The nucleus-encoded protein MOC1 is essential for mitochondrial light acclimation in *Chlamydomonas reinhardtii*

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SUMMARY

Mitochondrial respiration plays an important role in optimising photosynthetic efficiency in plants. As yet the mechanisms by which plant mitochondria sense and respond to changes in the environment are unclear, particularly when exposed to light. Here we describe the characterisation of the *Chlamydomonas reinhardtii* mutant *stm6*, which was identified on the basis of impaired state transitions, a mechanism that regulates light harvesting in the chloroplast. The gene disrupted in *stm6*, termed *Moc1*, encodes a homologue of the human mitochondrial transcription termination factor mTERF. *MOC1* is targeted to the mitochondrion and its expression is up-regulated in response to light. Loss of *MOC1* causes a high light sensitive phenotype and disrupts the transcription and expression profiles of the mitochondrial respiratory complexes causing, compared to WT, light-mediated changes in the expression levels of nuclear and mitochondrial encoded (COX) cytochrome c oxidase subunits and (NAD) UQ-oxidoreductase subunits. The absence of *MOC1* leads to a reduction in the levels of cytochrome c oxidase and of rotenone-insensitive external NADPH dehydrogenase activities of the mitochondrial respiratory electron transfer chain. Overall we have identified a novel mitochondrial factor that regulates the composition of the mitochondrial respiratory chain in the light so that it can act as an effective sink for reductant produced by the chloroplast.

INTRODUCTION

In plant cells, respiration within the mitochondrion not only provides ATP but also serves an important role in the maintenance of an appropriate reduction/oxidation (redox) state within the chloroplast (1). Through the use of metabolite shuttles within the inner envelope membrane of the chloroplast, reducing equivalents produced by photosynthetic electron transport activity can be exported and consumed within the mitochondrion through respiration (2). The respiratory chain in plant mitochondria possesses a variety of dehydrogenases and oxidases. Besides a typical rotenone-sensitive type I NADH:UQ oxidoreductase (complex I), the inner membrane also contains rotenone-insensitive internal and external facing type II NAD(P)H:UQ oxidoreductases (3,4,5). Two types of oxidases catalyse reduction of oxygen: A typical cyanide-sensitive cytochrome c oxidase and a cyanide-insensitive family of alterna-
tive oxidases. Cytochrome oxidase is coupled to ATP synthesis whereas the alternative oxidase is not, and possibly acts as a valve to allow oxidation of the ubiquinone pool (6).

A key signalling molecule in the chloroplast thylakoid membrane is plastoquinone (PQ) which according to its redox state appears to control gene transcription in the chloroplast (7) and to regulate light-harvesting which in turn appears to control the ratio of linear to cyclic electron flow in algal chloroplasts (8). Because the redox state of the plastoquinone pool is affected by mitochondrial respiration in the dark (9), and possibly in the light, the mitochondrion has the capacity to influence both chloroplast function and development. Important evidence for the role of the respiratory chain in optimising photosynthesis (10,11,12,13) and the development of the chloroplast (14) has come from analysis of mutants lacking various respiratory complexes, as well as through the use of various mitochondrial-specific inhibitors (15).

It is already known that the mitochondrial proteome changes in the light. For instance, in potato expression of a nucleus-encoded internal rotenone-insensitive NADH dehydrogenase (NDA1) is switched on by light, probably to oxidise NADH produced during photorespiration (16). However, there is no information about how expression of the mitochondrial genome is coordinated with the rest of the cell in response to light stress.

One approach to identify components that are important for the redox-controlled regulation of photosynthesis, including factors possibly involved in mitochondrial gene expression, is to screen for mutants that are perturbed in state transitions. This is a redox-controlled mechanism that balances the excitation of the two photosynthetic reaction centres involved in linear electron flow, photosystems I and II (17,18). When the PQ pool, which is a component of the electron transport chain linking PSII and PSI, becomes more reduced, mobile light-harvesting antennae detach from PSII, following redox-activated phosphorylation, and dock with PSI thus increasing the excitation of PSI and driving the PQ pool more oxidised (into so-called state 2). The reverse process occurs when the PQ becomes more oxidised (to achieve state 1) (19,20). We and others have developed a rapid chlorophyll-fluorescence based screen to identify colonies of the model organism, the green alga *Chlamydomonas reinhardtii*, that are blocked in state transitions (the interconversion of state 1 and state 2) (21,22). Mutants identified in such screens are beginning to identify the key genes involved in regulating state transitions (23). Here we identify another gene that is important for state transitions. Surprisingly the gene product is targeted to the mitochondrion, not the chloroplast, and is involved in regulating mitochondrial gene expression.
EXPERIMENTAL PROCEDURES

Strains and culture conditions
The *Chlamydomonas reinhardtii* strain CC 1618 (arg7 cw15, mt⁻) was obtained from the *Chlamydomonas* Genetics Center Collection (Duke University, USA) and used as WT control in all experiments. WT13 (mt⁺) is derived from the original WT 137c strain (Jacqueline Girard-Bascou, Paris, provided by Dr. Saul Purton, UCL, UK) and was used for segregation experiments.

All strains used were cultivated mixotrophically in TAP medium (Tris-acetate-phosphate, pH 7.0) by illumination with 40 µmol m⁻² sec⁻¹ white light at 20 °C (24) in a twelve hours light–dark cycle to a cell density of 2x10⁶ cells per ml.

When required (for the arginine auxotroph strain CC1618) the medium was supplemented with 110µg arginine per ml.

