A Nucleus-Encoded Chloroplast Phosphoprotein Governs Expression of the Photosystem I Subunit PsaC in Chlamydomonas reinhardtii

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The nucleo-cytoplasmic compartment exerts anterograde control on chloroplast gene expression through numerous proteins that intervene at posttranscriptional steps. Here, we show that the maturation of psaC mutant (mac1) of Chlamydomonas reinhardtii is defective in photosystem I and fails to accumulate psaC mRNA. The MAC1 locus encodes a member of the Half-A-Tetratricopeptide (HAT) family of super-helical repeat proteins, some of which are involved in RNA transactions. The Mac1 protein localizes to the chloroplast in the soluble fraction. MAC1 acts through the 5’ untranslated region of psaC transcripts and is required for their stability. Small RNAs that map to the 5’ end of psaC RNA in the wild type but not in the mac1 mutant are inferred to represent footprints of MAC1-dependent protein binding, and Mac1 expressed in bacteria binds RNA in vitro. A coordinate response to iron deficiency, which leads to dismantling of the photosynthetic electron transfer chain and in particular of photosystem I, also causes a decrease of Mac1. Overexpression of Mac1 leads to a parallel increase in psaC mRNA but not in PsaC protein, suggesting that Mac1 may be limiting for psaC mRNA accumulation but that other processes regulate protein accumulation. Furthermore, Mac 1 is differentially phosphorylated in response to iron availability and to conditions that alter the redox balance of the electron transfer chain.

INTRODUCTION

The photosynthetic electron transfer chain in the thylakoid membrane comprises several large pigment-protein complexes that function together with remarkable efficiency to convert light energy into chemical energy, which is in turn used to fuel metabolism. The assembly of the complexes of the photosynthetic electron transfer chain requires the concerted expression of genes in two separate compartments: the nucleus and the chloroplast. These genes encode not only the subunits of the photosynthetic complexes, but also a large cohort of proteins that are required for gene expression and complex assembly.

Photosystem I is composed of 12 to 19 polypeptide subunits, depending on the organism, that bind ~200 pigments and cofactors. In Chlamydomonas reinhardtii, four components of PSI are encoded in the chloroplast: the larger PsaA and PsaB subunits, as well as the smaller PsaC and PsaJ subunits (Redding, 2009). Ten other polypeptides are encoded in the nucleus and imported into the chloroplast where they assemble with the chloroplast-encoded subunits. Two other chloroplast-encoded proteins, Ycf3 and Ycf4, facilitate the assembly of the PSI complex (Boudreau et al., 1997; Naver et al., 2001; Ozawa et al., 2009). The expression of the chloroplast psaA and psaB genes is governed at the posttranscriptional level by a set of nucleus-encoded factors that are transcript specific. The psaA mRNA is assembled in two steps of trans-splicing from three separate precursors (Choquet et al., 1988; Kück et al., 1987). Trans-splicing of psaA depends on a chloroplast-encoded RNA, tscA, and at least 14 nucleus-encoded proteins (Goldschmidt-Clermont et al., 1990, 1991). The stability and translation of psaA mRNA further depends on Taa1, a nucleus-encoded member of the OPR family (octatrico peptide repeat) of RNA binding helical-repeat proteins (Lefebvre-Legendre et al., 2015). The stability and translation of psaB mRNA depend on Tab1, another OPR protein, and on Tab2, a protein that is widely conserved in oxygenic phototrophs but has no previously described RNA binding motifs (Daumville et al., 2003; Rahire et al., 2012; Stampacchia et al., 1997).

The expression of chloroplast genes encoding subunits of the other photosynthetic complexes similarly depends on numerous transcript-specific nucleus-encoded proteins. Likewise in flowering plants, a large group of nucleus-encoded proteins govern chloroplast gene expression at the levels of transcript processing, splicing and stability, C-to-U editing, and mRNA translation (Stern et al., 2010; Barkan, 2011). The somewhat surprising complexity of chloroplast gene expression and the large number of nuclear...
genes involved raises the question of whether this provides a route for regulation of the plastid by the nucleus. Alternatively it has been argued that part of the complexity may have arisen in a process of constructive neutral evolution, where preexisting nucleus-encoded proteins can suppress new mutations that appear in the chloroplast (Maier et al., 2008; Gray et al., 2010; Lukes et al., 2011; Barkan and Small, 2014). A typical example where this might be the case is provided by the nucleus-encoded editing factors that make specific C-to-U changes in the sequence of chloroplast mRNAs at a posttranscriptional step (Schmitz-Linneweber et al., 2005a). Another example may come from the numerous factors that are required for splicing in trans of the psaA mRNA in Chlamydomonas (Lefebvre-Legendre et al., 2014). In these cases, the nucleus-encoded proteins could be needed constitutively and would not be involved in chloroplast gene regulation in the strict sense.

This does not exclude the possibility that some of the nucleus-encoded proteins do participate in the regulation of chloroplast gene expression in response to environmental or developmental cues. For example, in Chlamydomonas, nitrogen deprivation leads to rapid decrease in the amount of cytochrome b_{6f} complex. This coordinate response involves the proteolytic degradation of its subunits and also of Mca1 and Tca1, nucleus-encoded proteins that determine the stability and translation of petA mRNA, which encodes the Cyt(f) subunit (Raynaud et al., 2007; Boulouis et al., 2011; Wei et al., 2014). The concerted response to nitrogen deprivation also involves other nucleus-encoded proteins that take part in the assembly of the complex and its hemes (Wei et al., 2014). An early response of Chlamydomonas to iron deprivation is the downregulation of PSI and the remodeling of its light-harvesting antenna (Moseley et al., 2002; Naumann et al., 2005). There is a concomitant downregulation of Taa1, which is required for the stability and translation of psaA (Lefebvre-Legendre et al., 2015).

Here, we describe the identification of Mac1, a nucleus-encoded protein that localizes to the chloroplast where it is required for the expression of psaC. Mac1 belongs to the TPR/HAT (tetratrico peptide repeat/half a tetratrico peptide) family of helical repeat proteins, whose members are involved in RNA transactions (Hammani et al., 2014). Mac1 acts through the 5’UTR (untranslated region) on the stability of psaC, and Mac1 protein expressed in bacteria binds RNA in vitro. The amount of Mac1 is regulated in response to iron deprivation. We present evidence that Mac1 is phosphorylated and that this posttranslational modification is modulated by iron availability and other environmental conditions.

**RESULTS**

**Identification of MAC1**

A collection of random insertional mutants of Chlamydomonas was previously generated by transformation with an expression cassette containing the aphVIII gene, which confers paromomycin resistance (Johnson et al., 2010; Houille-Vernes et al., 2011). The mutants were screened for defects in photosynthesis based on chlorophyll fluorescence induction kinetics (Figure 1A). One of these mutants, which had an apparent defect in PSI and could not grow photoautotrophically, was chosen for further study. Immunoblotting with antibody against the PsaA subunit of PSI, which accumulated in the mutant to less than 30% of the wild-type level, confirmed that the mutant has a defect in PSI (Figure 1B). The loss of one subunit of a photosynthetic complex can lead to the degradation of the other subunits. This applies in particular to chloroplast mutants lacking PsaC (Takahashi et al., 1991). To investigate which subunit is primarily affected in the PSI-deficient mutant, wild-type and mutant RNA were analyzed by agarose gel electrophoresis and blot hybridization with probes for the chloroplast genes encoding three major subunits of PSI, psaA, psaB, and psaC (Figure 1C). This revealed a defect in the accumulation of psaC transcripts in the mutant. The psaC gene is upstream of petL, with which it is cotranscribed in the Chlamydomonas chloroplast genome. The slower migrating band is the dicistronic psaC-petL RNA (1.1 kb), while the faster migrating band is the monocistronic psaC RNA (0.45 kb) (Takahashi et al., 1991). Both of these transcripts were missing in the mutant, while the monocistronic petL RNA was present at elevated levels relative to the wild type (Figure 1C). By contrast, psaA was trans-spliced normally and psaB mRNA accumulated to wild-type levels. Because of its specific defect in the accumulation of psaC RNA, the mutant was called mac1 (mRNA of psaC). For further analysis, the mac1 mutant was backcrossed three times to the wild type. The wild-type and mac1 progeny segregated 2:2, indicating a nuclear mutation, and paromomycin sensitivity or resistance segregated with the wild-type or PSI-deficient phenotype respectively in 92 progeny from 24 tetrads (some of them incomplete), suggesting that the aphVIII insertion was linked to the mac1 mutation.

