Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*

Raymond Surzycki*, Laurent Cournac†‡§, Gilles Peltier†‡§, and Jean-David Rochaix*¶

*Departments of Molecular Biology and Plant Biology, University of Geneva, 30 Quai Ernest Ansermet, 1211 Geneva, Switzerland; †Commissariat à l'Energie Atomique (CEA), Direction des Sciences du Vivant (DSV), Institut de Biologie Environmennetale et Biotechnologie (IBEB), Laboratoire de Bioénergétique et Biotechnologie des Bactéries et Microalgues (LB3M), F-13108 Saint-Paul-lez-Durance, France; ‡Centre National de la Recherche Scientifique (CNRS), Unité Mixte de Recherche (UMR) 6191, F-13108 Saint-Paul-lez-Durance, France; and §Université de la Méditerranée, UMR 6191, F-13108 Saint-Paul-lez-Durance, France

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An inducible chloroplast gene expression system was developed in *Chlamydomonas reinhardtii* by taking advantage of the properties of the copper-sensitive cytochrome c_6 promoter and of the nucleus-encoded Nac2 chloroplast protein. This protein is specifically required for the stable accumulation of the chloroplast *psbD* RNA and acts on its 5′ UTR. A construct containing the *Nac2* coding sequence fused to the cytochrome c_6 promoter was introduced into the *nac2-26* mutant strain deficient in *Nac2*. In this transformant, *psbD* is expressed in copper-depleted but not in copper-replete medium. Because *psbD* encodes the D2 reaction center polypeptide of photosystem II (PSII), the repression of *psbD* leads to the loss of PSII. We have tested this system for hydrogen production. Upon addition of copper to cells pregrown in copper-deficient medium, PSII levels declined to a level at which oxygen consumption by respiration exceeded oxygen evolution by PSII. The resulting anaerobic conditions led to the induction of hydrogenase activity. Because the *Cyc6* promoter is also induced under anaerobic conditions, this system opens possibilities for sustained cycling hydrogen production. Moreover, this inducible gene expression system is applicable to any chloroplast gene by replacing its 5′ UTR with the *psbD* 5′ UTR in the same genetic background. To make these strains phototrophic, the 5′ UTR of the *psbD* gene was replaced by the *petA* 5′ UTR. As an example, we show that the reporter gene *aadA* driven by the *psbD* 5′ UTR confers resistance to spectinomycin in the absence of copper and sensitivity in its presence in the culture medium.

copper | cytochrome c_6 | inducible promoter | photosystem II | RNA processing

Although the mechanisms of plastid gene expression have been studied intensively (1), few efforts have been devoted to establish an inducible chloroplast gene expression system that would be applicable to any plastid gene. Such a system would be of considerable interest for several reasons. First, an inducible chloroplast gene expression system would prevent problems arising from constitutive high-level expression of foreign proteins of commercial interest in transplastomic plants and algae. Some of these proteins, e.g., membrane proteins, may be toxic to the cells and could impair their growth. Second, the assembly of photosynthetic complexes could be studied in a new way because, by blocking the synthesis of a chloroplast-encoded core subunit, the entire complex would be degraded. Its assembly could be reinitiated *de novo* by restoring expression of the core subunit in the context of preformed thylakoid membranes. Third, the expression of essential chloroplast genes could be blocked, and the impact on cell function could be examined before the cells die. Besides genes involved in chloroplast protein synthesis, some plastid genes of unknown function such as *ycf1* and *ycf2* are thought to be essential because it has not been possible to obtain homoplasmic disruptions of these genes (1, 2). Fourth, such a system would allow one to control photosynthetic activity and the oxygen level within the cell by regulating photosystem II (PSII) accumulation. This system would open new possibilities for inducing anaerobic metabolism, e.g., hydrogen evolution under controlled conditions. An inducible chloroplast gene expression system was developed earlier in tobacco. The phage T7 RNA polymerase expressed from a nuclear inducible promoter was targeted to the chloroplast for expressing genes under the control of the phage T7 promoter (3–5). However this system has not been used widely and has not been developed in *Chlamydomonas*.