Mutant construction and genetic analysis
Nuclear transformation was performed as described following the methods of (25) and (26). Plasmids pARG7.8, containing a 7.8 kb genomic DNA fragment of the *Chlamydomonas reinhardtii* argininosuccinate lyase gene (27) and a 0.4 kb fragment of bacteriophage φX174 DNA (28), were used for transformation experiments. Prior to use pARG7.8 was linearised by digestion with BamHI.

Genetic crosses were performed with WT13 and the mutant as described by (24). DNA sequences flanking the tag were cloned by ligation-mediated suppression PCR (LMS-PCR) (29) and by plasmid rescue (30).

Complementation
For complementation a co-transformation strategy was established using the *Cryl* gene as a dominant selectable marker conferring resistance to emitine (plasmid p613) (31) together with a 37-kb Moc1-containing cosmid. The cosmid was isolated from a cosmid library (kind gift of Dr. Saul Purton UCL, UK) by hybridisation experiments using a specific probe derived from the 500-bp non-coding DNA region located between Moc1 and Toc1.

Cells were grown in TAP medium, harvested in the late logarithmic growth phase (OD₇₅₀nm=0.8) and resuspended in TAP-1/₁₀ N. The suspension was used in glass bead trans-
formations, transformed cells were collected in TAP-$\frac{1}{10}$N medium and incubated for 4 days. Cells were then resuspended in TAP medium and incubated for another 8 days. Again, cells were pelleted, resuspended in TAP, spread over TAP agar plates and grown for 12 days. Successful complementation was confirmed by PCR using $\text{Moc1}$-specific primers and by the recovery of state transitions as assayed by fluorescence video imaging.

**Fluorescence video imaging, PAM fluorescence spectroscopy and 77K fluorescence spectroscopy**

Screening of the mutants for state transitions defects was performed as described earlier (22) by video imaging with a FluorCam 700 MF apparatus (Photon System Instruments) at room temperature and at 77K with a fluorescence/luminescence spectrometer (Perkin Elmer LS50B). Samples were measured following illumination with 40$\mu$mol m$^{-2}$ s$^{-1}$ 620nm light to gain state 2. For state 1 images pictures were taken in the same white light briefly after an illumination period of 15min entirely with 80$\mu$mol m$^{-2}$ s$^{-1}$ 710nm (Schott filter, Germany) PSI light. Alternatively, cells were adjusted to state 2 by incubation in the dark for 30min under nitrogen atmosphere according to (9). Photochemical quenching was determined using pulse amplitude modulated (PAM) room temperature fluorescence spectroscopy (Walz) and fluorescence video imaging (Fluor Cam 700M™) and calculated as $1-q_p = 1-(F_m' - F_s)/(F_m' - F_o')$ according to (32).

**Chloroplast and mitochondria isolation**

Chloroplasts were isolated according to (33). Mitochondria were isolated according to (34) using the supernatant of the chloroplast isolation after breaking the cells with a BioNebulizer (kindly provided by J. Nickelsen, Bochum/Germany). Purity of the preparations was confirmed by immunoblotting with anti-D2 (for possible chloroplast contamination) and anti-Cyt c (for possible mitochondria contamination).

**Isolation of nucleic acids, Northern analysis and RT-PCR**

Northern analysis was performed according to (35). RT-PCR was used for semi-quantitative transcript analysis using the GeneCraft™ Life Technologies procedure. Prior to RNA isolation cells were cultivated in the dark until $\text{OD}_{750nm} = 0.6$ and, if desired incubated for further 4-6 hours under different light conditions.
RNA isolation was performed following a protocol of C. Admunsen ([http://biosci.cbs.umn.edu/~amundsen/chlamy/methods/RNA_prrep.html](http://biosci.cbs.umn.edu/~amundsen/chlamy/methods/RNA_prrep.html)) as described earlier (29) and DNA isolation using the DNeasy Plant Mini Kit (Qiagen, Germany).

**Immunoblotting**

Protein analysis by immunoblotting was performed as described earlier by electro-blotting onto nitrocellulose membranes (22). The polyclonal anti-phosphotheonine antibodies were obtained from Zymed. Anti-CytC, -AOX1 and –COX90 are *Chlamydomonas* antisera provided by F.A. Wollman (UPR-CNRS Paris, France), Sabeeha Merchant (UCLA, USA) and Saul Purton (UCL, UK), respectively. Anti-LCHII is a spinach antiserum provided by S. Jansson (Umea, Sweden). Anti-MOC1 was produced by GenoSys (U.K.) from a KLH-conjugated 13aa synthetic peptide identical to the MOC1 C-terminus. All antisera were polyclonal and used in conjunction with the anti-rabbit-IgG (Sigma)/anti-mouse-IgG (Sigma) alkaline phosphatase reaction or the CDP Star (Roche) reaction respectively.

**Protein phosphorylation**

Phosphorylated proteins were detected by western analysis using polyclonal anti-phosphotheonine (Zymed) in state1- and state2- adapted cells as described earlier (22). States were fixed by addition of *p*-benzoquinone according to (9).

**Lipid peroxidation**

Lipid peroxidation was measured indirectly by the concentration of malonyldialdehyde (MDA) and other carbonyl by-products of lipid peroxidation, which form a coloured complex in reaction with thiobarbituric acid that can be monitored at 532nm (36).

