Nickel and low CO₂-controlled motility in *Chlamydomonas* through complementation of a paralyzed flagella mutant with chemically regulated promoters

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**Abstract**

**Background:** *Chlamydomonas reinhardtii* is a model system for the biology of unicellular green algae. Chemically regulated promoters, such as the nickel-inducible *CYC6* or the low CO₂-inducible *CAH1* promoter, may prove useful for expressing, at precise times during its cell cycle, proteins with relevant biological functions, or complementing mutants in genes encoding such proteins. To this date, this has not been reported for the above promoters.

**Results:** We fused the *CYC6* and *CAH1* promoters to an HA-tagged *RSP3* gene, encoding a protein of the flagellar radial spoke complex. The constructs were used for chemically regulated complementation of the *pf14* mutant, carrying an ochre mutation in the *RSP3* gene. 7 to 8% of the transformants showed cells with restored motility after induction with nickel or transfer to low CO₂ conditions, but not in non-inducing conditions. Maximum complementation (5% motile cells) was reached with very different kinetics (5-6 hours for *CAH1*, 48 hours for *CYC6*). The two inducible promoters drive much lower levels of *RSP3* protein expression than the constitutive *PSAD* promoter, which shows almost complete rescue of motility.

**Conclusions:** To our knowledge, this is the first example of the use of the *CYC6* or *CAH1* promoters to perform a chemically regulated complementation of a *Chlamydomonas* mutant. Based on our data, the *CYC6* and *CAH1* promoters should be capable of fully complementing mutants in genes whose products exert their biological activity at low concentrations.

**Background**

*Chlamydomonas reinhardtii* is a unicellular green alga, capable of both photosynthetic and fermentative growth. A plethora of mutants in relevant biological processes are available, and nuclear and chloroplast transformation are easy to perform [1]. Its 120-megabase genome has been completely sequenced [2]. *Chlamydomonas* combines functions typical of higher plants, such as the presence of a chloroplast endowed with two photosystems [3], of protozoa, such as the presence of motile flagella for swimming [4], and of archaea, such as the presence of sensory rhodopsins mediating phototaxis [5].

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Several chemically regulated promoters have been described in *Chlamydomonas*: the Nitrate Reductase (*NIT1*) promoter, induced by ammonium starvation [11]; the Carbonic Anhydrase (*CAH1*) promoter, induced by low CO₂ [12]; and the Cytochrome C6 (*CYC6*) promoter, induced by copper (Cu) depletion or nickel (Ni) addition [13,14,12]. In all three cases, inducible expression has been demonstrated using reporter genes such as ariysulfatase or luciferase and, in the case of the *NIT1* promoter, through complementation of a paralyzed flagellar mutant, *pf14*, by expressing the wild type form of the RSP3 gene [15]. No data are available, to our knowledge, on the capacity of the *CAH1* and *CYC6* inducible promoters to drive complementation of *Chlamydomonas* mutants.

To assess the capacity of the *CYC6* and *CAH1* promoters to complement the *pf14* mutation in a chemically regulated fashion, we transformed the paralyzed *pf14* mutant with the RSP3 gene under the control of the above-mentioned promoters and scored the swimming phenotype. The strong constitutive *PSAD* promoter [16] was used as a control.

### Results

#### Constructs used for chemically inducible complementation

The complete RSP3 gene (including introns) was translationally fused to a 9-amino acid HA epitope at its 3’ end, to facilitate the immunodetection of the expressed protein [17]. The RSP3-HA hybrid gene was placed under the control of the *CYC6* and *CAH1* promoters, induced, respectively, by Ni and low CO₂ [13,14,12] and, as a control, of the strong constitutive *PSAD* promoter [16]. The constructs are schematically represented in Figure 1.

#### Constitutive complementation of the *pf14* mutant driven by the *PSAD* promoter

The *pf14* mutant strain was transformed with the *PSAD*: RSP3-HA plasmid and 68 paromomycin-resistant transformants were grown for 4 hours without shaking in the light. Upon microscopic examination, about 40% of the transformants showed swimming cells. The average percentage of swimming cells was about 80% (Table 1). This result shows that the RSP3-HA fusion protein is able to rescue the *pf14* mutant. The data of a representative transformant are shown in Figure 2. The majority of the cells (88%) were flagellated and motile (Panel A) and strong signals corresponding to the unphosphorylated (lower band) and phosphorylated (upper band) forms of the RSP3-HA protein were detected in a Western blot using the anti-HA antibody (Panel B).