To identify the site of the aphVIII insertion in the mac1 mutant, reverse PCR and sequencing were used to obtain a flanking sequence tag, which corresponded to gene Cre09.g389615 (g9646.t1) in Version 11 of the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). The insertion mapped to exon 2 of the predicted gene (Supplemental Figure 1), and mac1 is thus most likely a null mutant. Genomic transformation with both BAC clone 23A16 and a 5.6-kb subclone (pMAC1_gen3) containing the Cre09.g389615 gene efficiently rescued the photosynthetic deficiency of mac1 (Supplemental Figure 2). In representative complemented transformants, the accumulation of the PsaC protein and the psaC transcripts were restored (Figures 2A and 2B). Thus, mapping of the insertion site and complementation with the wild-type gene identified Cre09.g389615 as the MAC1 gene. To raise a rabbit antiserum against Mac1, a C-terminal domain of the protein was expressed in *Escherichia coli*. Although the serum recognized Mac1, it was not monospecific and also recognized several nonspecific bands including one that nearly comigrated with Mac1 (Figure 2B) or could sometimes barely be resolved (Figure 2C, marked with an asterisk). Therefore, to facilitate the detection of Mac1, the MAC1 gene was tagged with a triple hemagglutinin (HA) epitope (MAC1-HA). This construct rescued the mac1 mutant with high efficiency (Figures 2A and 2B; Supplemental Figure 2), showing that Mac1-HA is functional. As expected, the HA-tagged protein was also detected by the Mac1 antiserum and migrated more slowly than the untagged form (Figure 2B, mac1;MAC1-HA).
MAC1 encodes a predicted polypeptide of 982 amino acids. A striking feature of the predicted Mac1 polypeptide is the presence of two adjacent domains each with six or seven tetratricopeptide (TPR) repeats, most of which have features of the HAT subfamily (Half-A-TPR) (Supplemental Figure 1C). Members of this subfamily are implicated in RNA metabolism and some have been shown to associate with RNA in vivo or to bind RNA in vitro (Hammani et al., 2012, 2014; Loizeau et al., 2014). Indeed, we found the closest paralog of Mac1 in Chlamydomonas to be Mbb1, a TPR/HAT repeat protein involved in the stabilization or maturation of the psbB/T and psbH mRNAs (Vaistij et al., 2000a; Loizeau et al., 2014) (Supplemental Figure 3 and Supplemental Data Set 1). The ortholog of Mbb1 in vascular plants, HCF107, is required for the stability of psbH transcripts (Felder et al., 2001; Sane et al., 2005; Hammani et al., 2012).

Mac1 Is a Chloroplast Protein

Immunofluorescence and confocal microscopy were used to determine the subcellular localization of Mac1. In the mac1; MAC1-HA strain, immunolabeling with a monoclonal anti-HA antibody gave a signal in the chloroplast, which was absent in the wild-type control (Figure 3; Supplemental Figure 4). The chloroplast localization of Mac1-HA was confirmed by colabeling with a polyclonal antibody against the chloroplast stromal protein DnaK. Furthermore, the localization of Mac1-HA was distinct from that of Rpl37 (Figure 3A) and Rpl4 (Supplemental Figure 4), subunits of cytoplasmic ribosomes.

Cell fractionation experiments were used to confirm the localization of Mac1. The rabbit polyclonal antibody against the Mac1 protein was used to probe immunoblots of Chlamydomonas subcellular fractions (Figure 3B). Mac1 was found in the chloroplast fraction, together with the chloroplast markers PsA and DnaK, but not in the supernatant of the lysate which contained the Rpl37 subunit of the cytosolic ribosomes. Further fractionation of the chloroplasts yielded a stromal fraction with soluble proteins such as DnaK and a membrane pellet, which contained the PsA subunit of PSI, an integral protein of the thylakoid membrane. The Mac1 protein cofractionated with DnaK in the soluble fraction and showed no indication of membrane association.

To investigate whether Mac1 is part of a ribonucleoprotein complex, an extract of total soluble proteins was prepared from a strain expressing HA-tagged Mac1 (mac1; MAC1-HA). Half of the extract was treated with RNase, while the rest was mock-treated, and then the two samples were fractionated by sucrose gradient sedimentation (Figure 3C). Most of Mac1 remained close to the top of the gradient, in a position consistent with the expected sedimentation of the monomer. A minor fraction of Mac1 was distributed in larger complexes. Their sedimentation was not significantly affected by the RNase treatment.

Mac1 Is Required for psaC RNA Stability

The absence of detectable psaC transcripts in mac1 could be due to a defect either in transcription or in RNA stability. To distinguish between these two possibilities, transcription of psaC was evaluated in run-on transcription assays. Wild-type and mac1 cells were permeabilized and incubated with radiolabeled [α-32P]UTP for 5 or 15 min to allow extension of nascent transcripts (Klinkert et al., 2005). Under such conditions, there is no transcription reinitiation and the amount of radiolabel incorporated in nascent transcripts reflects the density of transcribing polymerases on the
Figure 2. Complementation of the mac1 Mutant.

(A) RNA gel blot hybridization analysis of the mac1 mutant and complemented strains. RNA was extracted from the wild type, the mac1 mutant, the mac1 mutant rescued by transformation with the genomic BAC clone (BAC23A16), with the genomic subfragment containing MAC1 (pMAC1-gen3), or with a derivative of the latter carrying a triple HA epitope (MAC1-HA), and a chloroplast mutant with an insertion in the psaC gene (psaC-D). The samples were analyzed by denaturing gel electrophoresis and RNA gel blot hybridization with radiolabeled fragments containing psaC and petL (upper panel) or atpB as a control (lower panel). The sizes of the transcripts are shown on the left (1.1 kb, asterisk). This precedent and the presence of a minor nonspecific control (lower panel). The sizes of the transcripts are shown on the left (1.1 kb, asterisk) runs just below Mac1.

(B) Immunoblot analysis of total proteins extracts from the same strains as in (A). The antisera used for immunoblotting are shown on the right. (C) Specificity of the antiserum against Mac1. Total protein extracts of the wild type and of the mac1 mutant were analyzed by SDS-PAGE and immunoblotting with a rabbit polyclonal antiserum raised against recombinant Mac1. A minor nonspecific band that is present in the mutant (marked with an asterisk) runs just below Mac1.

respective gene (Guerin and Bellemare, 1979; Monod et al., 1992). The radiolabeled RNA was extracted and hybridized to DNA probes spotted on a nylon membrane (Figure 4). There was no significant difference in the radioactive signal for psaC between the mac1 mutant and the wild type. These results indicate that transcription of psaC proceeds at normal rates in mac1 and, hence, that it is the stability of psaC RNA that is compromised in the mutant.

Some helical-repeat proteins are known to stabilize specific chloroplast mRNAs by binding to defined sequences in the 5′ UTR or the 3′ UTR and protecting the target transcript against exonucleases (Pfalz et al., 2009; Priiry et al., 2011). This mechanism can lead to the accumulation of RNA footprints, small RNA fragments that are protected by the respective RNA binding proteins (Pfalz et al., 2009; Ruwe and Schmitz-Linneweber, 2012; Zhelyazkova et al., 2012; Loizeau et al., 2014). Footprints that map to the 5′ end of the psaC transcripts could be identified in Chlamydomonas small RNA databases (Figure 5; Supplemental Figure 5). This was confirmed by RNA blot hybridization, which detected a small RNA of ~50 nucleotides that was present in the wild type, but absent in the mac1 mutant (Figure 5B, marked with an arrow). This suggests that Mac1 may bind the 5′ end of the psaC transcript or may indirectly promote the binding of a protein to this transcript, two alternatives that are not mutually exclusive.

Mac1 Acts through the 5′ UTR of psaC RNAs

Mbb1, the closest paralog of Mac1 in Chlamydomonas, protects the psbB transcript by associating with its 5′ UTR (Vaistij et al., 2000b; Loizeau et al., 2014). This precedent and the presence of Mac1-dependent footprints matching the 5′ end of psaC transcripts suggest that the target of Mac1 may be the 5′ UTR of psaC. To test this hypothesis, a chimeric reporter gene was constructed (psaC:lucCP) with the promoter and 5′ UTR of psaC fused to the coding sequence of firefly luciferase (Matsuo et al., 2006) followed by the 3′ UTR of rbcL. For biolistic chloroplast transformation, the chimeric luciferase reporter was introduced in the atpB-INT vector (Michelet et al., 2011), which carries a modified atpB gene as selectable marker, allowing selection of photoautotrophic transformants in a ΔatpB mutant host (Figure 6). The strain with the psaC:lucCP reporter was then crossed to the mac1 mutant, so that sibling progeny with either the wild-type or the mac1 mutant were recovered for analysis. Luciferase activity in the mac1 mutant (mac1/psaC:lucCP) was at background level and, thus, at least 30-fold lower than in the wild type (Figure 6B). Luciferase activity was restored to wild-type levels by transforming the mac1/psaC:lucCP progeny with the wild-type MAC1 gene (mac1/MAC1/psaC:lucCP). The mac1 mutation did not significantly affect the expression of a control reporter with the psbB promoter and 5′ UTR (mac1/psaB:lucCP). Luciferase expression with the psbB construct was much stronger than with the psaC construct, but such differences between chimeric genes expressed in the chloroplast are commonly observed in Chlamydomonas (Michelet et al., 2011). The results indicate that a genetic target of Mac1 is found in the promoter or 5′ UTR of psaC. Since the mac1 mutation affects psaC transcript stability, it is most likely that the target of Mac1 is in fact in the 5′ UTR rather than the promoter.