**Enzyme activity measurements**

Cytochrome oxidase activity was measured in isolated mitochondria using a cytochrome c oxidase assay kit (Sigma) following the technical instructions. NADH:UQ oxidoreductase activity and external NADPH oxidation activity were measured in isolated mitochondria according to (16). Mitochondria were osmotically burst in 1mM MOPS pH 7.2, 0.1mM EGTA for 6min at room temperature to permeabilize the membrane.
RESULTS

Isolation of state transition mutants
To identify genes involved in state transitions within C. reinhardtii, we screened a library of potentially tagged nuclear mutants that was generated through the random insertion of plasmid pArg7.8, carrying the Arg7 gene, into the nuclear genome of the arginine auxotrophic strain, CC1618 (27,28). By selecting on medium lacking arginine, only colonies containing Arg7 inserted into the nuclear genome were able to grow. A plate-based fluorescence video-imaging screen, which involves the recording of chlorophyll fluorescence from individual colonies before and after illumination with light that induces state transitions in WT, was used to identify potential state transition mutants (stm) (22). Of the $2 \times 10^4$ colonies screened, seven possible stm mutants were identified. The characterisation of mutant stm6 is described here.

stm6 is blocked in state 1 and impaired in PSII-LHC phosphorylation in the light
Chlorophyll fluorescence assays conducted by fluorescence emission spectroscopy at 77K (Figure 1a) and chlorophyll video imaging with actinic 620nm light at room temperature (Figure 1b) revealed that stm6 was blocked in state 1. In contrast to the WT, light preferentially absorbed by PSII was unable to drive the cells into state 2, which is monitored by an increase in the fluorescence coming from PSI at 720nm (Figure 1a). It should be noted that a transition to state 2 was blocked to a far less extent when the PQ pool was driven reduced in the dark through anaerbiosis (data not shown). This result indicated that the observed block in state transitions in stm6 was primarily a defect observed in the light.

Since state 2 is associated with the phosphorylation of threonine residues in the light harvesting (LHC) antenna proteins of PSII (19), anti-phosphothreonine antibodies were used to assess the level of phosphorylation in immunoblots. Stm6 showed a marked reduction in the phosphorylation levels of CP29 (P9) and LHC-P11 proteins upon illumination conditions (state 2) that promote phosphorylation of these proteins in WT (Figure 1c). Interestingly, the phosphorylation levels of the P13 and P17 proteins in stm6 in state 2 were comparable to WT levels, indicating regulatory differences in the phosphorylation mechanism between the various LHC proteins. Furthermore, no differences between WT and mutant were obtained when cultivated for 12hours in the dark (state 1). Control immunoblots confirmed that stm6 still contained WT levels of LHC proteins (Figure 1c).
As transition to state 2 in the light is controlled by the reduction state of the PQ pool, it was important to confirm that the PQ pool could be driven to a reduced state in the light. To test this, chlorophyll fluorescence measurements were performed to determine the photochemical quenching parameter, qP, from which 1-qP, which is a measure of the reduction state of the PQ pool, can be calculated (32). Under growth light conditions the PQ pool was in a more reduced state in stm6 compared with WT (1-qP of 0.77 in stm6 and 0.59 in WT) (Table 1).

Disruption of a single mterf-type gene encoding for a mitochondrial protein is responsible for the phenotype of stm6

To test whether the Arg marker carried by plasmid pArg7.8-φX174 was tightly linked to stm6, crosses were performed between WT13 (mt+) and stm6 and the progeny scored for the existence of φX174 DNA (by PCR) and the ability to do state transitions as assessed by video imaging. Of the 50 progeny that contained φX174 DNA, all showed impaired state transitions. In contrast all progeny lacking φX174 DNA showed normal state transitions (data not shown). These data suggested that stm6 was an Arg-tagged mutant.

A combination of plasmid rescue and ligation-mediated suppression (LMS)-PCR led to the identification of the site of insertion of the Arg marker in stm6 (Figure 2a). Sequencing and PCR analysis revealed that stm6 contained an insertion of two pArg7.8 plasmids at a single site (Figure 2a) and the deletion of approximately 2kb of genomic DNA (Figure 2a). Subsequent sequence analysis and homology searches revealed that two genes were affected by the random integration of the two pArg7.8 plasmids. One gene was previously described as a nuclear transposon (Toc1) (37); the second gene had not been previously described and was subsequently designated Moc1 (mterf-like gene of *Chlamydomonas*) (AF531421).

PCR analysis in stm6 and WT using a 5’-specific Moc1-primer and a second primer derived from the Arg marker resulted in the amplification of a 1005 bp PCR product in stm6. This confirmed that the insertion caused a partial deletion of Moc1 leaving the 5’ region of the gene untouched. The precise site of insertion of the pArg7.8 plasmids was confirmed through sequencing PCR fragments spanning the insertion sites. Overall approximately 2 kb of the genome was deleted in stm6, extending from the 3’region of Moc1 to the 5’region of Toc1 (Figure 2a).
To confirm that disruption of \textit{Moc1} caused the phenotype of \textit{stm6}, complementation experiments were performed using a cosmid containing the \textit{Moc1} gene, which had been isolated from a cosmid library (kind gift of Dr. Saul Purton, UCL, UK). DNA sequencing and PCR analysis revealed that this cosmid contained a 12 kb insert consisting of the entire \textit{Moc1} gene together with 2 kb and 5kb of upstream and downstream flanking sequences respectively. No other large open reading frames were identified in the insert of the cosmid. Complementation was performed using a co-transformation approach using the \textit{Moc1}-containing cosmid in combination with a second vector containing the \textit{Cry1} gene conferring emetine resistance as a dominant selectable marker (31). Of approximately 200 emetine-resistant colonies assessed, 4 were found to have incorporated the intact \textit{Moc1} gene into the genome. These four colonies were able to express MOC1 and perform WT-like state transitions as assessed by fluorescence video imaging. According to previous slightly different two-plasmid co-transformation approaches (65,66), a success rate of 2% with both transformed genes functionally expressed was expected. One of these colonies, \textit{B13}, was examined in more detail and found to exhibit a clear WT phenotype with no perturbations in light acclimation (see Figures 1a,1b; 4; 6a, Tab.1). In contrast nine out of the 200 emetine-resistant colonies that contained only fragments of the \textit{Moc1} gene as assessed by PCR were unable to perform state transitions. From these data, we conclude that \textit{Moc1} is required for functional light acclimation in \textit{C. reinhardtii}.