### Chemically inducible complementation of *pf14* driven by the *CYC6* and *CAH1* promoters

*pf14* cells were transformed with the *CYC6*:RSP3-HA and the *CAH1*:RSP3-HA plasmids and 68 transformed colonies were analyzed for each construct. Before analysis, the *CYC6*:RSP3-HA transformants were inoculated in TAP ENEA2 medium, allowing optimal expression of the *CYC6* promoter, and expression was induced in the mid-log phase (6 × 10⁶-8 × 10⁶ cells/ml) by adding 25 μM Ni [14]. We used a rather low Ni concentration, since higher concentrations cause detachment of flagella, preventing the scoring of the swimming phenotype (data not shown). The swimming phenotype was scored 48 hours after induction, when *CYC6* promoter expression is maximal [14]. Approximately 8% of the transformants displayed swimming (Table 1) and, in these transformants, an average of 5% of the cells were motile. This difference with respect to the *PSAD*:RSP3-HA transformants is due to two factors: a much lower percentage of cells are flagellated in the *CYC6*:RSP3-HA transformants (12% vs 90%) and, of these, a lower percentage are swimming (Table 1). As discussed below, we attribute this difference to a threshold effect. Movies of *PSAD*: RSP3-HA and *CYC6*:RSP3-HA transformants are available as Additional files 1 and 2.

In order to prevent loss of flagella at high cell densities (see below), cells were also induced with 25 μM Ni in early log phase (1 × 10⁶-2 × 10⁶ cells/ml), but no transformants showed swimming, and percentage of flagellated and swimming cells in the rescued transformants

| Construct | % rescued transformants | % flagellated cells in rescued transformants | % swimming cells in rescued transformants |
|-----------|-------------------------|---------------------------------------------|------------------------------------------|
| PSAD: RSP3-HA | 40 | 90 ± 10 | 90 ± 16 |
| CYC6: RSP3-HA | 8 | 12 ± 2 | 5 ± 0.8 |
| CAH1: RSP3-HA | 7 | 90 ± 10 | 5 ± 1.0 |

In this experiment 68 transformants were analyzed for each construct.
rescue was observed (data not shown). This is consistent with the observation of Quinn et al. [13] that activation of the CYC6 promoter is stronger when the cells are induced at mid-late log phase, probably because Ni uptake is higher.

The CAH1:RSP3-HA transformants were grown in air containing 5% CO2, in minimal medium supplemented with extra phosphate buffer to keep the pH stable. Expression of the CAH1 promoter was induced in early-log phase by transferring the plate to air and cells were scored for swimming 6 hours after induction, when the CAH1 promoter shows high expression [12]. Approximately 7% of the transformants showed swimming and, as for the CYC6:RSP3-HA transformants, approximately 5% of the cells were motile in the rescued transformants (Table 1).

The percentage of swimming cells in two representative CAH1:RSP3-HA transformants was determined 6 hours after transfer to low CO2 (Figure 4), when the CAH1 promoter shows high expression [12]. In this case, cell density was low (2 × 10^6-4 × 10^6 cells/ml) and the percentage of flagellated cells was high (approx. 90%). However, as for the CYC6:RSP3-HA transformants, the percentage of motile cells was low (5%-6% of total cells).

Figure 5 (Panels A and B) shows a Western blot of several CYC6:RSP3-HA and CAH1:RSP3-HA transformants, grown in the same conditions of Figures 3 and 4, and probed with an anti-HA antibody. Only transformants showing motility in the swimming assay (Figures 3 and 4) showed the two bands corresponding to the RSP3 protein. The signal of the two bands is very weak compared to the PSAD:RSP3-HA transformants, suggesting that the low percentage of swimming cells is probably due to low expression of the RSP3 protein. The swimming transformants were re-grown in the same conditions used in Figure 6, and probed 0 h and 48 h (CYC6 transformants) or 0 h and 6 h (CAH1 transformants) after induction. The results (Panel C) show that the RSP3 protein is completely absent in non-induced, and readily detectable in induced cells.

Kinetics of induction of the swimming phenotype
We then determined the kinetics of appearance of swimming cells in one representative CYC6:RSP3-HA and one CAH1:RSP3-HA transformant (Figure 6). In the case of the CYC6 promoter, swimming cells were observed as early as
24 hours after Ni addition. At 48 hours the number of swimming cells reached a maximum and then decreased at 72 hours. This is in agreement with the kinetics of activation of the CYC6 promoter, measured with the luciferase reporter gene, which reaches maximum activity after two days of induction and then decreases at three days [14]. In the CAH1:RSP3-HA transformant, swimming cells were observed as early as 2 hours after transfer to low CO2. The maximum number of swimming cells was reached 5 hours after transfer, and then declined at 8 hours. However, considering the standard deviations at 5 and 8 hours, this decline is not significant.

