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# Chloroplast unfolded protein response, a new plastid stress signaling pathway?

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**A unique feature of the ATP-dependent ClpP protease of eukaryotic photosynthetic organisms is that its catalytic subunit ClpP1 is encoded by the chloroplast genome. Attempts to inactivate this subunit through chloroplast transformation have failed because it is essential for cell survival. To study the function of ClpP we have developed a repressible chloroplast gene expression system in *Chlamydomonas reinhardtii*. This system is based on the use of a chimeric nuclear gene in which the vitamin-repressible *MetE* promoter and *Thi4* riboswitch have been fused to the coding sequence of *Nac2*. Upon entry into the chloroplast the Nac2 protein specifically interacts with the *psbD* 5'UTR and is required for the proper processing/translation of the *psbD* mRNA. This property can be conveyed to any chloroplast mRNA by replacing its 5'UTR with that of *psbD*. In this study we have chosen *clpP1* as plastid target gene and examined the cellular events induced upon depletion of ClpP through transcriptomic, proteomic, biochemical and electron microscope analysis. Among the most striking features, a massive increase in protein abundance occurs for plastid chaperones, proteases and proteins involved in membrane assembly/disassembly strongly suggesting the existence of a chloroplast unfolded protein response.**

**Keywords:** autophagy, chaperones, *Chlamydomonas*, chloroplast, ClpP, proteases, unfolded protein response

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The unfolded protein response was first discovered in the endoplasmic reticulum (ER) when inhibition of protein folding led to the transcriptional upregulation of several chaperones.[1] A similar pathway was found to be induced in mitochondria upon accumulation of unfolded proteins in the mitochondrial matrix with the increase of transcripts of nuclear genes encoding mitochondrial chaperones but not of genes of ER and cytosolic stress proteins.[2]

Recently a repressible chloroplast gene expression system was developed in the green unicellular alga *Chlamydomonas reinhardtii* which allows one to repress any plastid gene.[3,4] This system which is mainly based on a vitamin-repressible riboswitch, has been used for the depletion of several essential chloroplast proteins such as RpoA, the α subunit of the plastid RNA polymerase, the ribosomal protein Rps12 and ClpP1, the catalytic subunit of the ATP-dependent ClpP protease. In the latter case an extensive transcriptomic and proteomic analysis revealed that the progressive loss of this protease leads to the increased abundance of many stress proteins. They include several chaperones, proteases, proteins involved in lipid trafficking and also in autophagy. Indeed, upon ClpP1 depletion an extensive vacuolization and swelling of the cells occurs reminiscent of autophagy. Because autophagy can be specifically induced by rapamycin in *Chlamydomonas*,[5] it was possible to compare the autophagic response with that induced by the depletion of ClpP. Although these 2 responses overlap to a significant extent, there are marked differences in the respective gene expression programs.[4]

Genes whose expression is specifically increased upon ClpP1 deletion but not by rapamycin treatment include several chaperones, proteases, proteins with ubiquitin-related functions and proteins involved in thylakoid membrane assembly and lipid trafficking (**Table 1**). As an example

**Table 1.** List of proteins with specific increased abundance upon ClpP depletion in *Chlamydomonas reinhardtii*

| Gene ID Au10.2 | Gene name | Protein | Control | rapamycin | ΔClpP |
|---|---|---|---|---|---|
| **HSP proteins** | | | | | |
| Cre14.g617400.t1.1 | HSP22F | heat shock protein 22F | 1.19 | 6.8 | 8.42 |
| Cre07.g318800.t1.1 | HSP22A | heat shock protein 22A | −4.2 | −4.85 | 7.89 |
| Cre01.g060000.t1.1 | HSP22C | heat shock protein 22C | 1.06 | 1.19 | 7.69 |
| Cre07.g318850.t1.1 | HSP22B | heat shock protein 22B | −2.38 | −2.39 | 7.41 |
| **Proteases** | | | | | |
| Cre12.g498500.t1.1 | DEG11 | DegP-type protease | 1.34 | 5.18 | 7.01 |
| Cre10.g429001.t1.1 | — | E2 ub-conjugating enzyme | 1.48 | 2.30 | 6.01 |
| Cre16.g662450.t1.1 | — | de-ubiquitinating peptidase | 1.68 | 0.75 | 4.88 |
| Cre03.g179100.t1.1 | — | Ub- fusion degradation prot. | −0.07 | 0.18 | 2.26 |
| Cre19.g752550.t1.2 | MMP8 | Metalloproteinase | 1.03 | −4.69 | 2.1 |
| Cre16.g690900.t1.1 | RBL8 | rhomboid-like protein | 0.27 | 1.26 | 2.09 |
| Cre26.g772100.t1.2 | — | VMS1 | −0.56 | 0.71 | 2.04 |
| **Chaperones** | | | | | |
| Cre01.g030350.t1.1 | CGL41 | predicted protein | 1.72 | 1.56 | 5.98 |
| Cre02.g090850.t1.2 | CLPB3 | ClpB chaperone, Hsp100 family | 0.41 | 1.73 | 3.35 |
| Cre16.g690400.t1.2 | DNJ5 | DnaJ-like protein | 0.94 | 1.91 | 3.28 |
| Cre18.g746450.t1.1 | CLPB1 | ClpB chaperone, Hsp100 family | 0.52 | 0.07 | 3.11 |
| Cre07.g327450.t1.1 | DNJ34 | DnaJ-like protein | 0.33 | 1.95 | 2.62 |
| Cre16.g663350.t1.1 | CLPX | ATP-dependent Clp protease | 1.57 | 0.3 | 2.08 |
| **Membrane and vesicle assembly** | | | | | |
| Cre11.g468050.t1.1 | VIPP2 | | 0.74 | 4.66 | 7.01 |
| Cre04.g214750.t1.2 | VMPL2 | R-SNARE protein | 1.59 | 2.63 | 5.19 |
| Cre13.g583550.t1.1 | VIPP1 | Vesicle inducing plastid protein | −0.46 | 2.29 | 2.78 |