The \textit{Moc1} gene encodes a 35kDa protein containing, somewhat surprisingly, a putative mitochondrial transit sequence and two mitochondrial transcription termination factor domains (mterf) with leucine zipper motifs characteristic of DNA-binding proteins (38). RT-PCR experiments confirmed that \textit{Moc1} was not expressed in \textit{stm6} (Figure 2b). Anti-peptide antibodies raised against MOC1 were used to confirm that MOC1 was targeted to the mitochondrion rather than the chloroplast (Figure 2c).

Overall there were striking similarities to the 35kDa human mTERF protein (34% based on alignments covering the full length sequences) (Figure 3) and their homologues in \textit{Drosophila melanogaster} (DmTTF, (39)) and \textit{sea urchin} (mtDBP, (40)). Human mTERF binds downstream of tRNA genes and is thought to be involved in controlling the amount of tRNA and rRNA synthesised within the mitochondrion as well as the expression of other mitochondrial genes and consequently the functionality of the mitochondrial respiratory chain (41,42,43). Interestingly 9 homologues to MOC1 with mterf domains have been identified in the genome.
of Arabidopsis thaliana, four of which are predicted to be targeted to the mitochondrion (At1g61980, At2g44020, At4g02990, At2g03050) and one to the chloroplast (At5g55580). Analysis of the Chlamydomonas nuclear genome data-base, plus DNA hybridisation experiments (data not shown), suggest that there is only one copy of Moc1 in C. reinhardtii.

Growth of stm6 is sensitive to high light and MOC1 expression is light-regulated
Besides effects on state transitions, a striking phenotype of the stm6 mutant was its sensitivity to light stress. In contrast to WT, stm6 cells did not survive photoautotrophic long-term growth experiments on HSM agar plates incubated for 200 hours at a constant irradiance of 200 µmol m⁻² s⁻¹ (Figure 4a).
After 4 hours illumination by 1000 µmol m⁻² s⁻¹ of visible light, cells of the mutant showed a marked decline in viability whereas the WT and the complemented strain, B13, were nearly unaffected (Figure 4b). stm6 also showed a dramatic increase in singlet oxygen inside the cell upon illumination as assessed by the accumulation of lipid hydroperoxides (Table 1).
Importantly, expression of MOC1 in WT was up-regulated at both the RNA and protein level upon a dark to light transition (Figure 4c) suggesting a particular function of this protein in the light.

Mitochondrial respiratory complexes are expressed aberrantly in light-grown cultures of stm6
Since most mitochondrial genes in C. reinhardtii encode subunits of respiratory complexes, experiments were performed to assess whether the absence of MOC1 had affected the expression and the activity of mitochondrial respiratory complexes. Immunoblotting analysis of cells grown in the light revealed that accumulation of the nuclear encoded subunit 90 of cytochrome oxidase (44) (COX90) was heavily reduced in stm6 in light grown cultures (Figure 5a), whereas levels of a 30kDa homologue of the NAD9 subunit of plant complex I (64); identified on Scaffold 96, contig5 in the C. reinhardtii genome database http://genome.jgi-psf.org/chlre1/chlre1.home.html and the alternative oxidase(s) (AOX1, AOX2, (45)) were much higher in the mutant compared to WT.
Effect of Moc1 deletion on a homologue of the rotenone-insensitive NADPH dehydrogenase NDB1

Two rotenone-insensitive NAD(P)H dehydrogenases of the mitochondrial respiratory chain have been characterised recently in potato and A. thaliana by Rasmusson and colleagues (4,5,16). NDA1 functions as an internal NADH dehydrogenase, which is switched on by light, probably to oxidise matrix NADH generated through photorespiration. NDB1 is an external facing NADPH dehydrogenase and appears to be expressed constitutively in both the light and dark. Whether there are homologues of these two enzymes in C. reinhardtii mitochondria has not been investigated previously.

In BLAST searches of the Chlamydomonas genome database (http://genome.jgi-psf.org/chlre1/chlre1.home.html) we could identify a homologue of NDB1 on JGI-Scaffold 595. RT-PCR experiments identified mRNA of Ndb1 in WT and stm6 (not shown). Therefore we conclude that a homologue of NDB1 is indeed expressed in C. reinhardtii.

Northern analyses revealed that, compared to WT, the transcript levels of NDB1 were heavily reduced in standard light-grown cultures of stm6 (Figure 5c). Activity measurements in isolated mitochondria confirmed an up to 70% reduction in the activity of the external NADPH oxidation (Table 1). Overall these data provide strong support for the idea that stm6 is compromised in its ability to oxidise external sources of NADPH.

