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Manipulating RuBisCO accumulation in the green alga, *Chlamydomonas reinhardtii*

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Abstract The nuclear factor, Maturation/stability of *RbcL* (MRL1), regulates the accumulation of the chloroplast *rbcL* gene transcript in *Chlamydomonas reinhardtii* by stabilising the mRNA via its 5′ UTR. An absence of MRL1 in algal *mrl1* mutants leads to a complete absence of RuBisCO large subunit protein and thus a lack of accumulation of the RuBisCO holoenzyme. By complementing *mrl1* mutants by random transformation of the nuclear genome with the *MRL1* cDNA, different levels of *rbcL* transcript accumulate. We also observe that RuBisCO Large Subunit accumulation is perturbed. Complemented strains accumulating as little as 15% RuBisCO protein can grow phototrophically while RuBisCO in this range is limiting for phototrophic growth. We also observe that photosynthetic activity, here measured by the quantum yield of PSII, appears to be a determinant for phototrophic growth. In some strains that accumulate less RuBisCO, a strong production of reactive oxygen species is detected. In the absence of RuBisCO, oxygen possibly acts as the PSI terminal electron acceptor. These results show that random transformation of *MRL1* into *mrl1* mutants can change RuBisCO accumulation allowing a range of phototrophic growth phenotypes. Furthermore, this technique allows for the isolation of strains with low RuBisCO, within the range of acceptable photosynthetic growth and reasonably low ROS production. MRL1 is thus a potential tool for applications to divert electrons away from photosynthetic carbon metabolism towards alternative pathways.

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Abbreviations

MRL1	Maturation/stability of *rbcL* 1
UTR	Untranslated region
ROS	Reactive oxygen species
TAP	Tris-acetate-phosphate medium
MIN	Minimal medium
LSU	RuBisCO Large Subunit
Φ_{PSII}	Quantum yield of PSII

Introduction

The single chloroplast of Chlamydomonas cells contains some hundred copies of its genome, thus, regulation of the plastid transcriptome and proteome is heavily reliant on a number of post-transcriptional and post-translational strategies to integrate these elements. The discovery of nuclear encoded Maturation and Translation Factors (M and T factors) shed light on how the chloroplast orchestrates the biogenesis of its complexes but also on how these nuclear encoded factors potentially regulate chloroplast function (Barkan and Goldschmidt-Clermont 2000). Gene families with organellar targeting, containing identifiable repeated motifs have been identified in the nuclear genome of every eukaryotic species. These nuclear factors regulate post-transcriptional events in organelles by stabilising, maturing or editing mRNA and by regulating translation (Marín-Navarro et al. 2007; Stern et al. 2010; Wobbe et al. 2008).

The MRL1 protein (Johnson et al. 2010) is one of the rare representatives of the Pentatricopeptide Repeat (PPR) motif family which has conserved both sequence identity and at least partial function between green algae (*Chlamydomonas*

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reinhardtii) and higher plants (*Arabidopsis thaliana*). In Chlamydomonas, absence of MRL1 results in an absence of the chloroplast *rbcL* transcript while in Arabidopsis only the processed form of the *rbcL* transcript is absent. The MRL1 protein contains two conserved and essential domains, one of these domains is composed of 12 predicted PPR repeats in green algae and land plants, and the other, "C-domain" , is similarly composed of helix-coil-helix repeats and is found exclusively in MRL1 proteins. The conclusion is that the organisation of the MRL1 protein and its target specificity were fixed early in the evolution of Viridiplantae. The protein forms part of a high molecular mass complex in chloroplast stromal fractions. The complex contains an RNA, most probably the *rbcL* mRNA, because a mutant lacking the *RbcL* gene (Δ*rbcL*) presents a complex of lower molecular mass.

Eleven PPR proteins have been identified in Chlamydomonas, of which MCA1 the nuclear regulator of *petA* mRNA stability is well studied (Loiselay et al. 2008). In conjunction with TCA1, a T factor for translation of cytochrome *f* (cyt *f*), MCA1 regulates the accumulation of cyt *f* in response to environmental cues (Raynaud et al. 2007). MCA1 is a short-lived protein, rate-limiting for the accumulation of *petA*. Here the same line of reasoning is pursued; that a nuclear regulator protein required to stabilise an mRNA transcript could be required in stochiometric quantities for the stabilisation of its target. Thus, by producing less (or more) of the nuclear regulator, the cell could stabilise less (or more) of the target transcript. Here, as in Raynaud et al., we rely on the random insertion of the *MRL1* gene into the nuclear genome of *mrl1* mutants and the so-called positional effects of such random insertions to have an effect on *rbcL* accumulation.

RuBisCO is a hexadecamer composed of eight 55 kDa large subunits (LSU, encoded by the chloroplast gene *RbcL*) and eight 12 kDa small subunits (SS, encoded by a family of RBCS genes in the nucleus), which catalyzes competing carboxylation and oxygenation reactions. Because of these competing reactions, its very slow turnover rate and possible photoprotection activity, it is generally accepted that chloroplasts must accumulate large quantities of RuBisCO [for a recent review covering these aspects in Chlamydomonas see Wostrikoff and Stern (2009)]. In Chlamydomonas, strains lacking RuBisCO can achieve a growth rate similar to wild type cells when supplied with acetate under low light conditions while they are non-photosynthetic and show light-sensitivity when grown under mixotrophic conditions. This photosensitivity was observed to be due to the Mehler reaction occurring at the acceptor side of PSI, and we hypothesised that photosensitivity was caused by H_2O_2 production. The Mehler reaction provides a benefit to the cells in terms of survival capacity, as shown by enhanced cell death when cells are illuminated in anaerobic conditions (Johnson et al. 2010). In particular, it may permit electron flow even in the absence of a functional Calvin cycle, allowing generation of light-induced ΔpH as required for photoprotection purposes (Niyogi 1999).