Discussion
Through the use of the chemically regulated CYC6 and CAH1 promoters and of a genomic RSP3 clone fused to an HA epitope, we have achieved the chemically regulated motility of Chlamydomonas cells. While the vast majority of cells showed motility when RSP3 expression was driven by the constitutive PSAD promoter, only a minority (5%) of cells showed motility after induction of the CYC6 and CAH1 promoters. This is probably due to a threshold effect: the levels of RSP3-HA protein driven by PSAD are much higher than those driven by CYC6 and CAH1. The low levels of RSP3-HA protein expressed from the CAH1 promoter after 6 hours of induction contrast markedly with the high levels of CAH1 protein expressed from the endogenous gene (data not shown). Low expression of exogenously introduced constructs in Chlamydomonas is a well-known phenomenon, which has been attributed to gene silencing [18].
Figure 5 Western blot of CYC6::RSP3-HA and CAH1::RSP3-HA transformants, probed with the anti-HA antibody. Panels A and B: Screening of protein extracts CYC6::RSP3-HA and CAH1::RSP3-HA transformants, extracted, respectively, 48 h and 6 h after induction. Transformants that exhibit inducible swimming are labeled. Arrows point at the RPS3-HA bands. Cultures were grown and induced as in Figures 3 and 4, extracted, and 20 μg total proteins were loaded on each lane. Panel C: Re-analysis of transformants exhibiting inducible swimming (from Panels A and B). Cultures were grown and induced as in Figure 6, extracted, and 40 μg total proteins were loaded on each lane. For details, see Methods.

Figure 6 Time course of inducible swimming in one CYC6::RSP3-HA (Panel A) and one CAH1::RSP3-HA (Panel B) transformant. The CYC6::RSP3-HA transformant was grown in 6 ml in 6-well microtiter plates with shaking (120 rpm) and the CAH1::RSP3-HA transformant was grown in 150 ml in 250-ml Erlenmeyer flasks with bubbling.
The low levels of RSP3-HA protein expressed from the CYC6 promoter after 48 hours of induction are also puzzling, since, in TAP ENEA2 medium, the CYC6 promoter is able to drive levels of luciferase expression comparable to those driven by PSAD [14]. We attribute this difference in RSP3 vs luciferase expression to the fact that RSP3 accumulates over time when it is expressed from PSAD, while expression for 48 hours (from CYC6) or 6 hours (from CAH1) allows accumulation of low RSP3 levels (Figure 5). This implies that the RSP3 protein is more stable than luciferase (whose estimated half-life in Chlamydomonas is <2 hours [19]). Whatever the case, the low levels of expressed RSP3-HA protein are sufficient for achieving motility in 5% of the transformed cells. To our knowledge, this is the first example of the use of the CYC6 and CAH1 promoters for achieving chemically regulated complementation of a Chlamydomonas mutant, as well as the first example of metal- or CO2-regulated motility engineered in a living organism. The partial complementation observed is probably due to the fact that the RSP3 protein, to exert its function, is required in high concentrations. Although the number of radial spokes required to restore motility to flagella is not known, each wild type flagellum contains approximately 2,000 radial spokes [20].

Zhang and Lefebvre [15] have used the RSP3 gene under the control of the ammonium-repressible NIT1 promoter to complement the pf14 mutant in a nitrogen source-dependent fashion. In that study, 81 out of 2,000 cotransformants showed motility in permissive conditions, i.e. a fraction of about 4%, comparable to the 7-8% reported here for the CYC6 and CAH1 promoters. At least one of the transformants, containing multiple copies of the NIT1:RSP3 plasmid, showed full complementation, i.e. a large number of swimming cells, a fact we did not encounter in the case of the CYC6 and CAH1 promoters, probably due to the smaller number of colonies screened in our study and to the fact that the vast majority of the insertions, in our case, are single-copy (Additional file 3). Whatever the case, the frequency of swimming transformants obtained with the strong PSAD promoter is 40% (Table 1), i.e. much higher than what can be obtained using either the NIT1, CYC6, or CAH1 promoters in permissive conditions. A chemically regulated promoter system allowing such high complementation efficiencies in permissive conditions has yet to be worked out.

Conclusions
We have demonstrated low level, chemically regulated complementation of the paralyzed flagella pf14 mutant by the RSP3 gene, encoding a component of the flagellar radial spoke complex, cloned under the control of the CYC6 and CAH1 promoters. Maximum complementation is reached with very different kinetics (6 hours for CAH1, 48 hours for CYC6). In principle, these promoters should be capable of fully complementing mutants in genes whose products exert their biological activity at low concentrations (e.g. receptor/signalling protein kinases). Test of this hypothesis is under way, as well as the optimization of the CYC6 and CAH1 promoters, for full complementation of mutants in genes encoding abundant intracellular proteins.

Methods
Strains and culture conditions
The paralyzed flagella mutant pf14 [8] was used for all experiments. Nuclear transformation was performed as described [21]. Plasmids were digested with Sca I and 300 ng of DNA were used for each transformation. Transformants were selected on TAP agar plates containing paromomycin (10 μg/ml).