Absence of MOC1 perturbs regulation of the transcript profile of the mitochondrial genome upon exposure to light

Given that MOC1 encodes a transcription factor homologous to human mTERF, experiments were directed at detecting possible perturbations to the transcript profile of the mitochondrial genome, which for C. reinhardtii consists of a 15.8 kb linear genome (46). A number of respiratory subunits are encoded by the genome including subunit 1 of cytochrome oxidase (Cox1 gene product), 5 subunits of complex I (Nad1-2, Nad4-6 gene products), and apocytochrome b (Cob gene product).

Nad transcripts in Chlamydomonas are transcribed bi-directionally with Nad1, Nad2 and Nad6 together with Cox1 in one direction.

Against the background that mitochondrial RNA makes only 1% of the total RNA (47) highly sensitive semi-quantitative RT-PCR analysis was performed to analyse differences in the transcription profile of certain mitochondrial genes. The experiments confirmed differences in transcript levels of the Nad1, Nad2, Nad6 and Cox1 genes between WT and stm6 following
exposure of dark-grown cells to light. In WT, comparison between light and dark treated cells revealed only very moderate light-induced (10 to 25%) deviations of Nad and Cox1 transcript levels (Figure 5b). In stm6 differences in the RNA abundance of mitochondrial genes between dark-cultivated and light-treated cultures were far more dramatic. stm6 showed a ~60% reduced level of the Cox1 transcript in the light, a 80-90% decrease of the Nad1 and Nad6 transcripts and a 210% increase of Nad2 transcripts (Figure 5b).

Together these results suggest an important role for MOC1 in influencing the expression of both complex I (NADH:UQ oxidoreductase) and complex IV (cytochrome oxidase) at the level of transcription of the mitochondrial Nad genes and of Cox1. Since it is likely that COX1 is the first subunit to be assembled into the cytochrome oxidase complex, the level of COX1 expression is likely to be an important determinant of the amount of enzyme synthesized (48).

**Acclimation of the mitochondrial respiratory chain to light is compromised in stm6**

Enzyme activity measurements were also performed to investigate whether there were differences in the activities of the respiratory complexes upon acclimation of dark-grown cells to 6 hours of light.

Following the light treatment, there was a dramatic decrease in cytochrome oxidase activity in stm6 (Figure 6b). The NADH:UQ-oxidoreductase activities of both WT and stm6 declined in the light. However here, in contrast to the obtained differences in complex IV activity, the light-induced decrease of NADH:UQ-oxidoreductase activity was greater for WT than for stm6 (Figure 6b). Levels of ATP in light-grown stm6 cells were also determined to be at about 50% of the levels of the WT and B13 (Figure 6a), consistent with a role for cytochrome oxidase in the generation of ATP from photosynthetically derived reductant, in line with earlier suggestions on higher plants (1). In contrast, after the light treatment, the cyanide-insensitive respiration rate in stm6, assigned to the activity of the alternative oxidases, was much higher than the WT level (Table 1).
DISCUSSION

Mutants affecting state transitions in the chloroplast

State transitions represent an elegant mechanism to regulate photosynthetic electron transport within the chloroplast at the level of light harvesting. By changing the size of the antenna associated with either PSI or PSII, the state transition mechanism maintains optimal electron transport through PSI and PSII under varying light conditions. In addition, in the case of Chlamydomonas, and possibly vascular plants, state 2, in which LHC is associated with PSI, promotes cyclic electron transfer around PSI and the production of ATP (49). State transitions are therefore also in principle important for regulating the ratio of ATP/NADPH produced during the light reactions of photosynthesis (50).

The results presented here indicate that abnormal LHC state transitions can also be used as a marker to detect general perturbations in cellular light acclimation processes.

The functional control of state transitions is complex. It is clear that the chloroplast Cyt b6f complex plays an important role in regulating the LHC kinase activity (51,52,53,54) and more recently good candidates for this kinase have been identified in Arabidopsis thaliana (55) and C. reinhardtii (22).

Defects in state transition mutants can be caused directly by deletion of functional genes such as LHC kinases. However, state transitions seem to be mainly controlled by the redox state of the plastoquinone pool (19). The PQH2:PQ ratio is influenced not only by linear and cyclic photosynthetic electron transport but also by alternative proton/electron feeding mechanisms (56).

Consequently, perturbations in the control of the cellular redox homeostasis could cause indirectly defects in redox-regulated short term light acclimation of the chloroplast.

In this work we have now identified an additional gene product, MOC1, which is needed for functional state transitions. Most surprisingly we have shown that MOC1 is targeted to the mitochondrion, not the chloroplast, and so its effect on state transitions must be indirect.

MOC1 is a member of a family of proteins with transcription termination motifs

MOC1 encodes a homologue of the human mitochondrial transcription termination factor mTERF, which in humans plays a central role in an attenuation phenomenon at the border between the 16S rRNA and tRNA genes (57) and is reported to be important for controlling and coordinating mitochondrial gene transcription (42). Non-functional binding of mTERF to the mitochondrial DNA results in stress-related diseases such as MELAS encephalo-
myopathies (43) and special forms of diabetes and can cause cell death. Detailed molecular characterisation have shown that human mTERF is a multizipper protein and binds to mitochondrial DNA as a monomer (41). Although a number of homologues have now been identified in other systems such as sea urchin (mtDBP, (40)) and Drosophila melanogaster (DmTTF, (39)), the binding and activation sites have not been conserved, possibly reflecting the differences in the gene order of mitochondrial genomes.

The identification of MOC1 in Chlamydomonas reinhardtii and the in silico identification of several potential mitochondrial, and even one possible chloroplast-targeted protein, with mterf domains in Arabidopsis thaliana suggest that this family of transcription factors might play an important role in the expression of plant organelle genomes as well.