Here we report on newly isolated alleles for *mrl1*. Using these mutant strains we show that randomly transforming MRL1 into the nuclear genome of *mrl1* mutants affects the accumulation of *rbcL*, leading to changed levels of LSU protein. To our knowledge it is the first report of a mechanism whereby levels of a wild type RuBisCO enzyme and thus carbon fixation can be manipulated to achieve different levels by a partner interaction indispensable for RuBisCO biogenesis. This makes MRL1 a potential tool to modulate carbon metabolism.

Materials and methods

Media and growth conditions

Wild type, mutant and transformed strains of *Chlamydomonas* reinhardtii were grown in 200 ml TAP medium, pH 7.2 (Harris 1989) with shaking under continuous low light (5–10 μE m^{-2} s^{-1}). Growth tests were performed on solid TAP or Minimal medium by spotting 25 μl of cultures resuspended in H_2O (to approx. 5×10^5 cells/ml) or streaking a single layer of cells directly onto agarose plates containing Minimal media. Low light for growth tests was at 5–10 μE m^{-2} s^{-1} and high light at 150 μE m^{-2} s^{-1}.

Generation of strains

For the generation of *wcf3* (*mrl1-1*) and *wcf2* (*mrl1-3*) refer to (Johnson et al. 2007). For the strains 76.5EN and Δ*rbcL* refer to (Hong and Spreitzer 1994; Johnson et al. 2010). Generation of recipient strain (Jex4 *mt*−) and T222 *mt*+ for the mutant collection and for crosses was derived from strain 137C. This recipient strain is also the wild type used for all experiments shown in this paper. Mutants, *mrl1-4* and *mrl1-5,* were generated by electroporation of Jex4 with *Sca*I/*Kpn*I digested cassette of the vector pBC1 (100 ng/5×10^7 cells) containing the *aphVIII* resistance gene and selection on 10 μg/ml paromomycin on TAP medium at low light. Clones were then screened by fluorescence digital video imaging for those with kinetics dissimilar to the wild types. Genetic crosses were performed according to methods in (Harris 1989). The complemented strains *mrl1-4* C and *mrl1-5* C were produced by electroporation with proMRL1 cDNA construct and selection for phototrophy at 40 μE m^{-2} s^{-1}. Negative controls were used to validate complementation was not the result of a reversion event.

DNA and RNA analyses

The proMRL1cDNA construct and transformation protocol was previously reported in Johnson et al. (2010). RNA extraction was performed using the hot-phenol method on cell cultures and gel blot analyses were carried out as described in (Drapier et al. 1998). Probes were a 250 bp PCR fragment amplified from the *RbcL* chloroplast gene, a 240 bp fragment of the *PsbA* chloroplast gene and 1,500 bp PCR fragment of the *PsbB* chloroplast gene. mRNA was quantified in each lane from a PhosphorImager scan of the ^{33}P labelling by the program Image Quant (Molecular Dynamics). *RbcL* transcript was normalized against the constitutive control, either *PsbA* or *PsbB,* and then expressed as the percentage of total wild type *rbcL* transcript.

Protein isolation and analyses

Cell cultures were centrifuged, washed in 20 mM HEPES buffer, pH 7 and suspended in 0.1 M DTT, 0.1 M Na_2CO_3 buffer with protease inhibitors (EDTA-free cocktail, Roche), before boiling in 2% SDS and 12% Sucrose. Loading was based on equal chlorophyll and the equivalent of 10 µg (Fig. 3b) or 5 µg (Fig. 4b) chlorophyll was loaded and analyzed on 12% polyacrylamide SDS gels in the presence of 8 M urea and 8% polyacrylamide SDS gels. Immunoblotting was performed according to (Kuras and Wollman 1994). Protein quantification data shown in Figs. 3a and 5 was the average of two independent experiments showing the same trends. In the first experiment, blots were incubated with the RuBisCO LSU antibody (Agrisera) at 1/5,000 dilution and OEE2 antibody (to verify that charge was equal (de Vitry et al. 1989)) at 1/1,000 for 1 h in PBS-tween-milk. To immuno-detect the blot with the bound primary antibody, incubation with I^{125}-labelled protein-A was carried out for 1 h in PBS-tween-milk. The Typhoon phosphoimaging system (GE Healthcare Sciences) was used to reveal immunoreactive proteins and ImageQuant software to quantify LSU against wild type levels. In the second experiment, blots were reacted with RuBisCO LSU antibody (Agrisera) at 1/25,000 dilution and OEE2 antibody at 1/10,000 for 1 h in PBS-tween-milk followed by Rabbit-specific secondary antibody and ECL plus (GE Healthcare) to reveal the immunoreactive proteins. BioRAD chemidoc was used to determine non-saturating levels of signal and BioRAD chemidoc software to quantify LSU accumulation against the wild type.