Mac1 Binds RNA in Vitro

To investigate the RNA binding properties of Mac1, a recombinant protein corresponding to the full sequence (except for the
predicted transit peptide) was expressed in *E. coli*, with a tag of six histidines at the C terminus. After nickel-affinity chromatography, the preparation still contained contaminants, some of which were found to also bind RNA in preliminary experiments. Therefore, the proteins were further separated by gel filtration chromatography (Figure 7). In the eluted fractions, recombinant Mac1 protein was identified by SDS-PAGE by its expected size (96 kD; Figure 7A) and its recognition by Mac1 antiserum in immunoblots (Figure 7B). Mac1 peaked in the fraction expected for a monomer of its size (lane 8). To assay RNA binding, a radiolabeled probe was prepared corresponding to 51 nucleotides at the 5’ end of psaC mRNA, matching the footprint observed in vivo. For the electrophoretic mobility shift assay, aliquots of each protein fraction eluted from the gel filtration column were mixed with the radiolabeled probe, and the RNA-protein complexes that formed were separated from the unbound probe by non-denaturing gel electrophoresis. The major RNA-protein complex that formed matched exactly the elution profile of Mac1 protein, peaking in lane 8 (B2, Figure 7C). Minor complexes (B1 and B3, Figure 7C) with other proteins were clearly separated from Mac1, peaking in fractions 5 and 12, respectively. Mac1 bound the RNA probe with high affinity. The estimated $K_d$ was 40 to 60 nM for two independent preparations, comparable to the $K_d$ observed in vitro for HCF107 (70 nM) (Hammani et al., 2012).

**Regulation of Mac1 in Response to Iron Limitation**

In *Chlamydomonas*, early responses to iron limitation under mixotrophic conditions involve the dismantling of the photosynthetic electron transfer chain, and in particular of PSI and the

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**Figure 3.** Mac1 Localizes to the Chloroplast.

(A) Immunofluorescence of *Chlamydomonas* *mac1* mutant cells complemented with an HA-epitope-tagged *MAC1* gene (*mac1;MAC1-HA*) or wild-type cells as a control, fixed in methanol to remove chlorophyll. The samples were decorated with both a monoclonal mouse antibody against the HA epitope and a rabbit polyclonal antibody against either the chloroplast protein DnaK or the cytosolic subunit of the ribosome Rpl37. The anti-mouse and anti-rabbit secondary antibodies were labeled with Alexafluor 647 or Alexafluor 546, respectively. Immunofluorescence was observed by confocal microscopy and is shown separately (green and magenta respectively) or as a merged image (green + magenta in white; third panels). An image of the same cells observed by transmission microscopy is also presented (fourth panels).

(B) Cells from the cell wall-deficient mutant cw15 were lysed by nebulization. The lysate (total) was subjected to low-speed centrifugation and the supernatant was collected (supernatant) while the chloroplasts in the pellet were further purified by Percoll gradient centrifugation (chloroplast). The chloroplasts were further lysed by sonication and fractionated by high-speed centrifugation into a supernatant fraction (soluble) and a pellet (membrane pellet). Equal amounts of protein from each fraction were subjected to SDS-PAGE and immunoblotting with the polyclonal antisera indicated on the right.

(C) Sucrose gradient sedimentation analysis of Mac1. An extract of total soluble protein from the *mac1;MAC1-HA* strain was split in two aliquots, one was mock-treated and the other was treated with RNaseA. The protein complexes were then separated by sedimentation in sucrose density gradients. Twenty fractions were collected from the bottom and analyzed by SDS-PAGE and immunoblotting with either anti-HA monoclonal antibody or anti-Rubisco antiserum as indicated. The sedimentation of markers used for calibration in a parallel gradient is indicated at the top (thyroglobulin, 670 kD; aldolase, 160 kD). Rubisco holoenzyme has a molecular mass of ~550 kD.
cytochrome $b_6f$ complex (Moseley et al., 2002; Hohner et al., 2013). PsaC is the subunit of PSI that directly binds two of its three 4Fe4S iron-sulfur centers, $F_A$ and $F_B$. To investigate whether the response to iron limitation also involves the regulation of Mac1 protein accumulation, mac1/Mac1-HA cultures were grown through 10 division cycles in acetate-containing medium at three different iron concentrations: 20 $\mu$M Fe (iron replete), 1 $\mu$M Fe (limited), and 0.2 $\mu$M Fe (deficient) (Glaesener et al., 2013). As expected, in the iron-deficient culture (0.2 $\mu$M Fe) PSI decreased approximately 4-fold as estimated from the reduced accumulation of PsaA and PsaC compared with a dilution series of proteins from the iron-replete culture (Figure 8A; Supplemental Figure 6). There was a concomitant −50% decrease in the abundance of Mac1-HA (Supplemental Figure 6D). Similar results were obtained with a wild-type strain using the Mac1 antibody (Figure 8B).

However the accumulation of ATP synthase was not reduced under iron limitation (Figure 8A, CF1 antiserum), confirming previous observations that the response to iron starvation is not a general dismantling of the thylakoid membrane (Moseley et al., 2002; Hohner et al., 2013). Under the same conditions, the abundance of the pscC transcripts decreased −30% as estimated from a comparison to a dilution series of RNA from iron-replete conditions (Figure 8C; Supplemental Figure 6B). The amounts of atpB mRNA remained unchanged, indicating that the response is not a general degradation of chloroplast mRNA. The lack of PscC protein in the ΔpsaC mutant (Takahashi et al., 1991) did not affect the accumulation of Mac1 (Figure 2B), suggesting that the decrease of Mac1 in iron-deficient conditions is not a consequence of the reduced levels of PscA.

Under iron deficiency, we observed a concomitant decrease in Mac1-HA and pscC mRNA accumulation, with the former more pronounced than the latter (Supplemental Figures 6D and 6F). The question thus arises whether the amount of Mac1 could be limiting for pscC expression. To investigate this point, different transformants of mac1 with the MAC1:HA construct, expressing different levels of Mac1-HA, were compared under iron-replete conditions (Figure 9). In strains that expressed different levels of Mac1-HA, the accumulation of the pscC transcripts paralleled the amounts of Mac1-HA (Figure 9A). However the amount of PscC protein in the different transformants remained constant and similar to the wild type (Figure 9B). These observations suggested that Mac1-HA may be limiting for pscC mRNA accumulation, but that increased levels of pscC mRNA do not lead to increased accumulation of PscC. This could be due to translational or posttranslational regulation of PscC amounts.

**Figure 4.** Analysis of pscC Transcription in the mac1 Mutant.

For the run-on transcription assay, duplicate nylon membranes were decorated with spots of PCR fragments derived from the chloroplast genes indicated on the right (0.6 and 0.3 $\mu$g DNA of each probe, except a single spot of 0.25 $\mu$g for pscA ex1) or the bacterial plasmid vector pUC19 as a negative control. Wild-type or mutant indicated on the right (0.6 and 0.3 $\mu$g DNA of each probe, except a single spot of 0.25 $\mu$g for pscA ex1) or the bacterial plasmid vector pUC19 as a negative control. Wild-type or mutant cells were permeabilized by freezing and thawing and then radiolabeled with $^{32}$P-UTP for 15 min (left panel) or 5 min (right panel). The labeled RNAs were extracted and hybridized to the membranes (the same pair of membranes was used first with the 5 min samples, and after stripping used again with the 15 min samples). In the run-on transcription assay elongating RNA polymerases insert radiolabel in the nascent transcript, but transcription initiation does not occur. The hybridization signal is thus a measure of the density of transcribing polymerases on the respective gene.

**Figure 5.** Small RNA Footprints in the 5' UTR of pscC.

(A) sRNA sequence coverage graph of the pscC 5'-region. Per-base read coverage within this region was extracted from sRNA sequencing databases (Ibrahim et al., 2010; Loizeau et al., 2014). Bars represent the pscC 5' UTR and the coding sequence (the actual sequence is provided in Supplemental Figure 5). The arrowhead marks the 5'-end of the pscC 5' UTR. A line labeled pscC4as denotes the antisense probe used in (B).