Analysis of the genome sequence for Chlamydomonas plus Southern hybridisation analysis indicates that there is only one copy of Moc1. Previous studies had identified cDNA fragments of Moc1 in 3 independent Chlamydomonas EST libraries obtained from two different stress-treated cell cultures (http://www.biology.duke.edu/chlamy, http://www.kazusa.or.jp/en/plant/chlamy/EST) so it is possible that MOC1 has a general role in regulating mitochondrial gene expression in response to stress conditions.

Electron microscopy performed with WT and stm6 cells grown under different light conditions did not reveal differences in the number of mitochondria per cell between WT and mutant under the same growth conditions (data not shown). So it is unlikely that MOC1 has a role in determining the number of mitochondria per cell although effects on the copy number of the mitochondrial genome cannot be excluded at this stage.

**MOC1 is required for appropriate expression of mitochondrial respiratory complexes in the light**

It has been well established that the mitochondrion is important for optimal photosynthesis (10,11,58). Current ideas have emphasised the movement of metabolites between the two organelles so that perturbation of respiration would have a deleterious effect on chloroplast activity because of inappropriate redox conditions within the chloroplast. Our characterisation of MOC1 builds on these ideas and in addition suggests that changes in the transcription of the mitochondrial genome in the light, mediated by MOC1, is important for acclimation to high light.
In higher plants, the onset of photosynthetic activity is known to lead to changes, some dramatic, in the relative content of the respiratory complexes in the mitochondrion. For instance, expression of the internal NADH dehydrogenase, NDA1, in potato is switched on in light, possibly to maintain high rates of glycine oxidation in the photorespiratory pathway, and to prevent over-reduction of the matrix (16) and the external NADPH dehydrogenase NDB1 is reported to play an important role in regulating cellular redox homöostasis by transferring redox equivalents from cytosolic NADPH to the mitochondrial ubiquinone pool (5).

For WT _C. reinhardtii_, our work has also indicated marked differences in the composition of the respiratory chain in light-grown and dark-grown cultures. The reciprocal relationship in the expression of the type I and type II NAD(P)H dehydrogenases upon changing illumination conditions is quite similar to recent findings in potato and might reflect the need to increase rates of NAD(P)H oxidation but at the same time maintain an appropriate redox state for the UQ pool (see: (16) Fig.8, page 79).

In _stm6_ there is a major perturbation in the relative content of the respiratory complexes compared with the _WT_: most dramatically less cytochrome oxidase and rotenone-insensitive NADPH dehydrogenase, but higher levels of the alternative oxidases compared to _WT_ (Figure 5). Clearly the wide-ranging effect of the _Moc1_ mutation on the expression of both nuclear and mitochondrial-encoded proteins, would indicate some indirect effects on gene expression.

A question that will be addressed in future work is the mechanism by which _MOC1_ controls transcription in the mitochondrion. The 15.8-kb mitochondrial genome of _C. reinhardtii_ is transcribed bi-directionally to give two primary transcripts, which are then processed into smaller transcripts (46). Our RT-PCR and Northern data as well as activity measurements presented in Figures 5 and 6 indicate that _MOC1_ is needed in the light to adjust the respiratory chain and to maintain in particular complex IV activity as a functional site for ATP production and the activity of the external NADPH-DH when chloroplast photophosphorylation is perturbed.

This is furthermore relevant given that rotenone-insensitive mitochondrial NAD(P)H dehydrogenases might be induced in the light (4,5,16) so that there would be a need for the synthesis of more cytochrome oxidase. Initial RT-PCR experiments have yet to provide evidence to support this model; so it remains feasible that _MOC1_ binds at other sites (39), possibly even to RNA, and that its effects on transcription are indirect.
Chloroplast/mitochondrial interactions are perturbed in the Moc1 mutant

We have shown here that MOC1 is needed for appropriate transcription of the mitochondrial genome in the light and that in its absence there is a marked light-induced reduction of cytochrome oxidase activity and, compared to WT, a more moderate reduction of NADH:UQ oxidoreductase activity. Importantly we have also identified for the first time the presence of the NDB1 type II NADPH dehydrogenase and have shown that the activities of the external rotenone-insensitive NADPH dehydrogenase is drastically reduced in \textit{stm6}. These data strongly suggest that the mitochondria in \textit{stm6} are less able to function as electron sinks for the oxidation of reductant that is formed during photosynthesis. ATP levels are also dramatically reduced.

The influence of ATP levels for redox-controlled plastidic light adaptation mechanisms such as state transitions has already been described (9,15,59). It has been suggested that lower levels of ATP relieves the ATP-induced inhibition of phosphofructokinase activity in glycolysis, which then causes an increase in stromal NAD(P)H levels because of the activity of the downstream glyceraldehyde-3-phosphate dehydrogenase. Impaired oxidation of NAD(P)H by the mitochondrion in \textit{stm6} would also enhance NAD(P)H levels in the chloroplast. Levels of reduced PQ in the thylakoid could increase via reduction through a type-2 NAD(P)H:PQ oxidoreductase. Overall ATP depletion would be predicted to result in a shift towards state 2, as observed recently for mitochondrial mutants lacking one or more of the respiratory complexes 13. In contrast \textit{stm6} is shifted towards state 1 in the light, despite a reduced PQ pool. Recent evidence has pointed out that the activity of the LHC kinase is under two types of redox control in plants (60). Besides activation by the PQ pool, the kinase is switched off when levels of reduced thioredoxin in the stroma are high. Consequently the inability of \textit{stm6} to undergo a transition to state 2 in the light could be explained by the presence of an over-reduced stroma. This suggestion would also nicely explain why \textit{stm6} can be driven into state 2 in the dark upon anaerobiosis; under these conditions the stroma will be less reduced than in the light. It still remains possible though that state transitions might be regulated by other pathways that are dependent on the activity of the mitochondrion. It should be noted that under anaerobic conditions in standard growth light \textit{stm6} is unable to switch from linear to cyclic electron transfer most likely due to the block in state 1 (8,50).