Fluorescence screen and ROS detection

A new setup for chlorophyll fluorescence imaging (Johnson et al. 2009) has been used for the screening of mutant algal colonies. This setup allows the automatic detection of slight changes in the fluorescence kinetics in response to a continuous illumination. In addition, saturating pulses are used to probe F_M during the period of illumination and allow the quantification of the photosynthetic activity, here measured by the quantum efficiency of PSII ($\Phi_{PSII} = (F_M - F)/F_M$). By changing the wavelength of the light source (450 nm light emitting diode Luxeon LXK2-PR14-Q00 filtered through 1 mm thick Schott colored glass filter BG3) and the filter used to select the fluorescence (dielectric bandpass Semrock FF01.542/50–25), the setup has been adapted to the detection of ROS (hydrogen peroxide and peroxyl radical) using 2′,7′-dichlorofluorescein, a fluorescent dye peaking at around 530 nm formed from the cellular hydrolysis and oxidation of the colourless and non fluorescent 2′,7′-DichloroDiHydroFluorescein Diacetate (H_2DCFDA, purchased from Interchim). For the ROS production test, cells were initially grown in TAP to 2×10^6 cells/ml (mid-exponential phase), centrifuged and resuspended in water to a concentration of 5×10^6 cells/ml, the H_2DCFDA was added to a final concentration of 5 µM and the cells were incubated in darkness for 1 h. The cells were then subjected to very high light (1,500 µE m^{-2} s^{-1}) with constant shaking and air cooling. Samples were taken every 15 min by aliquoting 200 µl into a 96-well plate and ROS estimated using the adapted imaging set up.

Results

MRL1 deficient mutants accumulate different levels of *rbcL* transcript but are always non-phototrophic

Table 1 summarizes the different alleles of *mrl1* isolated so far. The previously characterised first allele, *mrl1-1*, was reported before along with the tentatively designated *mrl1-2*, a mutant that had been previously named 76.5EN for which the mutation was not identified (Hong and Spreitzer 1994). Genetic crosses with *mrl1-1* showed it to be allelic or closely linked to *mrl1-2* and it could be complemented to phototrophy by the *MRL1* gene. We find that the *mrl1-2* mutation allows for 10% *rbcL* transcript when compared to the wild type (Fig. 1). The mutant *mrl1-3* was isolated in the same screen as *mrl1-1*. Along with *mrl1-4* it is interrupted in the 5′ region of MRL1. The *mrl1-5* mutant contains an untagged mutation and accumulates no detectable *rbcL* mRNA.

The mutants *mrl1-4* and *mrl1-5* have recently been isolated in a new mutant screen based upon the detection of colonies showing unusual fluorescence profiles using a digital imaging fluorescence camera (Fig. 2). From approximately 12,000 transformants screened, two were missing RuBisCO, both of which were identified as *mrl1*.

Table 1 The different *mrl1* alleles isolated to date with details of the type of screen used to isolate the mutant, linkage analysis and characterisation of insertion of the antibiotic resistance gene and an estimation of *rbcL* transcript accumulating in each of these alleles

Allele	Name	Source	Screen	Mutation	*rbcL* transcript (%WT)
mrll.1	wcf3	Johnson et al. 2007	Phototrophy	Linked to *aphVIII* insert in 5′ region	0
mrll.2	76.5EN	Hong and Spreitzer 1994	Phototrophy	Unknown	10%
mrll.3	wcf2	Johnson et al. 2007	Phototrophy	Linked to *aphVIII* insert in 5′ region	0
mrll.4	L11A	This work	Fluorescence	Linked to *aphVIII* insert in 5′ region	~2%
mrll.5	L54B	This work	Fluorescence	Non-linked to *aphVIII* insert	0

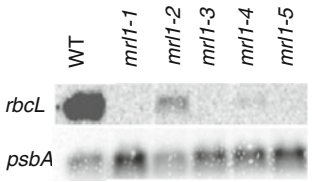

Fig. 1 The different alleles of *mrl1* accumulate very low or no *rbc*L transcript. Cells were grown in liquid culture in TAP at low light and total cell RNA was hybridised with *rbcL* and *psbA* as a control probe

As reported previously for *mrl1-1* (Johnson et al. 2010), while the initial (5 s of light) fluorescence kinetics of the two mutants resembled a wild type (hence the name *wcf* for wild type chlorophyll fluorescence), see left Fig. 2a for an example of a RuBisCO knock out, over a 5 min period of illumination at 130 µE m^{-2} s^{-1}, the stationary fluorescence (F) increases towards the maximum fluorescence (F_M), here probed with a saturating pulse every minute (Fig. 2b). This leads to a decreased quantum efficiency of PSII ($\Phi_{PSII} = (F_M - F)/F_M$) when measured 4 min after illumination. Mutants showing this fluorescence profile were then analysed by a western blot to identify those lacking LSU protein. Linkage analysis was undertaken to identify if *MRL1* was tagged by the *aphVIII*, paromomycin resistance cassette. In the case of *mrl1-4*, the 72 progeny tested showed a clear linkage between resistance to paromomycin, non-phototrophy and fluorescence profile. For *mrl1-5*, the analysis of 72 progeny showed non-linkage between antibiotic resistance and fluorescence profile although the latter correlated with non-phototrophic growth. The two new alleles were then complemented to phototrophy with the *MRL1* gene.