(B) Low molecular weight enriched RNA extracted from the mac1 mutant and the wild type was subjected to gel electrophoresis (left panel, ethidium bromide fluorescence image), transferred to nylon membranes, and hybridized with the radiolabeled antisense probe. The arrow marks a small RNA of ~50 bases that is present in the wild type but not in the mac1 mutant.
Phosphorylation is a posttranslational modification that is involved in the regulation of numerous proteins. To analyze whether Mac1 could be subject to protein phosphorylation, protein extracts of mac1:MAC1-HA were analyzed using electrophoresis in polyacrylamide gels containing a Phos-tag gradient (Phos-tag PAGE; Supplemental Figure 7). When chelated with a divalent cation such as Zn\(^{2+}\), Phos-tag binds phosphate groups and retards the migration of phosphorylated polypeptides (Kinoshita et al., 2006; Kinoshita and Kinoshita-Kikuta, 2011; Longoni et al., 2015). Immunoblotting with monoclonal anti-HA antibody revealed two bands for Mac1, suggesting that the upper one (P\(_1\)) could represent phosphorylated Mac1 (Figure 10). Treatment of the sample with λ protein-phosphatase led to the disappearance of the slowly migrating band, confirming that it corresponds to a phosphorylated form of Mac1. As a control, a strain expressing HA-tagged Sedoheptulose Bis Phosphatase (Sbp-HA) (Loizeau et al., 2014) was analyzed in parallel and there was no evidence that this protein was phosphorylated.

The availability of iron had a strong effect on the phosphorylation of Mac1 (Figure 10B). In cells grown under iron-replete mixotrophic conditions, Mac1 was strongly phosphorylated. After growth under conditions of iron limitation, the ratio of phosphorylated (P\(_1\)) to nonphosphorylated Mac1 (U) decreased and was even lower under iron depletion. As already observed in the previous section (Figure 8), the total amount of Mac1-HA concomitantly decreased under iron depletion compared with iron sufficiency. To control for the possibility that the lower apparent ratio of phosphorylation, which paralleled the reduction in the total amount of Mac1, could be due to an artifact of Phos-tag PAGE and immunoblotting, a dilution series of the sample from iron-replete condition was similarly analyzed (Figure 10C). The ratio of the phosphorylated band to the unphosphorylated form did not change significantly at lower total protein concentrations, thus corroborating the validity of the observation that phosphorylation of Mac1-HA is lower under iron limitation.

Phosphorylation of light-harvesting complex II (LHCCI) subunits in the thylakoid membrane by the kinase Stt7 is regulated in the redox state of the electron transfer chain (Lemeille and Rochaix, 2010). Under anaerobic conditions in the dark, which lead to a reduction of the plastoquinone pool (state 2), Mac1-HA was largely phosphorylated (Figure 10D, lane 2). The two slowly migrating bands (labeled P\(_1\) and P\(_2\)) could represent different degrees of phosphorylation of Mac1. Conversely under aerobic conditions in low light, which favor plastoquinone oxidation (state 1), Mac1 was partly unphosphorylated (Figure 10D, lane 1, band labeled U). To determine whether this phosphorylation depends on the Stt7 kinase, the MAC1:HA construct was transformed into the stt7-7 mutant. In this strain, the phosphorylation patterns of Mac1-HA in state 1 and state 2 conditions were the same as in the wild-type MAC1:HA strain, indicating that Stt7 is not involved in the phosphorylation of Mac1 (Figure 10D, lanes 3 and 4). Proteomic surveys of Chlamydomonas protein phosphorylation have indicated that Mac1 can be phosphorylated at serines 137 and 139 (Wang et al., 2014). To determine whether these are the residues that account for the phosphorylation of Mac1 detected by Phos-tag electrophoresis, a mutant version of MAC1:HA in which both serines 137 and 139 were replaced by alanines was constructed, MAC1-AA-HA. The MAC1 mutant strain transformed with MAC1-AA-HA grew normally on minimal medium, indicating that the modified protein is functional. The migration of phosphorylated Mac1-AA-HA in Phos-tag gels was unaffected compared with wild-type Mac1-HA in state 1 or state 2 conditions (Figure 10D, lanes 5 and 6), indicating that the major sites of phosphorylation that can be detected in this way are not serines 137 or 139.

**DISCUSSION**

**Mac1 Controls the Stability of psaC mRNA**

Mac1 was identified through the analysis of a nonphotosynthetic mutant deficient for PSI. The primary defect in mac1 is its failure to accumulate psaC mRNA (Figures 1C and 2A) and the PsaC

![Figure 6](image)
In the chloroplast genome of Chlamydomonas, psaC is transcribed in a polycistronic unit that also contains the downstream petL gene (Takahashi et al., 1994). In the mac1 mutant, the accumulation of the monocistronic psaC mRNA and the dicistronic psaC-petL transcript are specifically affected, while the amount of monocistronic petL mRNA is slightly elevated. This suggests that the mac1 mutation affects the stability rather than the transcription of psaC. Indeed, in a run-on transcription assay the activity of psaC appeared comparable in the mutant and in the wild type (Figure 4). Furthermore, a fragment containing the promoter and 5’ UTR of psaC was sufficient to confer dependence on MAC1 to a chimeric psaC-lucCP luciferase reporter (Figure 6). Although an accessory effect on transcription cannot be ruled out, taken together these results indicate that Mac1 is involved, directly or indirectly, in stabilizing transcripts containing psaC through its 5’ UTR. This interpretation is supported by the identification in existing databases of small RNAs (sRNA) corresponding to the 5’ end of the psaC transcripts. Using RNA gel blot hybridization, the existence of a sRNA from psaC (approximately 50 nucleotides) could be confirmed in vivo and was shown to depend on the presence of Mac1 (Figure 5). Such sRNA footprints were first identified in plant chloroplasts at the position of RNA binding proteins such as PPR10, which protect the bound RNA against exonucleolytic degradation from both sides (Pfälz et al., 2009; Prikryl et al., 2011). Likewise in Chlamydomonas, small RNAs at the 5’ end of psbB and psbH are formed in the presence of Mbb1, a nucleus-encoded protein that is required for the stable accumulation of the two mRNAs (Loizeau et al., 2014) and is the closest paralog of Mac1 (Supplemental Figure 3). Interestingly, HCF107, the ortholog of Mbb1 in Arabidopsis thaliana and maize (Zea mays), can bind psbH RNA in vitro and forms a sRNA footprint in vivo (Hammani et al., 2012). Mac1 is only found in Chlorophyta, whereas in maize, the pentatricopeptide repeat (PPR) protein CRP1 binds the 5’ UTR of psaC, where it generates a sRNA footprint and is required for its efficient translation (Fisk et al., 1999; Schmitz-Linneweber et al., 2005b; Ruwe and Schmitz-Linneweber, 2012).

Mac1 contains two domains with tandem repeats of 34-amino acid residues that belong to the HAT/TPR family. Members of the HAT repeat proteins, which in turn belong to the helical-repeat superfamily, are involved in RNA interactions (Preker and Keller, 1998). Indeed, Mac1 protein expressed in bacteria binds RNA with high affinity in vitro (Figure 7). As mentioned above, HCF107 also belongs to this family and binds RNA in vitro with similar affinity (Hammani et al., 2012). In the PPR proteins, which form the most prevalent helical-repeat protein family in plant organelles (Barkan and Small, 2014), the 35-amino acid repeats are composed of two antiparallel α-helices that stack onto one another to form a superhelical backbone (Ke et al., 2013; Yin et al., 2013). Each modular repeat of a PPR protein interacts with one nucleotide of the bound RNA through specific residues that determine the recognition of the target base, allowing the definition of a “PPR code.” The HAT repeats also form a superhelical scaffold, and it can be predicted that TPR/HAT domains of Mac1 will bind RNA in a similar way (Bai et al., 2007; Hammani et al., 2014). In the Chlamydomonas sRNA databases that we queried (Figure 5), a series of sRNAs corresponding to the 5’ end of psaC delineate a relatively long footprint (approximately 50 nucleotides), which is roughly the size of the sRNA detected in vivo, and two...
subpopulations of shorter small RNAs map to the same region. There are two TRP/HAT domains in Mac1 (Supplemental Figure 1C), each of which consists of six or seven TRP repeats and is thus expected by analogy with PPR proteins to bind six to seven bases in the RNA (Barkan and Small, 2014). The relatively large size of the protected RNA fragment could be due to binding of two adjacent sites by the two TPR/HAT domains of Mac1 with a possible intervening RNA loop, such as was proposed for the binding of CRP1 to the petB-petD site in maize (Barkan et al., 2012). Alternatively, it is also possible that the large footprint is generated by one or more other partner proteins in a Mac1-dependent manner. It is conceivable that the minor fraction of Mac1 that sediments with large complexes in sucrose gradients could reflect its transient or labile association with other partners.