Control (column 4) refers to the *Chlamydomonas* strain treated with vitamins without ClpP depletion; Rapamycin (column 5) refers to the same strain treated with rapamycin, ΔClpP (column 6) refers to the strain in which *clpP1* was repressed by vitamin treatment. Numbers in columns 4 and 6 refer to the $\log_2$ ratio of protein level after 43 h of vitamin treatment/ protein level at 0 h. Numbers in column 5 refer to the $\log_2$ ratio of protein level after 8 h of rapamycin treatment / protein level at 0 h. Note that the increase of Vipp2 and Deg11 upon rapamycin treatment results mainly from the change in light irradiance between the 0 and 43 h time points (data taken from ref. 4). Gene ID Au10.2; gene identifier from Augustus 10.2. Ub, ubiquitin.

Vipp2, a chloroplast protein that appears to be required for thylakoid biogenesis,[6] is only upregulated by ClpP1 depletion in cells grown in constant dark or constant light but not by rapamycin under the same conditions nor by tunicamycin which induces a stress response in the ER or by nitrogen starvation.[4] Its level is however increased when cells are exposed to high light which induces photooxidative damage. Similar responses were obtained with the Deg11 protease which increased upon ClpP1 depletion but not with rapamycin treatment. In contrast to Vipp2, Deg11 abundance also increased upon ER stress indicating interactions between ER and the chloroplast. These examples point to a specific response due to the depletion of ClpP1which cannot be recapitulated by most other stresses such as inhibition of the TOR pathway. ClpP1 depletion appears to induce a response which is closely related to an unfolded protein response in the chloroplast. ClpP deficiency leads to protein aggregation and accumulation of damaged proteins in the chloroplast compartment which are sensed through a signaling chain by the nucleus resulting in the upregulation of genes of chloroplast chaperones and proteases. For many of these genes a number of isoforms exist which are present in several locations in the cell. Depletion of ClpP1 leads mainly to increased abundance of chloroplast-localized isoforms. The massive increase of these proteins can then counteract protein aggregation and protein damage in the chloroplast compartment. The components of this signaling chain are still largely unknown but the identification of specific genes upregulated under the particular conditions of ClpP1 depletion provides powerful tools for a genetic dissection of this signaling pathway. In particular it will be interesting to compare it with the mitochondrial unfolded protein response. A stress responsive system for mitochondrial protein degradation has been identified which involves the CDC48 ATPase chaperone.[7] This protein is recruited together with VMS to the mitochondrial outer membrane where it is capable of extracting mitochondrial proteins from the inner membrane and to transfer them to the proteasome for degradation. Interestingly, both CDC48 and VMS1 are also induced during ClpP1 depletion in *Chlamydomonas* raising the possibility that a similar process may occur.[4] Moreover chloroplast proteins such as Rubisco have been identified in cytoplasmic vacuoles upon certain stress conditions in this alga. The detection in these studies of protrusions of the outer membrane of the chloroplast envelope that enclosed stroma suggest that chloroplast proteins were extruded from the chloroplast in membrane-bound structures to the vacuoles. Similar observations were made with the light-harvesting protein LHCII found in the chloroplast near the envelope and also in granules within vacuoles in the cytosol.[8] This pathway appears to be triggered when light-harvesting proteins are synthesized in excess

relative to the capacity of integration of these proteins into the thylakoid membranes.[8,9]

In conclusion it appears that algal cells have developed several different mechanisms to maintain chloroplast protein homeostasis. Under adverse stress conditions such as high light, nutrient starvation, elevated temperature, proteins are damaged and may aggregate. A first response is proteolysis. Although a large number of plastid proteases have been identified with a partial overlap in substrates, the ClpP protease appears to play a major role in this process as its loss cannot be compensated by other proteases and leads to cell death. In parallel the accumulation of misfolded proteins elicits a massive induction of chaperones and proteases not only in the chloroplast but also in the cytosolic compartment. This process has the hallmarks of an unfolded protein response. In addition proteins are extruded from the chloroplast envelope and targeted to the vacuoles for degradation under conditions where the integration capacity of the chloroplast for newly imported proteins is overwhelmed.[8,9] Most of the molecular mechanisms underlying these processes are still poorly understood and remain challenging for the future.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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