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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.

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