In Chlamydomonas, there are several examples of nucleus-encoded proteins that bind the 5′ end of their respective target transcripts and offer protection against 5′ to 3′ exonucleolytic degradation. Apart from Mbb1, the closest paralog of Mac1 mentioned above, these also include the TPR/HAT protein Nac2, which is required to stabilize psbD RNA (Kuchka et al., 1989; Nickelsen et al., 1999). Other examples are provided by members of the OPR family of helical-repeat proteins, such as Taa1 and Tab1, which are required for the stability and translation of psaA and psaB, respectively, and Mbi1, which is necessary for the stable accumulation of psbI mRNA (Lefebvre-Legendre et al., 2015; Wang et al., 2015). Also of interest is another OPR protein, Mcg1, which is required for stabilization of petG mRNA and generates a corresponding sRNA footprint at its 5′ end (Wang et al., 2015). There are also examples among the relatively few PPR proteins of Chlamydomonas: Mca1 binds the 5′ UTR of petA, hinders its 5′ to 3′ exonucleolytic degradation, and, in association with Tca1, promotes its translation (Loiselay et al., 2008), while Mrl1 ensures the stability of rbcL mRNA (Johnson et al., 2010). The properties of Mac1 are consistent with the model that emerges from these comparisons: Mac1 could bind the 5′ UTR of petA, hinder its 5′ to 3′ exonucleolytic degradation, and, in association with Tca1, promotes its translation (Loiselay et al., 2008), while Mrl1 ensures the stability of rbcL mRNA (Johnson et al., 2010). The properties of Mac1 are consistent with the model that emerges from these comparisons: Mac1 could bind the 5′ UTR of psaC and protect the downstream transcripts from exonucleolytic degradation. The combined action of 5′ and 3′ exonucleases would eventually generate a sRNA footprint. It remains an open question whether Mac1 is also involved in translation.

**Mac1 Is Downregulated in Response to Iron Deficiency**

The low availability of iron can be a severe limitation for the growth of photosynthetic organisms, be it in aqueous environments or on land. Both the mitochondrial respiratory chain and the photosynthetic electron chain comprise proteins that contain iron, heme, and iron-sulfur clusters as cofactors. In Chlamydomonas grown in photoheterotrophic conditions, where both respiration and photosynthesis are normally active, iron deficiency leads to the preferential allocation of the metal to mitochondrial respiration subcomplexes.

**Figure 8.** Mac1 and PsaC Levels Coordinately Respond to Iron Availability.

(A) Cultures of the mac1 mutant complemented with an HA-epitope tagged MAC1 gene (mac1;MAC1-HA) were grown mixotrophically (acetate-containing medium in the light) for 10 doublings in the presence of different concentrations of Fe: 20, 1, or 0.2 μM. Total cell extracts were analyzed by SDS-PAGE and immunoblotting with the antisera indicated on the right. A dilution series of the iron-replete sample (20 μM) is presented in the first three lanes.

(B) Wild-type cells were grown and analyzed as in (A), and Mac1 was detected with the polyclonal antiserum (see Figure 2C).

(C) RNA was extracted from the same wild-type cultures as in (B) and subjected to denaturing gel electrophoresis and blot hybridization with radiolabeled probes for psaC (upper panel) or atpB as a control (lower panel).
at the expense of photosynthesis (Moseley et al., 2002; Terauchi et al., 2010; Urzica et al., 2012). PSI, which contains three 4Fe4S centers, is an early target of this response: It is disconnected from its light-harvesting antenna and rapidly dismantled, like other complexes of the photosynthetic electron chain, while ATP synthase remains more stable (Moseley et al., 2002; Naumann et al., 2005). The iron that is released is bound by ferritin, which is upregulated at the level of translation (Busch et al., 2008). The response to iron deficiency also involves changes in the abundance of numerous other proteins and in particular of Taa1, which is degraded (Hohner et al., 2013; Lefebvre-Legendre et al., 2015). As previously mentioned, the latter is a nucleus-encoded protein of the OPR family that is specifically required for the stability and translation of psaA mRNA in the chloroplast. There is thus a clear parallel with Mac1, which controls psaC mRNA stability and is downregulated under iron deficiency. Hence, the response to iron limitation involves not only the degradation of the PSI complex, but also the downregulation of nucleus-encoded factors that regulate the expression of chloroplast-encoded PSI subunits. As was observed with Taa1, the decrease in Mac1 abundance is most likely regulated at the posttranscriptional level, since data from high-throughput RNA sequencing indicate that under iron limitation there is a moderate increase in MAC1 RNA levels (Urzica et al., 2013; Phytozome v11 at https://phytozome.jgi.doe.gov/). It is interesting to compare this response to a similar response that occurs under nitrogen limitation in Chlamydomonas with the downregulation of the cytochrome b6f complex. This latter response involves the coordinate degradation of the b6f complex itself, of proteins that regulate the expression of its chloroplast genes, and of proteins involved in its biogenesis (Raynaud et al., 2007; Wei et al., 2014).

In response to iron deficiency, there is a correlation between the reduced accumulation of PSI subunits and of factors that govern their expression. This correlation raises the question whether the decrease of the nucleus-encoded factors has a causal role in the decrease of the chloroplast-encoded proteins. Alternatively, it cannot be excluded that the nucleus-encoded factors and the PSI subunits could be responding independently to the same nutritional cues. In other words, the question is whether Mac1 truly exerts anterograde regulation on psaC expression in response to iron availability. As a step toward answering this question, we sought to determine whether the amounts of Mac1 can be limiting for the accumulation of psaC mRNA and PsaC protein. In an allelic series of transformants expressing different levels of Mac1-HA, we observed that the accumulation of psaC mRNA paralleled that of Mac1-HA (Figure 9). Thus, the amounts of Mac1 do seem to be limiting for the accumulation of psaC mRNA. However the amounts of PsaC protein were similar in all the strains of the allelic series. This may be due to the negative feedback regulation that is exerted by unassembled PsaC on translation of its own mRNA

![Figure 9. psaC RNA Accumulation Correlates with Mac1 Levels.](image-url)
In the Mac1-HA overexpressors, any excess of PsaC that cannot be assembled with PsaA and PsaB is expected to inhibit its own translation and eventually to be degraded (Choquet and Vallon, 2000). Thus, the biosynthesis of PsaC would not be affected by increases in the amount of \( \text{psaC} \) mRNA above what actually is required for translation (Hosler et al., 1989).

**Mac1 Is Phosphorylated to Different Extents Depending on Growth Conditions**

Protein phosphorylation is a prevalent posttranslational modification that plays an important role in the regulation of numerous processes in biology. Hundreds of phosphoproteins that are known or predicted to localize to the chloroplast have been identified in large-scale proteomic studies, both in Chlamydomonas and in vascular plants (Lohrig et al., 2009; Reiland et al., 2009; Wang et al., 2014). In plastids, protein phosphorylation plays regulatory roles in photosynthesis, gene expression, and metabolism (Baginsky and Gruissem, 2004). Thylakoid proteins such as LHCII and PSII are some of the most abundant chloroplast phosphoproteins. The phosphorylation of LHCII, which is largely dependent on the protein kinase Stt7 in Chlamydomonas and STN7 in plants, plays a role in a regulatory response to changing light quality and to metabolic demands of the cell known as state transition (Depege et al., 2003). Reversible phosphorylation regulates the dynamic allocation of LHCII to PSI or PSII. The phosphorylation of the PSII core subunits by the protein kinase STN8, a paralog of STN7, plays a role in the organization of the thylakoid membranes and in the repair cycle of photodamaged PSII in Arabidopsis (Bonardi et al., 2005; Tikkanen et al., 2008; Fristedt et al., 2009).

Focusing on the role of protein phosphorylation for gene expression in the chloroplast of vascular plants, there is evidence for regulation both at the level of transcription and at posttranscriptional steps. The chloroplast sensor kinase, which is related to bacterial two-component sensor kinases, is reported to play a role in the regulation of plastid transcription as a function of environmental conditions. This is illustrated in Figure 10, which shows the phosphorylation of Mac1 under different environmental conditions.

**Figure 10.** Mac1 Is Phosphorylated in Response to Environmental Conditions.

(A) Protein extracts of mac1;MAC1-HA (the mac1 mutant complemented with an HA-epitope tagged MAC1 gene) grown mixotrophically were analyzed by electrophoresis in polyacrylamide gels containing a gradient of Phos-tag. Prior to loading on the gels, the samples were incubated in the presence (+) or absence (−) of λ protein phosphatase as indicated at the top (lanes 1 and 2). For comparison, protein extracts of SBP-HA were analyzed after incubation with (+) or without (−) λ protein phosphatase (lanes 3 and 4).

(B) Top panel: Cultures of mac1;MAC1-HA were grown mixotrophically in the presence of different initial concentrations of Fe as described for Figure 8A. Protein extracts were analyzed by Phos-tag gel electrophoresis and immunoblotting with HA antibodies. The extent of phosphorylation (estimated as the intensity of the upper band divided by the sum of the intensities of both bands) is shown below each lane as a percentage (+/− SD, \( n = 3 \)). Lower panels: The same samples were analyzed by SDS-PAGE in normal gels and immunoblotting with the antisera indicated on the right.