Complemented *mrl1-4* and *mrl1-5* show different levels of accumulation of *rbcL* transcript

The two mutants *mrl1-4* and *mrl1-5* were transformed with the construct proMRL1cDNA (a chimeric fusion of genomic sequence from 400 bp upstream of the proposed *MRL1*

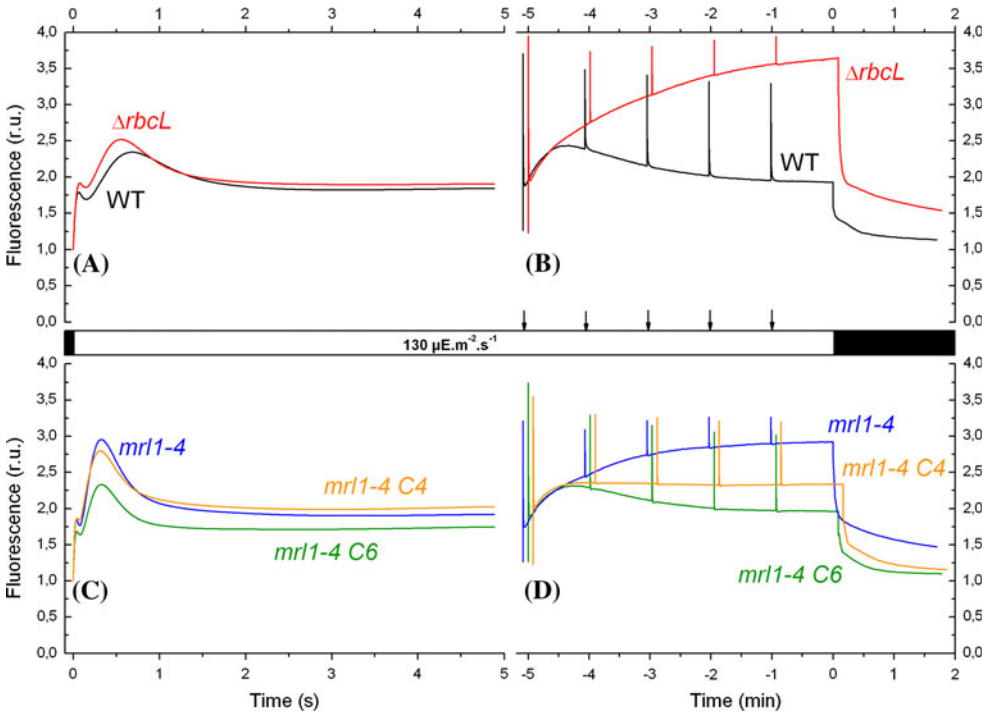

Fig. 2 Fluorescence kinetics of wild type, *mrl1-4*, *mrl1-4* C4, C6 and Δ*rbcL* cells grown on TAP and dark adapted. **a** and **c** show fluorescence induction response during a 5 s illumination (130 µE m^{-2} s^{-1}), followed by **b** and **d**, a 5 min illumination at the same light intensity and probed by a saturating pulse every minute to determine F_m. Curves are normalised on their F_0. Fluorescence units are arbitrary units

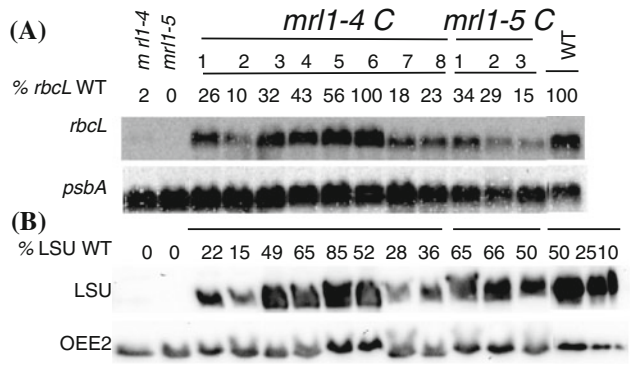

Fig. 3 Complemented *mrl1-4* and *mrl1-5* C strains show different levels of **a** *rbcL* mRNA accumulation and **b** LSU protein accumulation in comparison to the wild type. For RNA extractions, cells were grown in liquid culture in TAP at low light and total cell RNA was hybridised with *rbcL* and *psbA*/*psbB* as a control probe. For the protein blot, total cell extracts from the same cultures were reacted with the anti-LSU and anti-OEE2 antibodies

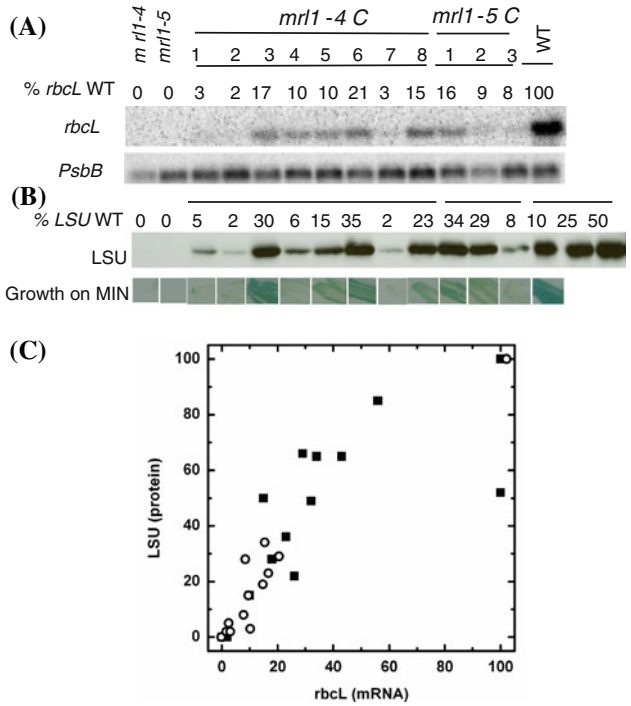

Fig. 4 Storing complemented *mrl1-4* and *mrl1-5* C strains on non-selective media results in reduced levels of **a** *rbcL* mRNA accumulation and **b** LSU protein accumulation and growth on Minimal media at 40 μE m^{-2} s^{-1} in comparison to the wild type. Cell cultures for RNA and protein were treated as for Fig. 3. **c** The relationship between accumulation of *rbcL* mRNA versus LSU protein for freshly complemented strains (*squares*) and stored strains (*circles*)