Genetic analysis in *Chlamydomonas*, maize, and *Arabidopsis* has revealed a large set of nucleus-encoded factors which act mostly at posttranscriptional steps of chloroplast gene expression such as RNA processing and stability, splicing, and translation (1). Among these proteins, the Nac2 protein of *Chlamydomonas reinhardtii* is of particular interest for establishing an inducible chloroplast gene expression system. This protein is specifically required for the stable accumulation of the *psbD* mRNA encoding the D2 reaction center polypeptide of PSII (6, 7). Here, we have taken advantage of the properties of this protein and of the *Cyc6* promoter of the cytochrome c_6 gene, which is induced by copper depletion (8) as well as by anaerobiosis (9), to develop an inducible gene expression system that is applicable to any chloroplast gene.

This inducible chloroplast gene expression system may open new possibilities for producing hydrogen in *Chlamydomonas*. It is known since the early work of Gaffron and Rubin (10) that this alga can produce hydrogen under anaerobic conditions in the light. However, this production is transient because the hydrogenase is rapidly inactivated by oxygen produced by photosynthesis (11). To circumvent this problem, Melis *et al*. (12) used sulfur deprivation, which leads to the gradual inactivation of PSII. Under these conditions, oxygen evolution ceases and oxygen is depleted through respiration. This process leads to anaerobiosis, which in turn induces the synthesis of hydrogenase. Moreover, sulfur starvation leads to the accumulation of carbohydrates, which is important for sustained hydrogen production in the long term (13, 14). It also leads to the inhibition of the Calvin–Benson cycle and thereby removes an important electron sink, thus favoring hydrogen production (15). However, cells can survive only for a few days in sulfur-depleted medium and will eventually die. We have tested whether the copper-repressible system in *Chlamydomonas* can be used to turn off PSII activity and thereby create anaerobic conditions suitable for hydrogen production.

Results

Inducible Chloroplast Gene Expression System Based on Nac2. No natural inducible chloroplast gene expression system is available for

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Abbreviations: PSII, photosystem II; TAP, Tris-acetate-phosphate; F_V/F_M, variable/maximal fluorescence; HSM medium, high-salt minimal medium.

¶To whom correspondence should be addressed. E-mail: jean-david.rochaix@molbio.unige.ch.

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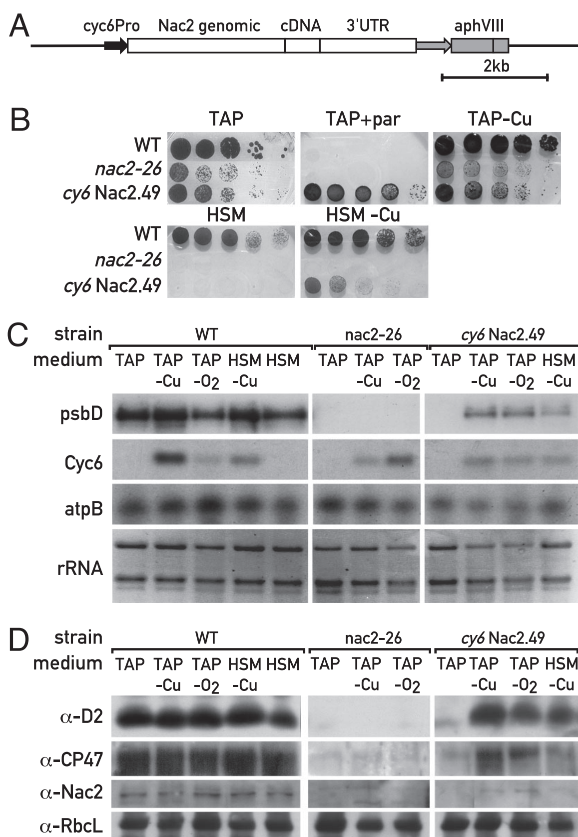