(C) Three different amounts of total protein from cultures with 20 \( \mu \)M Fe were analyzed by Phos-tag gel electrophoresis and immunoblotting with HA antibodies, and phosphorylation was calculated as in (B). The estimated phosphorylation is approximately constant over the 4-fold range of total protein, indicating that in (B), the decreased phosphorylation of Mac1 observed with the 0.2 \( \mu \)M culture is not an artifact due to the lower amount of Mac1.

(D) Cultures of mac1;MAC1-HA, of stt7-7;MAC1-HA (the stt7-7 kinase mutant transformed with MAC1-HA), or of mac1;MAC1-AAA-HA (with serines 137 and 139 changed to alanines) were treated under conditions that favor oxidation of the plastoquinone pool (state 1, 10 \( \mu \)M DCMU in the light; lanes 1, 3, and 5) or its reduction (state 2, anaerobiosis in the dark; lanes 2, 4, and 6). Protein extracts were analyzed by Phos-tag gel electrophoresis and immunoblotting with HA antibodies (upper panel) or by SDS-PAGE in normal gels and immunoblotting with phospo-specific antiserum against phosphorylated Lhcb2 of Arabidopsis (At P-Lhcb2; lower panel). That the conditions were effective in promoting state 1 or state 2 is shown by the widely different levels of phosphorylation of LHCII. The exposure shown for stt7-7;MAC1-HA is longer than for mac1;MAC1-HA because of different levels of Mac1-HA expression in the two strains.
photosynthetic activity (Puthiyaveetil et al., 2008). The chloroplast casein kinase 2 (cpCK2), also known as Plastid Transcription Kinase (PTK) because of its association with RNA polymerase complexes, is involved in the regulated phosphorylation of sigma factors that play a role in promoter recognition (Schweer et al., 2010). At the posttranscriptional level, chloroplast ribonucleoproteins are phosphorylated by cpCK2, and in the case of 25RNP, phosphorylation can influence RNA binding in vitro (Kanekatsu et al., 1995; Lisitsky and Schuster, 1995). Phosphorylation by cpCK2/PTK also regulates the activity of the chloroplast endoribonuclease p54 in vitro (Liere and Link, 1997). The targets of cpPK2 are not limited to proteins involved in gene expression and, for example, also include the β-subunit of ATP-synthase in the thylakoid membrane (Kanekatsu et al., 1998). The fact that the kinase STN7 is itself a phosphoprotein and the presence in the plastid of numerous other protein kinases are suggestive of a complex regulatory phosphorylation network in the chloroplast of vascular plants (Baginsky and Gruissem, 2004; Reiland et al., 2011; Bayer et al., 2012).

Little is known about the phosphorylation of proteins involved in chloroplast gene expression in Chlamydomonas. Using electrophoresis in Phos-tag gradient gels and immunoblotting, we revealed that Mac1 is phosphorylated (Figure 10). Two phosphorylated forms of Mac1-HA were resolved with this gel system (P1 and P2), which could represent mono- and diphosphorylated Mac1-HA or two different forms with different degrees of multiple phosphorylation. A first indication that phosphorylation of Mac1 changes with the environmental conditions came with the observation that under conditions of iron deficiency, there was a decrease in the proportion of the phosphorylated form, concomitant with a reduction in the total amount of Mac1-HA. This raises the interesting but open question of whether phosphorylation has an influence on the proteolytic degradation of Mac1. A second indication came with Chlamydomonas cells that were incubated under anaerobiosis in the dark, a condition that induces state 2, with the reduction of plastoquinone and the consequent activation of Stt7 (Wollman and Delepelaire, 1984). In this condition, a strong phosphorylation of Mac1-HA was observed, with the appearance of the slower migrating form (P2). Conversely, in a condition that induces the oxidation of the plastoquinone pool and state 1, incubation in dim light in the presence of DCMU, phosphorylation of Mac1 was less extensive. However, in the stt7-7 mutant background, the strong phosphorylation with the presence of two phosphorylated forms of Mac1-HA was still observed in state 2 conditions. Hence, the kinase, or possibly the kinases, responsible for Mac1 phosphorylation remain elusive. A possible candidate could be Stl1, the paralog of Stt7 in Chlamydomonas, for which no mutant has yet been described. The physiological significance of Mac1 phosphorylation will need to be investigated in future experiments, in particular, to determine whether phosphorylation might affect its RNA binding activity or its proteolytic turnover.

Among the proteins involved in posttranscriptional steps of chloroplast gene expression, two general groups can be distinguished. One includes the proteins that bind a large set of chloroplast RNAs with little gene specificity, such as the cpRNP proteins that intervene at multiple steps of gene expression (Kupsch et al., 2012). The other consists of the proteins that bind to a restricted subset of plastid transcripts or to a single one and have more specific roles. As discussed above, it is known that some of the proteins of the former group are phosphorylated. Our current work establishes that a member of the latter group, namely, Mac1, is also subject to phosphorylation. This observation opens many new questions about the posttranscriptional regulation of chloroplast gene expression.

METHODS

Strains and Growth Conditions

Cells were grown in Tris acetate phosphate (TAP) or high salt minimal (HSM) media (Kropat et al., 2011) in the dark, under low light (6 μmol m−2 s−1) or normal light (60 μmol m−2 s−1) from fluorescent tubes. The original mutant isolate was backcrossed three times to a wild-type strain (137c), and a spore from the last cross was used in this work as mac1 (mating type minus).

For growth under iron limitation, the glassware was treated with sterile 10 mM EDTA for 15 min and rinsed three times with sterile MilliQ water before use. Precultures were grown for at least 10 doublings under normal light in TAP medium, collected by centrifugation, and washed three times in TAP without Fe. Cells were then diluted to 2 × 105 cells/mL, supplemented with 0.2, 1.0, or 20 μM FeCl3, and grown for 10 doublings with intermediate dilutions to maintain the concentration below 2 × 106 cells/mL. The state transitions were obtained with cells grown in TAP and normal light. The cells were shifted to HSM medium with three steps of centrifugation and washing, and incubated with shaking in the dark for 2 h. They were then collected by centrifugation and resuspended in HSM medium to a concentration of 2 × 107 cells/mL and split in two aliquots. State 1 was induced during 1 h and 30 min by gentle agitation in low light with 10 μM DCMU. State 2 was obtained by incubating 1 h and 30 min in the dark in a sealed 2-mL syringe without air bubbles on a rotating wheel.

Identification of MAC1

The mutant was generated by random insertional mutagenesis of an aphVIII cassette into the wild-type line jex4 and screened for aberrant chlorophyll fluorescence kinetics as described previously (Houille-Vernes et al., 2011). The mutation was identified by inverse PCR as follows. Total DNA from the mutant was extracted with the DNeasy plant mini kit (Qiagen), and 100 ng was digested with BstU1 for 5 h. The enzyme was inactivated by heat treatment and the DNA was purified through phenol-chloroform extraction followed by ethanol precipitation. The DNA from the pellet was ligated with T4 ligase overnight at 16°C in a volume of 70 μL, and 5 μL aliquots were used as templates for nested PCR. The first PCR was with primers IP2 and IP3 (Supplemental Table 1) in a final volume of 25 μL (45 s at 95°C, 45 s at 55°C, and 2 min at 72°C, 35 cycles). A 1-μL aliquot was then used for a second round with primers IP1 and IP4. Sequencing of the fragment and a BLAST search of the Chlamydomonas reinhardtii genome showed that the insertion mapped to exon 2 of Cre09.g388615 (g9646.t1) in version 11 of the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). This locus is included in the BAC clone 23A16 (Lefebvre and Silflow, 1999).

DNA Constructs

The plasmids MAC1_gen3 and MAC1-HA were obtained as illustrated in Supplemental Figure 8A. A 4-kb BspHI fragment containing the 3′ flank and most of the coding sequence was cloned in the TOPO vector pCR2.1 from a digest of BAC 23A16. This plasmid (Mac1-genomic-TOPO) was then extended with a PCR fragment (prepared with primers IF_EcorV-Sbf1-forward and IF_Rev; Supplemental Table 1) containing the beginning of the gene...
and the 5' flanking promoter region using Gibson assembly (Gibson et al., 2009). The final construct (MAC1-gen3) contains ~1 kb upstream of the predicted coding sequence and 1 kb downstream. To insert the triple HA (hemagglutinin) epitope tag at the C terminus of the coding sequence, a synthetic BsmI-BsaW1 fragment (obtained from Biomatik) was cloned into the corresponding sites of Mac1-genomic-TOPO. The beginning of the gene and the 5' flanking promoter region were then added as above. A transformant expressing the HA-tagged Mac1 at a level similar to the wild type (#6 in Figure 9) was selected and designated in this work as mac1; MAC1-HA. The MAC1-AA-HA mutant plasmid was derived from MAC1-HA by replacing the BsrEI-NruI fragment with two overlapping PCR fragments (obtained with oligonucleotides mut5 and mut5 R and S137A_S139A_F with mut-mac-rev, respectively, and MAC1-HA as template; Supplemental Table 1) using Gibson assembly.