Current models would suggest that the over-reduced stroma in \textit{stm6} in the light could be due to a combination of low ATP levels, which promotes glycolysis and the formation of...
NAD(P)H (9), and the reduced mitochondrial capacity to oxidise chloroplast stromal reduc-
tant (through the malate valve and the DHAP shuttle) (56). An inability to keep the stroma
more oxidised would tend to enhance the production of reactive oxygen species in the chloro-
plast, as suggested from measurement of lipid hydroperoxides (Table 1). In addition perturba-
tions to mitorespiration in *stm6* might also inhibit photorespiration (61), which again could
cause sensitivity to photoinhibition. The reduced levels of ATP in *stm6* would also impair the
normal ATP-dependent repair processes leading to the observed sensitivity of growth to high
light.

It should be noted that a reduced cytochrome oxidase activity and an increased AOX activity
as shown for *stm6* is similar to that reported by (62) for stress-induced nitric oxide (NO) sig-
nalling in plants. However 2-(4-carboxylphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-
oxide (cPTIO), which is known to prevent the killing effect of NO (63) did not prevent light-
induced cell death in *stm6* indicating that NO signalling is not involved.

In conclusion, we have identified a nuclear-encoded protein, MOC1, which is important for
regulating mitochondrial respiration in response to light and possibly other stresses. As such
the identification of MOC1 is an important step in the search for factors that coordinate the
redox poise between organelles.

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Figure legends:

Figure 1: Analysis of light-induced short-term adaptation mechanisms in the chloroplast:

a. State transitions analysed by 77K fluorescence emission spectroscopy in WT, B13 and stm6.

b. Fluorescence video image of WT, B13 and stm6 on TAP-agar plates in state 2 and state 1 (Illumination with actinic light at 620nm ± 15min illumination with 710nm PSI light).

b. In vivo phosphorylation protein pattern of thylakoid membranes isolated from state2 (S2)- and state1(S1)- (dark-) adapted WT and stm6 cells. Arrows indicate P9 and P11 proteins

Figure 2: Identification and localisation of MOC1 in C. reinhardtii.

a. Identification of the genomic DNA site in stm6 affected by the tandem nuclear insertion of two pArg7.8 plasmids by sequence analysis after chromosome walking (LMS-PCR) and by PCR analysis with specific moc1, toc1 and pArg7.8 primers. The 2 black arrow sets mark the primer pairs used for PCR. The 2 grey arrows mark the integration sites of the nuclear insertion in WT.

b. RT-PCR with a specific cDNA probe of Moc1 to demonstrate that the Moc1 gene is not transcribed in stm6.

c. Localisation of MOC1 as a mitochondrial protein by western blotting with anti-MOC1. Mitochondrial Cytc and chloroplast D2 were used as controls. (Antisera dilution 1: 1000)
**Figure 3:** Protein sequence of MOC1 and alignment with the human transcription termination factor mTERF. The model and the boxes mark the locations of the mitochondrial transit sequences and the identified mTERF domain structures in MOC1. Grey frame: Potential transit sequence; black frame potential mTERF motifs; black labelled nucleotides are homologous or identical.

**Figure 4:** *stm6* is sensitive to light stress and *Moc1* is light-regulated.

a. *WT* and *stm6* cells cultivated for 200 hours on HSM agar plates in different light intensities

b. *WT*, *B13* and *stm6* strains, cultivated to their mid-logarithmic growth phase in TAP medium, exposed to 1000 µmol m^{-2} s^{-1} white light for 4h, subsequently inoculated on TAP agar plates and grown for 7d in 40 µmol m^{-2} s^{-1} white light. High light cell rates surviving high light treatment over a period of 4 hours in *WT*, *B13* and *stm6* strains. Cells were diluted to 1x10^4 cells/ml prior to light treatment.

c. Evidence for light regulation of *Moc1*:

*Moc1*-Northern Blots of isolated RNA from dark- and light-incubated (200 µmol m^{-2} s^{-1}) *WT*.

Anti-MOC1 immunoblots of mitochondria isolated from dark cultivated *WT* cells (D) exposed to moderate high light (200 µmol m^{-2} s^{-1}) for 0 and 6 hours and from mitochondria isolated from standard light-cultivated *WT* cells (L) (96 hours, 40 µmol m^{-2} s^{-1}).

**Figure 5:** Evidence for the role of MOC1 in light-induced control of mitochondrial respiration:

a. Immunoblot of dark and light grown *WT* and *stm6* cells using antibodies specific for cytochrome oxidase subunit COX90, the UQ-oxidoreductase subunit NAD9 and the alternative oxidase AOX.

b. Semi-quantitative RT-PCR of *Nad1*, *Nad2*, *Nad6* and *Cox1* in (D) dark-grown *WT* and *stm6* cultures exposed to 200 µmol m^{-2} s^{-1} light (L) for 6 hours. mRNA levels were normalised to that of Actin. Different PCR cycles of *Cox1* and Actin are shown in three independent approaches to give evidence that the assay is quantitative.
Column bars represent changes in expression levels (in percent) upon light incubation in dark grown *WT* and *stm6* (100% = no changes).

c. Northern analysis of a *Ndb1* homologue in light-grown *WT* and *stm6* cultures.