transcription start site and through to the first intron, fused to the cDNA) and clones were selected for phototrophy by plating on Minimal medium and exposing to light at 40 μE m^{-2} s^{-1}. Accumulation of *rbcL* transcript was tested by northern hybridisation for all complemented strains. Compared to the wild type strain, we observed a varying amount of *rbcL* transcript from 10 to 100% when normalised against the *psbA* loading control (Fig. 3a).

Transformed and complemented strains were stored at 18°C at <5 μE m^{-2} s^{-1} and on non-selective media (i.e acetate-containing media) for 6 months, where they were re-cultured every 1–2 months. These strains were then retested for growth and accumulation of *rbcL* mRNA transcript (Fig. 4). While the complemented strains still showed variations in their accumulation of *rbcL* transcript with the same trends as before, the strains now showed less accumulation of *rbcL*, from 2 to 21% WT levels (Fig. 4a). In line with this reduction in *rbcL*, now, 5 of the 11 strains, *mrl1.4* C1, C2, C4, C7 and *mrl1.5* C3 had very compromised photosynthetic growth when compared to WT growth under the same conditions (Fig. 4b).

Complemented strains accumulate different amounts of RuBisCO LSU leading to different growth phenotypes and photosynthetic capacities

Protein accumulation in the complemented strains showed that LSU levels varied from 15 to 85% of wild type levels (Fig. 3b and red bars in Fig. 4) and after 6 months of storage on non-selective media, 2–35% (Fig. 4b). Figure 4b shows that Chlamydomonas has severely compromised phototrophic growth with a RuBisCO level less than 15% that of the wild type strain. A graph comparing *rbcL* mRNA accumulation versus LSU protein accumulation is shown in Fig. 4c. This graph describes a roughly linear relationship between mRNA and protein (squares), except for the outlier *mrl1.4* C6. The relationship stays the same when less *rbcL* is accumulated as seen for the strains stored on non-selective media (circles).

The initial complemented strains were tested for phototrophic growth in high light conditions (growth after 1 week is shown in the green squares, Fig. 5) and these strains fell roughly into three categories (a, b and c in Fig. 5): those accumulating more than ~50% RuBisCO (A) had close to wild type growth, those that accumulated less than ~30% RuBisCO (C) had low levels of phototrophic growth, and those accumulating intermediate amounts of RuBisCO (B) had growth that ranged from affected to wild-type like (Fig. 5). This led us to conclude that photosynthesis was affected in these transformants due to a differential accumulation of the LSU protein. In order to further understand the effects of differing amounts of RuBisCO on cell growth, we searched for a correlation between the growth and the photosensitivity of the strains. The same strains grown on TAP medium with high light grew equally well as the wild type and better than on TAP with low light. This led us to conclude that the strains were not photosensitive at this light intensity. To further investigate the mechanisms that limit

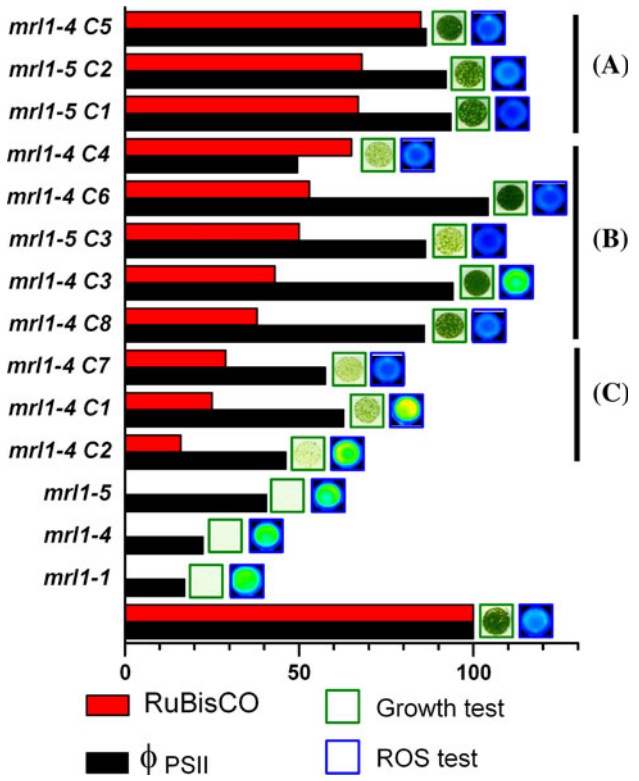

Fig. 5 Different levels of LSU accumulation and Φ_{PSII} in complemented *mrl1-4* and *mrl1-5* C strains determine phototrophic growth. LSU accumulation (*red bars*) and Φ_{PSII} (*black bars*) are presented as a percentage of wild type levels and the histogram is presented from *top* to *bottom* as those accumulating highest LSU (*A group*), those accumulating intermediate levels of LSU (*B group*) and those accumulating lowest LSU (*C group*). Three *mrl1* alleles and the wild type are also shown. The Φ_{PSII} was taken from cells grown on solid TAP media at low light for 1 week and the growth images (*green squares*) displayed come from the same cells grown on minimal medium at 150μE m^{-2} s^{-1} for 1 week. ROS production (*blue squares*) is also shown as an image of the signal generated after 1 h of exposure to very high light