The pscC::lucCP::rbcL reporter construct was obtained by replacing the acrV gene in the atpB-INT-psaA::acrV vector (Michelet et al., 2011) with the lucCP gene (Matsuo et al., 2006) using Ncol and Sphi. The pscA 5' flank (promoter and 5' UTR) was amplified by PCR with primers (psaC-prom3 & psaC-prom5; Supplemental Table 1), subcloned in the Topo vector pCR 2.1 and inserted as a XbaI-BspHI fragment in a XbaI-NcoI digest of the vector.

The transformation vector containing MAC1-HA used for transformation of stt-7 was obtained by inserting the aph 7' hygromycin resistance cassette (Berthold et al., 2002) as a PCR fragment (obtained with primers MacA_hyg_F and MacA_hyg_R; Supplemental Table 1) at the XbaI site of MAC1-gen3 using Gibson assembly. The final construct was transformed using a helium gun into the stt-7 mutant (Depege et al., 2003) with selection on 20 μg/mL hygromycin (Calbiochem). A transformant expressing Mac1-HA was identified by immunoblotting.

**Transformation**

Nuclear transformation by electroporation was modified from Shimogawara et al. (1998). A volume of 300 μL of cells suspended at 10^9 cell/mL in either HSM + 40 mM sucrose or TAP + 40 mM sucrose (for selection for photoautotrophy or for antibiotic resistance, respectively) were incubated with 2 μg DNA at 16°C for 20 min, and then 250 μL of the mix was transferred to a 4-mm-gap electroporation cuvette and pulsed at 750 V (C = 50 μF).

After 2 min incubation at room temperature, the cuvettes were transferred to 16°C for 20 min. The cell suspension was plated with 1 mL of HSM containing 25% starch (for photoautotrophy) or transferred to 50 mL TAP (without sucrose) overnight in low light and collected by centrifugation prior to plating with TAP starch (for antibiotic resistance).

Chloroplast transformation was described previously (Vaistij et al., 2000b).

**Mac1 Antiserum**

The Mac1 antiserum was raised in a rabbit with the C-terminal region of Mac1 prepared as follows. The MAC1 cDNA was amplified by PCR from a cDNA library (with the primers mac1-half-for and rev-xho; Supplemental Table 1), cloned in TOPO vector pCR2.1, and transferred to pET28a (Novagen) (Supplemental Figure 8B). This vector (pET28Mac1ceter) was used to transform Escherichia coli BL21 cells and the protein (tagged with six histidines encoded by the vector) was purified using Ni-NTA affinity chromatography and imidazole elution, followed by gel filtration on Sephadex S200. The rabbit serum was used at 1/5000 dilution for immunoblots.

**Immunoblotting**

Cell pellets were resuspended in lysis buffer containing 50 mM Tris, pH 6.8, 5% SDS, 10 mM EDTA, and 1× Protease inhibitor cocktail (Sigma-Aldrich). Samples (25 μg each) were supplemented with 0.2 volumes of sample buffer (10% SDS [w/v], 250 mM Tris, pH 6.8, 50% glycerol [v/v], 500 mM DTT, and bromophenol blue) and heated at 55°C for 15 min. Proteins were separated by SDS-PAGE on 15 or 7% acrylamide gels and transferred to nitrocellulose membranes. The total protein on the membrane was visualized by amido black staining, and the membrane was blocked in Tris-buffered saline Tween (TBST; 20 mM Tris, pH 7.5, 150 mM NaCl, and 1% [v/v] Tween 20) supplemented with 5% (w/v) nonfat milk for 1 h. The membrane was incubated with primary antibody in TBST and 1% milk. The primary antiserum (and their sources) were as follows: monoclonal anti-HA (Covance; MMS-101R), antiphospho-Lhc2 (Agrisera; AS13-2705), anti-Rpl4 (gift of W. Zerges), anti-PsaA (Redding et al., 1998), anti-Cyt (gift of F.-A. Wollman), anti-PsaC, anti-D1 (gifts of J.-D. Rochais), and anti-Rps12 (Ramundo et al., 2013). The membranes were washed three times for 10 min and then incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (Promega). After three washes, the detection was performed by enhanced chemiluminescence and imaging with the GE LAS4000 system (General Electric).

**RNA Hybridization**

Cells were grown in the indicated conditions until they reached 2 × 10^8 cells/mL. They were then centrifuged 2000 g for 5 min, aliquoted as pellets of 2 × 10^7 cells, frozen in liquid nitrogen, and kept at ~80°C until their use. RNA from the frozen pellet was extracted using the RNaseasy Kit (Qiagen) and analyzed by 1.2% agarose MOPS formaldehyde gel electrophoresis and capillary transfer to nylon membranes Hybond N+ (Amersham) (Sambrook et al., 1989). The hybridization was done in Church and Gilbert hybridization buffer with 32P-labeled probes as described (Rio et al., 2011). The probes were obtained by PCR with the oligonucleotides listed in Supplemental Table 1 and total genomic DNA as template, except for the psaA exon7 probe, which was a 280-bp HindIII fragment.

Probes were stripped in 0.1% SDS, 1 mM phosphate buffer, pH 7, and 1 mM EDTA at 98°C for 5 min, and the membranes were checked for residual signal by phosphor imaging. The RNA gel blots presented in Figures 1, 2, 8, and 9 and the run-on transcription assay in Figure 4 were all obtained using one membrane for each that was repeatedly stripped, checked for absence of residual signal, and reprobed.

**Immunofluorescence**

The protocol for immunofluorescence was described previously (Lefebvre-Legendre et al., 2015). The HA signal was revealed with goat anti-mouse antibodies coupled to Alexafluor 647 (excitation, 647 nm; emission, 660 to 750 nm). The other antibodies were revealed with goat anti-rabbit antibodies coupled to Alexafluor 546 (excitation, 546 nm; emission, 560 to 600 nm).

**Cell Fractionation and Sucrose Gradient Sedimentation Analysis**

Cell fractionation was described previously (Lefebvre-Legendre et al., 2015), using the protocol of Percoll gradient chloroplast isolation from Rivier et al. (2001).

For sucrose gradient sedimentation analysis, cells from a 250-mL culture of mac1; MAC1-HA (2.10^6 cells mL^-1) were collected and resuspended in 1.1 mL HKM buffer (20 mM HEPES, pH 7.2, 50 mM KCl, and 10 mM MgCl_2) supplemented with protease inhibitor cocktail (Roche EDTA-free tabs). The sample was frozen as 100 μL drops in liquid nitrogen and ground in a 50-mL compartment of a MM400 bead-beater (Rettsch) for 2 min at 30 cycles s^-1. The powder was then collected, melted on ice, and centrifuged for 20 min at 15,000g. Aliquots of the supernatant (800 μL) were mixed with 100 μL RNase buffer (100 mM Tris, pH 7.5, and 10 mM sodium acetate) with or without (mock treatment) 10 μg mL^-1 RNaseA and incubated for 10 min at 22°C. The samples were loaded on 10-mL sucrose gradients (HKM buffer, 5 to 45% grad.
sucrose) as described previously (Lefebvre-Legendre et al., 2015). Twenty fractions (500 µL) were collected from the bottom. Aliquots (150 µL) were precipitated (Wessel and Flugge, 1984) by sequential addition with thorough mixing of 600 µL methanol, 150 µL chloroform, and 450 µL water, followed by centrifugation for 5 min at 14,000g. The clear upper aequorin layer was discarded and the bottom phase with the white protein interface was re-suspended with 1 mL 50% (v/v) methanol, vortexed, and centrifuged again. The bottom phase with the protein interface was re-suspended with 650 µL methanol, inverted three times, and centrifuged for 5 min. The pellets were dried and re-suspended in 100 µL buffer (100 mM Tris-HCl, pH 7.5, 3% SDS [w/v], 10% glycerol [v/v], and 80 mM DTT), treated 10 min at 55°C, and finally analyzed by SDS-PAGE (7% acrylamide) and immunoblotting with anti-HA monoclonal antibody (Covance) or anti-Rubisco (gift of Jean-David Rochaix, University of Geneva).