**Figure 6:** ATP level, cytochrome c oxidase and NADH:UQ oxidoreductase activity measurements in *WT*, *stm6* and *B13*.

a. ATP levels in cells of *WT*, *stm6* and *B13* grown in the light and dark.

b. Specific activities of cytochrome c oxidase and NADH:UQ oxidoreductase in isolated mitochondria from *WT* and *stm6* cells cultivated in the dark and subsequently exposed to 6 hours 200 µmol m⁻²s⁻¹ light.

Error bars indicate 6 different independent measurements.

**Table 1:** Redox state of plastoquinone (1-qp)), lipid peroxidation rates (malonyldialdehyde, MDA), respiration rates and external NADPH oxidation activity (NDB1) of *WT*, *stm6* and *B13* strains cultivated in standard growth light.

|                         | *WT*                  | *stm6*            | *B13*            |
|-------------------------|-----------------------|-------------------|------------------|
| cyanide-insensitive     | set to 100            | 130 ± 5           | 105 ± 5          |
| oxygen uptake           |                       |                   |                  |
| External NADPH          | set to 100            | 40 ± 10           | no data          |
| oxidation activity      |                       |                   |                  |
| MDA concentration       | set to 100            | 230±20            | 110±15           |
| 1-qp                    | 0.59 ± 0.02           | 0.77 ± 0.02       | 0.60 ± 0.02      |
| 1-( Fm’-Fs)/(Fm’-Fo’)   |                       |                   |                  |
Figure 2

a.

```
stm6
5'-----------------------3'
TCAACATGAA             GCCGCTGACCTATGATACGG
Moc1  pBR329  Arg  pBR329  Arg  Toc1
5'-----------------------3'
TCAACATGAAACGTCAGAGGC  TACGTGCCCCTTATGATACGG
Moc1  Toc1
```

b.

```
bp
Moc1  actin
Moc1  actin

1018  517
stm6  WT
```

c.

```
anti-Cytc  anti-MOC1  anti-MOC1  anti-D2
kDa
-12  -35  -35  -28
mito  mito  chloro  chloro
```

WT  stm6
Figure 3

Transit sequence  mTerf  mTerf

MOC1 1 47 177 374

MOC1
MQQQLYRQT---------IHQHHOLLHARGPSLASTSSGRFCARPGCDSRRRLRCYSSYYTGGSSSL
MQSLSLGQTSISKGLNYLTMAPGNLHMRNNFLFGSRCWMTREFSANEITFKSQSFIFEGVKCHNT

mTERF

ESPVKRAFSLSGVSIN--DIERAAARELFESVIALVDKLDRLHGMIDLLLASPSDSIIGQVLLLAYP
DSEPLKNEDLLKNLLTMGVIDIDMARKRQFGVHEHR-MITNEQDLKMFILSSKGASKEVLASLISRYP

FAEQLSLELAEREVLDFELFDDMLHESEQCVRTVTLTRYPSILMNVKGLRPQVAYLSLGVYPESSLR
RAITRTPENLSKRWDLWFKIVTSDEITVNEILERSPEFERSNNNNLNLSENIFELSYVGLLRTKCLC

ELVLSRPLVGLFGIDT---VITFLKRLVEFQSMMHMLRSCPILDYRVEKSFSAAAPGSSHSSSS
RLLTPAPRTESNSLDLNKMVEFLQAAGLSLGHNDPADEVKFIKNFEILLQSTKRVKANIEFL

SGGMMGRN-----YWRQGAGCAGGRMVDCUAFLAATAAAPRHVKCTSAVINILLLSGCSRILAYRP
RSTFNLNESSLLVLICGPAEILDLSNWNDLARKSSHYSYANIKELKFLSLGCSEEVQKFWLSYPDVIFLA

CGLPPARKAPCIGAMCIAHVG-APRSPRWSEIERTVGDQORCIRITASGLERLIQRMAAHTGPRWRWA
EKKFNKDIIIICMENISROQIENPRVLDSSISTLKSRIKELVNAGCNLSTLNITLLWSKKRYEA
Figure 4

(a) 

|  10 |  200 |  800 μmol |
|-----|------|----------|

STM6  WT  STM6  WT  STM6  WT

(200 hours illumination, HSM medium)

(b) 

B13  WT  STM6

dark  4h (1000 μmol)

(c) 

WT

Moc1

|  0  |  2   |  4 h |
|-----|------|------|

(200μmol)

D → L

MO1C

|  0  |  6   |  96 h | M |
|-----|------|-------|---|

kDa  34  25

cells / ml

0  60  120  180  240 

time (min)
Figure 6

a.

![Graph showing ng ATP/100μg Chl for B13 and WT in light and dark grown conditions.]

b.

![Graph showing specific activity of cytochrome oxidase and NADH-UQ-oxidored.]

- B13 and WT are compared under light grown and dark grown conditions.
- Specific activity is measured in different treatments: D, D+6h L (200μmol).
The nucleus-encoded protein MOC1 is essential for mitochondrial light acclimation in Chlamydomonas reinhardtii
Christine Schönfeld, Lutz Wobbe, Rüdiger Borgstädt, Alexandra Kienast, Peter J. Nixon and Olaf Kruse

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