growth, we measured the quantum yield of PSII (Φ_{PSII}) and the generation of ROS (blue squares in Fig. 5) using the DCF test for oxidative stress under very high light conditions. In this test, fluorescence is generated when the molecular probe (DCF) is metabolised and oxidised in the cell by ROS. Blue represents low levels of fluorescence while green, yellow, red and white, increasingly strong levels of fluorescence. The Φ_{PSII} (black bars in Fig. 5) is presented as a percentage of the wild type strain from the fluorescence kinetics shown in Fig. 2d. Again, there was a correlation for the high accumulating (A) and low accumulating (C) RuBisCO strains. The three highest accumulating strains (A) had a Φ_{PSII} approaching the wild type and did not produce detectable levels of ROS. The three low LSU accumulating transformants (C) were like *mrl1-4* and *mrl1-5* mutants, in that although they accumulated some RuBisCO, they had impaired growth, a very reduced Φ_{PSII} with *mrl1-4* C1 and C2 producing quantifiable levels of ROS. Also, like *mrl1-1*, when cells pre-grown on TAP low light were transferred to 200 μE m^{-2} s^{-1} light they became bleached i.e. they were photosensitive under these conditions (not shown). Those falling between the two categories (B) showed different levels of Φ_{PSII} and ROS. Among these strains it was evident that those with the highest Φ_{PSII} had the highest levels of phototrophic growth even when significant ROS production was detected as in the case of *mrl1-4* C3.

Discussion

Here we report new alleles of *MRL1* isolated in a screen for photosynthetic mutants using a novel fluorescence screen approach. We now have a collection of stable *mrl1* mutants in different genetic backgrounds. Three alleles *mrl1-1*, *mrl1-3* and *mrl1-4* are tagged, with *aphVIII* gene cutting off the PPR region from the C-domain or inserting before the PPR region. In the mutants *mrl1-1*, *mrl1-3* no *rbcL* is stabilised while the *mrl1-4* allele accumulates very low but quantifiable amounts of the *rbcL* transcript thus it can be described as a weaker allele of the *mrl1-1* phenotype previously reported.

Here we show that complementation using a "minimal" construct containing 436 bp upstream of the translation start site through to the first intron and fused to the *MRL1* cDNA and randomly transforming the nuclear genome results in different accumulation levels of *rbcL* for the 11 transformants reported, in some cases near to wild type and in others severely reduced. This construct gives different results in comparison to transformation with a genomic fragment of *MRL1* spanning a region 468 bp upstream of the MRL1 translational start site until 2,372 bp downstream of the identified polyadenylation site where accumulation of *rbcL* and LSU on the four clones tested resembled wild type levels (Johnson et al. 2010). It is interesting to note that we were unable to complement the non-photosynthetic phenotype of *mrl1-1* by transformation with the *MRL1* cDNA. These different transformation results may be best explained by the presence of introns in the constructs. Reports in Chlamydomonas and the closely related *Volvox carteri* show the improved expression of nuclear constructs containing introns (Eichler-Stahlberg et al. 2009; Gruber et al. 1996). This may be related to introns being tightly coupled with subsequent mRNA maturation steps especially nucleoplasmic export (Zhu et al. 2010). In the complemented strains shown, the genome insertion site and the numbers of copies inserted into the genome probably contribute to the expression of *MRL1* which we infer leads to different accumulations of *rbcL* and LSU. We cannot state definitively that LSU

accumulation is exactly reflective of active RuBisCO holoenzyme however, the relationship between LSU accumulation and Φ_{PSII} in Fig. 5 suggests that a comparable level of active RuBisCO holoenzyme is formed in the complemented strains. The overall conclusion is that different levels of LSU can be achieved by using a relatively simple technique based on random transformation of a nuclear factor required for stabilising an RNA target.

We have observed that Chlamydomonas is capable of dividing and growing phototrophically at RuBisCO levels of around 15% of the wild type. The lowest RuBisCO accumulations reported in plants transformed with antisense RBCS constructs were estimated at 40% (Rodermel et al. 1988) and 18% (Hudson et al. 1992) downregulation of RuBisCO content in comparison to wild type, showing severe effects to plant growth and photosynthetic activity. The ~15% reported here, may represent the lower limit for photoautotrophic growth in Chlamydomonas. None of the transformants tested accumulated more than ~85% LSU in comparison to the wild type while *rbcL* mRNA accumulated up to wild type levels. Roughly, LSU accumulation seems to correlate with our quantifications of *rbcL* transcript level (Fig. 4c). However, multiple factors could influence the RNA-to-protein ratio, like in our hands the conditions for storage of the strains. After the cells were maintained for several months on non selective medium (containing acetate as a reduced carbon source), a possible transcriptional silencing of the MRL1 transgene reduced *rbcL* accumulation (see Fig. 4 vs. Fig. 3). Other factors have been reported to be involved in *rbcL* translation in Chlamydomonas (Cohen et al. 2006; Shapira et al. 1997; Uniacke and Zerges 2009; Winder et al. 1992) thus we do not expect that *rbcL* accumulation is a simple determinant for LSU accumulation. Furthermore, RuBisCO biogenesis is a tightly coupled process, requiring chaperones (RbcX) and relying on interactions with its partner SS (Mishkind and Schmidt 1983). For example, we would expect that using our technique, an over-accumulation of RuBisCO may be difficult to obtain and would require the tandem upregulation of translation and assembly partners.