**Run-on Transcription Assay**

The protocol was modified from Klinkert et al. (2005). A total of 10⁶ cells in early exponential phase were harvested by centrifugation and washed with resuspension buffer (10 mM HEPES, pH 7.5, 150 mM KCl, 250 mM sucrose, 1 mM EDTA, and 0.1 mM PMSF). The pellet was adjusted to 100 µL with the same buffer and frozen in liquid nitrogen. Then, 40 µL 4× run-on buffer (100 mM HEPES, pH 7.5, 1 M sucrose, 120 mM MgCl₂, 30 mM DTT, and 100 mM NaF) was mixed with 5 µL each of 10 mM rATP, rGTP, and rCTP, 15 µL RNasin (Promega), 20 µL [32P]rUTP (200 µCi, 5 µL final), and 80 µL of cell pellet (defrosted in a water bath at 20°C). The reaction mixture was incubated at 26°C for 5 or 15 min. Total RNA was immediately extracted with TRI Reagent (Sigma-Aldrich), precipitated with isopropanol, and re-suspended in 1 mL 1× TE (10 mM Tris and 1 mM EDTA, pH 8) prior to separation on a Sephadex G50 column. The RNA fractions were then used for hybridization. The membrane was prepared by spotting DNA probes prepared by PCR (see above) onto a Hybond N+ nylon membrane (Amersham). The membrane was then dried and cross-linked using the automatic mode of a UV cross-linker (Stratalinker). Hybridization of the labeled RNA probes to the membrane was performed as described above for RNA gel blots.

**sRNA**

Published Chlamydomonas sRNA sequencing data (Ibrahim et al., 2010) were mapped to the chloroplast genome (NC_005353) as described (Loizeau et al., 2014). Read coverage within the 5′ UTR and the 5′ end of the psaC coding sequence were visualized using the Integrated Genomics Viewer (Figure 5A). For experimental verification of identified sRNAs, total RNA from dark-grown cells was extracted with TRI Reagent (Loizeau et al., 2014). Read coverage within the 5′ UTR and the 5′ end of the psaC coding sequence were visualized using the Integrated Genomics Viewer (Figure 5A). For experimental verification of identified sRNAs, total RNA from dark-grown cells was extracted with TRI Reagent (Loizeau et al., 2014). Enriched sRNAs (10 µg) were separated by denaturing PAGE, blotted, and hybridized to an end-labeled DNA oligonucleotide named psaC4as (Supplemental Table 1) situated antisense to the putative sRNA within the 5′ UTR of psaC.

**Luciferase Assay**

The different strains were grown in TAP medium under normal light to 2 × 10⁶ cells/mL. For transformations of psaB:LucCP, 50-µL aliquots of each culture were plated in the wells of a white-walled microtiter plate sitting on dry ice. Cultures of psaC:LucCP were concentrated 10-fold by centrifugation before freezing. Frozen plates were kept at −20°C prior to the measurement. For the assay, 100 µL luciferase reagent (50 mM potassium phosphate buffer, pH 7, 150 mM NaCl, 1× Protease inhibitor cocktail [Sigma-Aldrich], 2.5 mM ATP, 2.5 mM MgCl₂, and 4 mM Luciferin [Promega]) was added to the frozen sample and the luminescence emission kinetics monitored at 28°C for 30 min using a Synergy 2 plate reader (Biotek). The maximum luminescence was used for the quantification.

**Mac1 Purification and RNA Binding Assays**

The sequence encoding MAC1 lacking the transit peptide was amplified by PCR with the oligonucleotides cdna_pet_inf_for and cdna_pet_inf_rev (Supplemental Table 1). The PCR fragment (2695 bp) was cloned between the Ncol and XhoI sites of pET28a using Gibson assembly. The construct was freshly transformed into E. coli BL21 (DE3) cells for each purification. The bacteria were cultivated in 2 liters Luria-Bertani medium containing 50 µg mL⁻¹ kanamycin until the culture reached an absorbance of 0.25 at 600 nm. Cultures were transferred to ice for 15 min, and IPTG was added to 100 µM and ethanol to 1% (v/v). After 15 more minutes, the culture was transferred to an incubator at 18°C and agitated for 20 h. Cells were harvested by centrifugation and the pelleted was used immediately. The cells were resuspended on ice in 25 mL lysis buffer (50 mM HEPES, pH 7.7, 750 mM NaCl, 5 mM MgCl₂, and 4 mM DTT) containing proteinase inhibitors (Roche EDTA-free tabs), and lysed by three passages in the EmulsiFlex C-3 at 20,000 p.s.i. The resulting extract was centrifuged 30 min at 12,000g and the supernatant was loaded on a 1-mL Ni-NTA column, washed with lysis buffer supplemented with 30 mM imidazole, and eluted with 5 mL of 25 mM HEPES, pH 7.7, 750 mM NaCl, 5 mM MgCl₂, 4 mM DTT, and 300 mM imidazole. The extract was then directly injected onto the size exclusion column (HiLoad Sephadex 200 16/60, AKTA system; GE Healthcare) and eluted in column buffer (50 mM Tris, pH 7.7, 250 mM NaCl, 4 mM DTT, and 10% [v/v] glycerol) at 0.6 mL min⁻¹.

A vector for the preparation of the RNA probe was obtained by cloning the following sequence in the TOPO pCR2.1 (Invitrogen) vector: TAA-TACGACTCAAGGAGAAGTCGATTCTCAATCTTCTTTTGATAGTGAGATGACATATTTAGCACAATCGATATG. The T7 promoter is underlined, the 5′ part of the psaC UTR is highlighted in bold, and a Clal site is shown in italics. The vector was digested with Apal (in the vector) and Clal (shown above in italics), leading to a fragment of 139 bp, which was purified by agarose gel electrophoresis and transcribed in vitro with T7 RNA polymerase (Promega) for 2 h at 30°C in a 20-µL reaction mixture containing: 4 µL transcription buffer (Promega), 1 µL 1 mM rUTP, 1 µL each 10 mM stocks of the three other nucleotide triphosphates, 3 µL [32P]rUTP at 10 mCi mL⁻¹ and 3000 Ci mmol⁻¹, 1 µL RNasin (Promega), 1 µL 20 mM DTT, 1 µL T7 polymerase, and 200 ng probe. This produced a 62-nucleotide RNA containing the first 51 nucleotides of the psaC5 UTR. After treatment with RNase (Promega) for 30 min, the labeled RNA was separated from the free nucleotides on a size-exclusion column (Sephadex G25 fine).

The binding conditions for electrophoretic mobility shift assay were previously described (Williams-Carrier et al., 2008). The buffer used was the column buffer supplemented with 0.04 mg/mL BSA (Applichem), 0.5 mg/mL Heparin (Sigma-Aldrich), and 4000 cpmp radiolabeled RNA probe (40 PM) in a final volume of 20 μL. The Kds were determined as the Mac1 concentration at which half of the probe was bound by the protein, which is a valid approximation when the protein is in large excess over the RNA in the assay (30 to 100 nM versus 40 PM).

**Phos-Tag Gel Electrophoresis**

Chlamydomonas culture (10 mL) was added to 40 mL cold acetone and precipitated overnight at −20°C. After centrifugation at 3000g for 20 min, the pellet was re-suspended in 200 µL sample buffer (50 mM HEPES, pH 7.8, 200 mM NaCl, 1× complete protease inhibitor [Roche EDTA-free], and 0.5% Triton X-100). The suspension was transferred to a 1.5-mL microtube. 500 μL acid-washed glass beads (0.4 to 0.6 mm) were added, and the samples were homogenized two times for 15 s in a Silamet shaker. The mixture was then centrifuged at 20,000g for 20 min, and the supernatant was transferred to a new tube. For dephosphorylation, an aliquot containing 25 µg protein was brought to 50 μL in lambda protein phosphatase reaction mix following the instructions of the manufacturer (New England Biolabs) and treated for 1 h at 30°C.
Phylogenetic Analysis

The phylogenetic tree was constructed with the MEGA6 software (Tamura et al., 2013), using ClustalW (with default parameters) to derive the protein sequence alignment, which was manually curated to remove divergent N-terminal sequences (Supplemental Data Set 1). The phylogenetic reconstruction used the maximum likelihood method (with default parameters) and 1000 bootstrap replicates.

Accession Numbers

Sequence data from this article can be found in public databases under the following accession numbers: MAC1, Cre09.g389615 (g9646.11) (Phytozone v11); Mbb1, XP_001686751.1 (GenBank); HCF107, NP_188329 (GenBank).

Supplemental Data

Supplemental Figure 1. Identification of the MAC1 gene.
Supplemental Figure 2. Complementation of the mac1 mutant.
Supplemental Figure 3. Phylogenetic analysis of Mac1.
Supplemental Figure 4. Localization of Mac1-HA by immunofluorescence confocal microscopy.
Supplemental Figure 5. Small RNA footprints in the 5′ UTR of psaC.
Supplemental Figure 6. Quantification of Psac, Mac1, and psaC mRNA under iron limitation.
Supplemental Figure 7. Phos-tag gel electrophoresis.
Supplemental Figure 8. Construction of DNA clones and vectors.
Supplemental Table 1. Oligonucleotides used in this work.
Supplemental Data Set 1. Text file of the alignment used for the phylogenetic analysis in Supplemental Figure 3.

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AUTHOR CONTRIBUTIONS


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Functional


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