Fig. 1. Inducible expression of the chloroplast *psbD* gene. (*A*) Map of the *Cyc6-Nac2* construct with the paromomycin resistance cassette. (*B*) Growth properties of the *cy6Nac2.49* strain. Cells from WT, *nac2-26*, and *cy6Nac2.49* (*nac2-26* transformed with *Cyc6-Nac2*) were grown on TAP medium, TAP with paromomycin, TAP lacking copper, HSM (minimal) medium, and HSM lacking copper. In each case, 5 μl of a liquid culture were spotted on agar plates with 5-fold serial dilutions. Cells were grown under 60 μmol·m^{-2}·s^{-1} light. (*C*) RNA blot analysis. RNA was extracted from cells of WT, *nac2-26*, and *cynac2.49*, fractionated by agarose gel electrophoresis, and hybridized with the gene probes indicated on the left. (*D*) Immunoblot analysis. Proteins from WT, *nac2-26*, and *cyNac2.49* were fractionated by PAGE and immunoblotted with antibodies indicated on the left.

Chlamydomonas. To develop such a system, we have taken advantage of the properties of the nucleus-encoded chloroplast Nac2 protein. This protein is required for processing and stable accumulation of the *psbD* mRNA, which encodes the D2 reaction center polypeptide of PSII (6). The target site of Nac2 is comprised within the 74 nucleotide *psbD* 5′ UTR (7). Fusion of this 5′ UTR to another coding sequence renders expression of this gene dependent on Nac2. We have fused the *Nac2* coding sequence to the *Cyc6* promoter of the cytochrome c_6 gene, whose expression is induced by copper depletion and anaerobiosis and also by addition of nickel, but which is repressed under copper-replete conditions (8, 9, 16). Because of the specificity of Nac2 for the *psbD* 5′ UTR, this system can be used in principle for the inducible expression of any chloroplast gene by fusing its coding sequence to the *psbD* 5′ UTR.

The *Cyc6-Nac2* construct was inserted into a plasmid containing the *aphVIII* gene conferring resistance to paromomycin (17) (Fig. 1*A*). This plasmid was used for transformation of the *Chlamydomonas nac2-26* mutant using paromomycin resistance for selection. Among 55 transformants tested, two displayed proper control of *Nac2* expression by copper. The growth properties of one of these transformants (*cy6Nac2.49*), of WT, and of the *nac2-26* mutant are shown in Fig. 1*B*. As expected, all three strains grow on Tris-acetate-phosphate (TAP) medium with and without copper, and the transformants also grow in the presence of paromomycin because they contain the selectable marker *aphVIII*. Only WT cells grow on minimal medium containing copper. However, growth of the *cy6Nac2.49* strain is restored on minimal medium lacking copper. Growth can also be restored by adding nickel because the *Cyc6* promoter is induced by this metal (16) (data not shown). The level of *psbD* expression was determined by RNA blot hybridization under different growth conditions (Fig. 1*C*). As expected, *psbD* RNA is undetectable in the *nac2-26* mutant strain. In contrast, in the *cy6Nac2.49* strain, expression of *psbD* follows that of *Cyc6* and is induced in the absence of copper or under anaerobic conditions (Fig. 1*C*). The level of the *psbD* product D2 was examined by immunoblotting using D2 antiserum (Fig. 1*D*). D2 protein is undetectable in *nac2-26* cells grown on TAP plates under all conditions. However, in *cy6Nac2.49*, it accumulates to 20% of WT levels when cells are grown in the absence of copper or under anaerobic conditions [supporting information (SI) Fig. 6]. On minimal medium, the induction of D2 is slightly lower. As expected, other PSII proteins such as CP47 follow a similar pattern as D2 because it is known that these proteins are unstable in the absence of the D2 protein (18). In contrast, the level of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein (RbcL) is not affected in *nac2-26* (Fig. 1*D*).