In previous work (Johnson et al. 2010), we showed that *mrl1-1* was photosensitive and that DCMU addition can alleviate this photosensitivity in light up to 80 $\mu E\ m^{-2}\ s^{-1}$. This data and the finding that photosynthetic yield in *mrl1-1* showed a lower affinity for oxygen than wild type cells lead us to attribute photosensitivity to ROS generated by an enhancement of the Mehler reaction which in this case substitutes for the Calvin Cycle. By using the DCF ROS detection test, sensitive to hydrogen peroxide, we now show that absence of RuBisCO in *mrl1* alleles leads to the generation of H_2O_2, an intermediate of the Mehler reaction and that partial restoration of RuBisCO levels reduces this phenomenon. This lends support to our previous observations (Johnson et al. 2010) showing that RuBisCO-deficient cells could survive only in the presence of oxygen in high-light (200 $\mu E\ m^{-2}\ s^{-1}$), so that the water-water cycle allows for cell survival. At the same time, ROS production would also explain our observed increase in photoinhibition of both photosystems in RuBisCO-less cells. We hypothesise that although the need for ATP in the chloroplast may be reduced in RuBisCO mutants (protein synthesis is costly in ATP and RuBisCO is a very abundant protein) alternative pathways generate the building of a transmembrane proton gradient for photoprotective purposes in the absence of RuBisCO. This includes the Mehler reaction but also cyclic electron flow, which would appear to be stimulated in the absence of NADPH consumption by the Calvin cycle (Alric et al. 2010).

It is generally argued that ROS production is detrimental for cells. Although it is certainly the case for the complemented strains accumulating low amounts of RuBisCO which show photobleaching at 200 $\mu E\ m^{-2}\ s^{-1}$, we also detect a significant ROS production in *mrl1-4* C3 which has wild type like growth and Φ_{PSII}. To us this strain exemplifies that Φ_{PSII} is determinant for growth, by comparison with *mrl1-4* C8 and *mrl1-5* C3 which have similar amounts of RuBisCO but lower Φ_{PSII} and that, in the right conditions, the water-water cycle is not detrimental but may be on the contrary beneficial for the photosynthetic cell. Cytotoxic effects of ROS depend upon their concentration, itself depending on the ratio between ROS formation and detoxification. In our experimental protocol we measure ROS production at 1,500 $\mu E\ m^{-2}\ s^{-1}$ every 15 min. In these conditions, we consider that we imbalance the ROS formation to detoxification ratio, but under the 150 $\mu E\ m^{-2}\ s^{-1}$ light conditions at which growth was measured, detoxification mechanisms are probably efficient enough to deactivate ROS. On the other hand we also observed for a number of transformed strains, the "B" strains (see Fig. 5); no ROS production, wild type-like growth and a high Φ_{PSII}. A possible explanation is that during the transformation process these strains have accumulated alterations to other alternative pathways which would alleviate PSI acceptor side limitations, interestingly, such mutations which suppress light sensitivity have been reported to accumulate at high frequency in RuBisCO mutants (Spreitzer and Ogren 1983). An increased malate shunt, the pathway by which NADPH reducing equivalents are transferred to mitochondria to produce ATP, or an overexpression of plastid terminal oxidase, leading to the reoxidation of plastoquinols producing ATP and proton gradient by an alternative water-water cycle, would both increase the photosynthetic yield of the cell.

What is the potential utility of controlling RuBisCO levels? Most biotechnological studies with RuBisCO have attempted to overexpress or engineer the protein to improve carbon fixation [for review see (Wostrikoff and

Stern 2009)]. Rather, we envisage that MRL1 could be used as a switch, turning RuBisCO on and off. In a similar manner to the nuclear translation factor, nac2, which is specifically required for the stable accumulation of the chloroplast *psbD* mRNA coding for the D2 protein of PSII complex (Schwarz et al. 2007). These authors used the inducible copper-sensitive cytochrome c(6) promoter fused to the nucleus-encoded nac2 chloroplast protein, to produce loss of PSII by adding copper to the medium. It took 20 h to deplete the cells of PSII and this provided a correct environment for hydrogen production (Surzycki et al. 2007). We could anticipate a similar biotechnological application for MRL1. Under the control of an inducible promoter to initially allow maximum photosynthetic growth without light stress, followed by MRL1 repression to up-regulate secondary metabolic pathways.

As we endeavour to understand more about photosynthetic carbon metabolism in green algae, a simple technique using MRL1 such as that reported here, should prove useful. The complemented *mrl1* strains are a good tool for observing the positive and negative effects of the water-water cycle and could serve as a background for global studies on how metabolism is affected when RuBisCO is limiting for carbon metabolism. Furthermore, the MRL1 protein represents a potential mechanism to down-regulate RuBisCO accumulation to favour energy transfer to other metabolic pathways.

