66]. They include the chloroplast small heat-shock proteins Hsp22C, Hsp22E and Hsp22F, the chaperones DnaK, ClpB3 DnaJ5, DnaJ34 and Cgl41. The latter is an uncharacterized protein with an RbcX domain identified in a chaperone required for the assembly of hexadecameric Rubisco [76]. Importantly, the transcriptomic analysis made it possible to identify which, amongst these proteins, are specifically induced upon ClpP depletion but not by rapamycin treatment. As a striking example, a 50% decrease in ClpP1 accumulation is sufficient to induce a significant upregulation of Vipp2, a nuclear gene encoding a chloroplast protein closely related to Vipp1. Remarkably, Vipp2 gene expression is not induced by nitrogen starvation or treatment with rapamycin or tunicamycin, a chemical agent that induces protein misfolding in the ER (endoplasmic reticulum) [66]. Instead, Vipp2 abundance increases upon exposure of cells to high light, a condition where chloroplast proteins can be easily damaged by the formation of reactive oxygen species. Thus expression of Vipp2 appears to be highly sensitive to perturbations in chloroplast protein homeostasis. However, its expression is not enhanced by heat shock, another abiotic stress known to trigger the cytosolic protein unfolding response [39]. Possibly, several sensing mechanisms may respond to different types of protein unfolding/misfolding in distinct cellular compartments. Loss of ClpP may induce a stronger and more persistent chloroplast response to which Vipp2 is sensitive.

The gene of the chloroplast ATP-independent Deg11 protease is also induced in the early phase of ClpP1 depletion. As for Vipp2, expression of this protease can be induced through high light treatment, but not by TOR inactivation with rapamycin. However, in contrast to Vipp2, Deg11 abundance also increases upon impairment of protein glycosylation in the ER raising the possibility of a functional and/or structural link between ER and the chloroplast. In this view perturbation of ER protein homeostasis might require specific adjustments in chloroplast protein homeostasis.

The analysis of reporter genes fused to the promoter and 5'UTR of Vipp2 and Deg11 indicates that the increased abundance of these proteins upon ClpP depletion or high light treatment is mediated through their promoter and 5'UTR [66]. In the case of ClpP depletion, this induction also occurs in the dark. As such, it is most likely triggered by unfolded proteins and/or damaged membranes.

In summary, it appears that perturbations of chloroplast protein homeostasis are sensed and the information is transmitted to the nucleus through a specific signaling pathway which leads to the upregulation of nuclear genes encoding plastid factors involved in protein quality control. Possibly, this signaling cascade is evolutionarily conserved as a similar response also occurs in land plants. A decrease of ClpR in the *clpR2-1* mutant of *Arabidopsis* results in an increase in abundance of chloroplast proteins involved in plastid protein import, folding, maturation and unfolding [65].

1.3. Additional pathways for chloroplast protein degradation

Transcripts specifically induced by the depletion of ClpP include those of proteins predicted to be associated with the chloroplast and related to E2 ubiquitin-conjugating enzymes. These findings raise the possibility that under these stress conditions when the internal plastid proteolytic system is impaired and unable to fully recycle aggregated or misfolded proteins for supplying free amino acids for plastid protein synthesis, proteolysis may be activated in the cytosol. Amongst the proteins involved in proteolysis whose abundance is increased under these conditions, Cdc48 and Vms1 are of particular interest as they are associated with the mitochondrial outer membrane upon mitochondrial stress in yeast [77]. Vms1 is induced by oxidative stress and interacts with Cdc48, a chaperone-like ATPase that forms a homohexamer complex and is involved in a stress-responsive system for mitochondrial protein degradation. Vms1 appears to act similarly as Udf1 which recruits Cdc48 to the membrane of the ER during ERAD (endoplasmic reticulum associated protein degradation) and mediates the extraction, ubiquitination and delivery of ER proteins to the proteasome in the cytosol [78]. This raises the possibility that ClpP depletion induces a cellular proteolytic response which may also involve mitochondria. A further possibility is that an unknown protein recruits Cdc48 to the chloroplast envelope in a similar way as Udf1 and Vms1 recruit Cdc48 to the mitochondria and ER, respectively, for delivery of damaged proteins to the proteasome.