Unless indicated differently, cells were grown photo-mixotrophically in TAP medium at 25°C under irradiation (16 L: 8 D) with fluorescent white light (200 μE m⁻² s⁻¹). For the initial screening, 68 transformants for each construct were grown in 200 μL in 96-well microtiter plates with shaking (900 rpm). For quantitative measurements of motility (Figures 2, 3, 4, 5A and 5B), transformants were grown in 2 mL in 24-well microtiter plates with shaking (500 rpm). For the experiments described in Figure 5C and in Figure 6 cells were grown in 6 ml 6-well microtiter plates with shaking (120 rpm) (CYC6 transformants) or in 150 ml in 250-ml Erlenmeyer flasks (CAH1 transformants) with bubbling. The plates were covered with Breathe-Easy membrane (Diversified Biotech, cat. BEM-1), to prevent evaporation without limiting gas and light exchange. For Ni induction, cells were grown in TAP ENEA2 medium [14] and induced at mid-log phase (6 × 10⁶-8 × 10⁶ cells/ml) by adding 25 μM Ni. For low-CO₂ induction, cells were grown in minimal medium with doubled phosphate buffer concentration, to keep the pH stable in high CO₂ conditions [22], in air containing 5% CO₂ and induced by shifting to air in early log phase (1 × 10⁶-2 × 10⁶ cells/ml).

Plasmid construction
The complete RSP3 gene (including introns) was amplified using the following oligonucleotides:

Forward:
GCTCTAGAATGGTGCAAGGCTAAGGCGCAGC

Reverse: GAAAGATCTTTAGGGCGTATGTCGGGCACGTCGTAAGGGGTACGGCGCCCTCGCCCTCGCGGCAGAC

The forward oligonucleotide inserts an Xba I restriction site, the reverse oligonucleotide inserts a 9- amino acid HA-tag (the corresponding nucleotide sequence is in italics) followed by a TAA stop codon and a Bgl II...
Finally the pellets were resuspended in 800 μl of 0.2 M NaOH and the absorbance measured at 615 nm. To calculate protein concentration a standard curve with BSA was used.

Western blot analyses were performed on total protein extracts obtained as described above. About 30 μg protein/sample were separated on an 8% SDS-PAGE gel [23]. Proteins were blotted on nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, 20% ethanol for 2 hours at 250 mA using a Hoefer TE22 apparatus. A commercial anti-HA antibody (Ascites Fluid Mono HA 11, 16B12, Covance) was used in a 1:250 dilution. The secondary antibody (anti-mouse, phosphatase conjugated, Thermo Scientific 31325) was used in a 1:2500 dilution. Detection was performed placing the nitrocellulose membrane in 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris (pH 9.5) containing Nitro-tetrazolium Blue chloride (NBT) 0.33 mg/ml and 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) 0.165 mg/ml. To stop the reaction, the membrane was rinsed with Phosphate-Buffered saline (PBS) containing 20 mM EDTA.

Microscopy
Cell motility and the presence of flagella were assessed using an Olympus BX41 microscope with 16 X and 40 X objective lenses, respectively. Movies were recorded with a Cool Snap HQ camera (Photometrics) on a Nikon Eclipse TE2000 inverted microscope using a 10 X objective lens.

Additional material

Additional file 1: Motility of a PSAD:RSP3-HA transformat. Movie showing the motility of a PSAD:RSP3-HA transformat.

Additional file 2: Motility of a CYC6:RSP3-HA transformat, 48 hours after Ni induction. Movie showing the motility of a CYC6:RSP3-HA transformat, 48 hours after Ni induction.

Additional file 3: Estimation of transgene copy number by quantitative Real-Time PCR. Figure showing the estimation of transgene copy number by Real Time PCR.

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Authors’ contributions
PF performed experiments. DRD and GG supervised experiments. All authors designed experiments, interpreted data and wrote the manuscript.

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Proteins electrophoresis and Western blotting
Two ml of Chlamydomonas culture were centrifuged at 10,000 × g at room temperature for 1 minute and the cell pellets were resuspended in 300 μl of 60 mM DTT, 60 mM Na₂CO₃, 2% SDS, 12% sucrose and shaken for 20 minutes at room temperature to extract the proteins. The protein extracts were centrifuged at 10,000 × g for 1 minute and the supernatant collected. To measure protein concentration, 10 μl of protein extracts were mixed with 800 μl of 0.5% Amido Black in 90% methanol and 10% glacial acetic acid. The samples were vortexed and centrifuged at 10,000 × g at 4°C for 10 minutes. The pellets were washed two times with 90% methanol and 10% glacial acetic acid at 4°C.
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