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References

Alric J, Lavergne J, Rappaport F (2010) Redox and ATP control of photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii* (I) aerobic conditions. Biochim Biophys Acta 1797(1):44–51

Barkan A, Goldschmidt-Clermont M (2000) Participation of nuclear genes in chloroplast gene expression. Biochimie 82(6–7):559–572

Cohen I, Sapir Y, Shapira M (2006) A conserved mechanism controls translation of Rubisco large subunit in different photosynthetic organisms. Plant Physiol 141(3):1089–1097

de Vitry C, Olive J, Drapier D, Recouvreur M, Wollman FA (1989) Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthesis mutants from *Chlamydomonas reinhardtii*. J Cell Biol 109(3):991–1006

Drapier D, Suzuki H, Levy H, Rimbault B, Kindle KL, Stern DB, Wollman F (1998) The chloroplast atpA gene cluster in *Chlamydomonas reinhardtii*. Functional analysis of a polycistronic transcription unit. Plant Physiol 117(2):629–641

Eichler-Stahlberg A, Weisheit W, Ruecker O, Heitzer M (2009) Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. Planta 229(4):873–883

Gruber H, Kirzinger SH, Schmitt R (1996) Expression of the Volvox gene encoding nitrate reductase: mutation-dependent activation of cryptic splice sites and intron-enhanced gene expression from a cDNA. Plant Mol Biol 31(1):1–12

Harris EH (1989) The chlamydomonas sourcebook: a comprehensive guide to biology and laboratory use. Academic Press, San Diego, Elsevier

Hong S, Spreitzer RJ (1994) Nuclear mutation inhibits expression of the Chloroplast gene that encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase. Plant Physiol 106:673–678

Hudson GS, Evans JR, von Caemmerer S, Arvidsson YB, Andrews TJ (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. Plant Physiol 98(1):294–302

Johnson X, Kuras R, Wollman FA, Vallon O (2007) Gene hunting in chlamydomonas. In: Proceedings of PSO7 the 14th international congress of photosynthesis research

Johnson X, Vandystadt G, Bujaldon S, Wollman FA, Dubois R, Roussel P, Alric J, Beal D (2009) A new setup for in vivo fluorescence imaging of photosynthetic activity. Photosynth Res 102(1):85–93

Johnson X, Wostrikoff K, Finazzi G, Kuras R, Schwarz C, Bujaldon S, Nickelsen J, Stern DB, Wollman FA, Vallon O (2010) MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of rbcL mRNA in *Chlamydomonas* and *Arabidopsis*. Plant Cell 22(1):234–248

Kuras R, Wollman FA (1994) The assembly of cytochrome b6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. EMBO J 13(5):1019–1027

Loiselay C, Gumpel NJ, Girard-Bascou J, Watson AT, Purton S, Wollman FA, Choquet Y (2008) Molecular identification and function of cis- and trans-acting determinants for petA transcript stability in *Chlamydomonas reinhardtii* chloroplasts. Mol Cell Biol 28(17):5529–5542

Marín-Navarro J, Manuell A, Wu JP, Mayfield S (2007) Chloroplast translation regulation. Photosynth Res 94(2–3):359–374

Mishkind ML, Schmidt GW (1983) Posttranscriptional regulation of ribulose 1, 5-bisphosphate carboxylase small subunit accumulation in *Chlamydomonas reinhardtii*. Plant Physiol 72(3):847–854

Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. Annu Rev Plant Physiol Plant Mol Biol 50:333–359

Raynaud C, Loiselay C, Wostrikoff K, Kuras R, Girard-Bascou J, Wollman FA, Choquet Y (2007) Evidence for regulatory function of nucleus-encoded factors on mRNA stabilization and translation in the chloroplast. Proc Natl Acad Sci USA 104(21):9093–9098

Rodermel SR, Abbott MS, Bogorad L (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell 55(4):673–681

Schwarz C, Elles I, Kortmann J, Piotrowski M, Nickelsen J (2007) Synthesis of the D2 protein of photosystem II in *Chlamydomonas* is controlled by a high molecular mass complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. Plant Cell 19(11):3627–3639

Shapira M, Lers A, Heifetz PB, Irihimovitz V, Osmond CB, Gillham NW, Boynton JE (1997) Differential regulation of chloroplast gene expression in *Chlamydomonas reinhardtii* during photoacclimation: light stress transiently suppresses synthesis of the Rubisco LSU protein while enhancing synthesis of the PS II D1 protein. Plant Mol Biol 33(6):1001–1011

Spreitzer RJ, Ogren WL (1983) Nuclear suppressors of the photosensitivity associated with defective photosynthesis in *Chlamydomonas reinhardii*. Plant Physiol 71(1):35–39

Stern DB, Goldschmidt-Clermont M, Hanson MR (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol 61:125–155

Surzycki R, Cournac L, Peltier G, Rochaix JD (2007) Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. Proc Natl Acad Sci USA 104(44):17548–17553

Uniacke J, Zerges W (2009) Chloroplast protein targeting involves localized translation in *Chlamydomonas*. Proc Natl Acad Sci USA 106(5):1439–1444

Winder TL, Anderson JC, Spalding MH (1992) Translational regulation of the large and small subunits of ribulose bisphosphate carboxylase/oxygenase during induction of the CO(2)-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Physiol 98(4):1409–1414

Wobbe L, Schwarz C, Nickelsen J, Kruse O (2008) Translational control of photosynthetic gene expression in phototrophic eukaryotes. Physiol Plant 133(3):507–515

Wostrikoff K, Stern DB (2009) Rubisco. In: The *Chlamydomonas* source book, Stern DB, Harris E (eds), vol 2, 2 edn. p 1071. Academic Press, San Diego, Elsevier

Zhu J, He F, Wang D, Liu K, Huang D, Xiao J, Wu J, Hu S, Yu J (2010) A novel role for minimal introns: routing mRNAs to the cytosol. PLoS One 5(4):e10144