Interestingly, a pathway for protein degradation which may act in a similar way has been proposed for *Chlamydomonas* in which proteins destined for degradation are extruded from the chloroplasts and targeted to the vacuoles for degradation [79,80]. Abundant pulse-labeled polypeptides synthesized on chloroplast ribosomes were recovered in vacuoles. The presence of protrusions of the outer membrane of the chloroplast envelope that enclosed stroma suggested that stromal

proteins were extruded from the chloroplast in membrane-bound structures to the vacuoles. Moreover light-harvesting proteins were detected inside the chloroplast near the envelope and also in granules within cytosolic vacuoles during the greening of the *C. reinhardtii y-1* mutant in the light at 38 °C. These observations suggest that algal cells use this pathway when light-harvesting proteins are synthesized in excess with regard to the capacity of the thylakoid membrane to integrate these proteins. A similar pathway may be induced upon depletion of ClpP in *Chlamydomonas*.

1.4. Altered lipid metabolism during ClpP depletion

It is intriguing that amongst the proteins induced in the early phase of ClpP depletion besides Vipp2, a protein encoded by Cre16.g683350 is also upregulated that is not predicted to be localized in the chloroplast. It is a member of the thioesterase superfamily. Because these enzymes catalyze the hydrolysis of long-chain fatty acyl-CoA thioesters to free fatty acids and CoA, an interesting possibility is that this protein induced in the early phase of ClpP depletion may act in a signaling pathway mediated in part by lipid molecules.

Vipp1 was originally identified as an envelope lipid transfer protein in land plants [81]. In *Chlamydomonas* Vipp1 and Vipp2 appear to be functionally redundant based on the observation that depletion of Vipp1 led to a compensatory increased abundance of Vipp2 and that the total Vipp protein pool in *C. reinhardtii* could not be reduced below 25% of wild-type levels although the relative contributions of Vipp1 and Vipp2 to this pool could be varied [39]. Similar findings with Vipp1 were also reported in *Arabidopsis* [82] and cyanobacteria [83]. Rather than being a vesicle-inducing protein as suggested by earlier studies, recent work with *Chlamydomonas* raises the possibility that the Vipp proteins could be involved in the formation of thylakoid centers found in cyanobacteria where thylakoid biogenesis is initiated [39]. Vipp1 interacts closely with the chaperone Hsp70B and Hsp90C. These proteins are required for assembly of Vipp1 in ring- and rod-like structures thought to help in providing structural lipids for the assembly of the photosystem core complexes [39] together with the assembly factor Alb3.2 [38]. Depletion of Vipp1 slows down this assembly process and results in the formation of prolamellar body-like structures at the origin of the thylakoid layers resulting in an impaired incorporation of structural lipids into thylakoid membrane core complexes which affects their structural organization and function and ultimately leads to a higher susceptibility to photoinhibition [39].

1.5. Conclusions and perspectives

The availability of a repressible chloroplast gene expression system has opened new possibilities for investigating the function of essential plastid genes. Here we have reviewed the use of this system in *C. reinhardtii* for the depletion of the essential proteins *rpoA*, *rps12* and ClpP1. The gradual depletion of these proteins elicits stress responses that have provided new insights into chloroplast and retrograde signaling pathways. Thus cessation of chloroplast transcription or translation through depletion of *rpoA* and *rps12*, respectively, leads to a marked increase in abundance of the transcripts of most RNA polymerase and ribosomal protein genes. This process reveals the existence of a plastid negative feedback system in which expression of these genes is repressed through factors that act at the transcriptional or translational level (Fig. 1). The identity of these factors is still largely unknown except in the case of ClpP1 in which the down-regulation of the ClpP1 protein correlates with the up-regulation of its mRNA suggesting that ClpP could act as a negative regulator of its own synthesis. Yet, it is not clear whether ClpP acts directly or indirectly. Such negative feedback loops may be especially important for genetic systems with multiple copies of genes to prevent over-accumulation of their transcripts.