To assess the time required to deplete cells of PSII upon arrest of Nac2 synthesis, cells of *cy6Nac2.49* were first grown in copper-depleted TAP medium. Under these conditions, PSII is synthesized and accumulates. The culture was split in half, and one culture was maintained under copper deprivation whereas copper was added to the other culture. The time course of cell density and the F_V/F_M ratio (variable/maximal fluorescence), which provides an estimate of PSII quantum yield, was determined at various time points (Fig. 2*A*). In the presence of copper, the F_V/F_M ratio declined to a minimal value within 32 h. During this period, cells divided 3- to 4-fold under both conditions and reached stationary phase. Cell extracts were prepared at various times for RNA and protein analysis. The levels of *Cyc6* and *psbD* RNA were significantly decreased 8 h after copper addition and were undetectable thereafter (Fig. 2*B*). Other chloroplast RNAs (*atpB*, rRNA) were stable under these conditions. Immunoblotting revealed that the amount of D2 diminished after copper addition with a lag compared with the decrease of its mRNA (Fig. 2*C*) and the other PSII core protein D1 also decreased. As expected, a decrease in Nac2 was also observed although the low amount of this protein made its detection difficult. In contrast, chloroplast proteins from PSI (PsaA) and Rubisco were stable (Fig. 2*C*).

In a reciprocal experiment, cells grown in the presence of copper were transferred to TAP medium lacking copper, and the time course of cell density and of F_V/F_M was determined. F_V/F_M started to increase only after a lag of 25 h, which is presumably due to the time needed to deplete the internal cellular copper reserve (Fig. 3*A*). RNA and protein from cell extracts at different time points were examined by RNA blot analysis and protein immunoblotting (Fig. 3 *B* and *C*). Although the *Cyc6* RNA was detectable after 16 h, there was a delay in *psbD* RNA accumulation and PSII activity presumably because of the fact that a threshold level of Nac2 is required for the accumulation of *psbD* mRNA and D2 synthesis.

Inducible Expression of Chloroplast Genes Unrelated to PSII. Although the Nac2 system can be used to deplete PSII in a reversible manner, we tested whether it can be extended to any other chloroplast gene. This extension is in principle possible because the Nac2 protein acts specifically on the *psbD* 5′ UTR and can drive chimeric *psbD* 5′ UTR reporter genes (7). Therefore, it should suffice to fuse the *psbD* promoter and 5′ UTR to the gene of interest. However, under those conditions, PSII does no longer accumulate because of the *nac2-26* mutation, which leads to the loss of *psbD* RNA. To circumvent this problem, the *petA* promoter and 5′ UTR were fused to the *psbD* coding sequence, and this construct was introduced into

Fig. 2. Time course of copper-mediated repression of PSII synthesis in *cy6Nac2.49*. (*A*) Cells were grown in TAP medium depleted of copper to a cell concentration of 2×10^6 cells per ml. At time 0, copper was added, and F_V/F_M and cell concentration were determined at different time points. (*B*) RNA was isolated from cells at different times, fractionated by agarose gel electrophoresis, blotted, and hybridized with the gene probes indicated. (*Left*) Cells grown in TAP lacking copper. (*Right*) Cells were grown in TAP lacking copper; at time, 0 copper was added to the culture. (*C*) Immunoblot analysis of proteins from *cyNac2.49* was performed as described in Fig. 2*C*. The antibodies used are indicated on the left.