Although there is some redundancy amongst the different plastid proteases, loss of the catalytic ClpP1 subunit of the ATP-dependent ClpP protease leads to cell growth arrest and cell death. Proteomic analysis of Clp mutants suggests that this protease may have many different substrates in the chloroplast amongst the proteins whose abundance increases upon ClpP depletion [65,66]. However some of the most up-regulated proteins are involved in protein folding and degradation and their increase could result from a plastid unfolded protein response although these processes are not mutually exclusive. In fact the cellular changes induced by the depletion of ClpP have all the hallmarks of an unfolded protein response with the appearance of protein aggregation and a massive induction of chaperones, proteases and in addition with proteins involved in lipid trafficking and membrane assembly/disassembly (Fig. 2). This response was first characterized in the ER in which interference with protein folding led to increased transcription of genes of chaperones resident in this organelle [84]. More recently, a similar response has also been found in animal and plant mitochondria with an increase of nucleus-encoded mRNAs of mitochondrial chaperones but not of mRNAs of stress proteins from the ER and cytosol [85,86].

Another intriguing question raised by the results of ClpP depletion is whether an impairment of chloroplast proteolysis elicits a salvage pathway in the cytoplasm which could operate through protein extraction from the plastid and delivery to the proteasome or which could activate other proteolytic systems in the cell. This may be particularly relevant for *Chlamydomonas* cells in which the chloroplast occupies nearly half of the cell volume.

Further important insights into plastid protein quality control have arisen from the analysis of second site suppressors of the FtsH *var2* variegation mutant in *Arabidopsis* [87]. This study revealed that mutations in ClpC2 suppress the requirement for FtsH in thylakoid membrane biogenesis and that ClpC2 is a negative regulator of this process. A functional link beween FtsH and ClpP is also revealed in another *var2* suppressor with a mutation in the ClpR1 subunit [88]. Furthermore, some other suppressors of *var2* are affected in genes involved in chloroplast gene expression such as a chloroplast-localized enzymes that catalyzes the isomerization of uridine to pseudouridine in non-coding RNAs [88], the chloroplast elongation factor G and translation initiator factor 2 [89]. The suppression is explained by a reduced rate of chloroplast protein synthesis that in turn decreases the demand for FtsH and

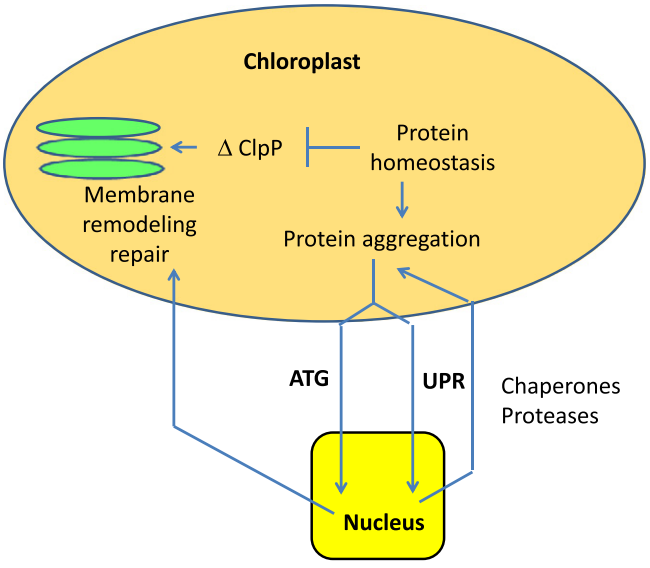

Fig. 2. Scheme showing how ClpP depletion affects chloroplast protein homeostasis and triggers various cellular responses including autophagy (ATG), a chloroplast unfolded protein response (UPR) and membrane remodeling. Induction of autophagy may occur through modulation of TOR signaling upon ClpP depletion. These responses lead to a bulk degradation of macromolecules and to an increase of several chloroplast chaperones, proteases and proteins involved in thylakoid membrane assembly/repair.

allows more plastids to overcome the threshold for turning green rather than white in the *var2* genetic background.

Taken together the analysis of the fate of cells depleted of essential chloroplast proteins and the search and characterization of additional second site suppressors of mutations affecting plastid proteases are likely to provide further important insights into the networks of protein quality control in plastids.

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