Fig. 3. Time course of accumulation of PSII in *cy6Nac2.49* after copper depletion. (*A*) Cells were grown in TAP medium to a concentration of 2×10^6 cells per ml, centrifuged, and resuspended in TAP medium depleted of copper. F_V/F_M and cell concentration were determined at different time points. (*B*) RNA blot analysis. RNA was extracted from cells at different times under either copper-replete (left) or copper-deficient (right) conditions and subjected to RNA blot analysis. The probes used are indicated on the left. (*C*) Immunoblot analysis of proteins from the samples described in *B*. The antibodies used are indicated on the left.

a modified version of the p108-14 chloroplast transformation vector (7) (for details, see *Materials and Methods*). In this vector, the recyclable *aadA* cassette (19) is inserted upstream of the *psbD* gene, which is driven by the *petA* promoter and 5′ UTR (SI Fig. 7*A*). This DNA was inserted into the chloroplast genome by biolistic transformation using the *aadA* cassette as selectable marker. In this way the endogenous *psbD* gene was replaced by the *petA-psbD* construct, and thus accumulation of its transcript was no longer dependent on Nac2. Transformants were restreaked three times on spectinomycin plates, and the homoplasmicity was tested by DNA blot and PCR analysis (data not shown), and one of the transformants, Ind41, was selected. The *aadA* cassette used was flanked by two repeats. To allow for the excision of the cassette (19), the homoplasmic transformant Ind41 was plated repeatedly on medium lacking spectinomycin. In this way, a strain was obtained, Ind41_18, which is sensitive to spectinomycin because it lacks the *aadA* cassette. The growth properties of *Ind41*, *Ind41_18* and *cy6Nac2.49* were tested on different media (SI Fig. 7*B*). As expected, *Ind41* grows in the presence of spectinomycin in contrast to *Ind41_18*, which is sensitive to the antibiotic. Moreover, both *Ind41* and *Ind41_18* grow on high-salt minimal (HSM) medium with or without copper. RNA blot analysis revealed that the chimeric *petA-psbD* RNA in *Ind41_18* accumulates under all conditions independent of the *Cyc6* RNA level (SI Fig. 7*C*). The *psbD* RNA is larger because the size of the *petA* 5′ UTR exceeds that of the *psbD* 5′ UTR. Immunoblot analysis revealed that D2 and D1 proteins accumulate to the same level under all conditions tested, in particular when Nac2 is not expressed (SI Fig. 7D).

Next, the *aadA* cassette fused to the *psbD* promoter, and 5′ UTR was introduced into this strain by transformation using the *cg12* vector (7) (Fig. 4*A*). Growth of the transformants *Ind_aadA-117* and of *Ind41_18* was tested on TAP plates containing increasing amounts of spectinomycin with and without copper (Fig. 4*B*). All strains grow in the absence (Fig. 4*B*) or presence (data not shown) of copper. As expected, the *Ind_aadA-117* grows on spectinomycin plates at concentrations of 250 μg/ml or higher only in the absence of copper. A faint growth was also observed in the presence of copper on plates containing 100 μg/ml spectinomycin. RNA blot analysis revealed that *aadA* RNA accumulates only under inducing conditions for the *Cyc6* promoter (Fig. 4*C*). Protein levels of D2, D1, CP47, and RbcL were largely unaffected, but Nac2 was detected only under inducing conditions (Fig. 4*D*). Because of a lack of reliable AadA antibody, the amount of this protein was assayed by measurements of aminoglycoside adenyl transferase activity. The activity was significantly elevated under conditions when Nac 2 is expressed (Table 1).

Fig. 4. Expression of the *psbD-aadA* gene is induced by copper depletion and repressed by copper in the *Ind_aadA-117* transformants. (*A*) Map of *aadA* construct in the *atpint* vector. (*B*) Cells of the transformants were grown with or without copper on plates with increasing amounts of spectinomycin (0, 100, 250, and 1,000 μg). (*C*) RNA blot analysis of *Ind_aadA-117* grown under different conditions as indicated. The probes are shown on the left. (*D*) Immunoblot analysis of the same samples as in *C* with the antibodies indicated on the left.

The Inducible Chloroplast Gene Expression System Can Be Used to Trigger Hydrogen Production. *Chlamydomonas* is able to induce hydrogenase and produce hydrogen under anaerobic conditions in the light. It was therefore tempting to test whether the inducible Nac2 system could be used to turn off PSII activity and O_2 evolution, so that respiration would lead to anaerobic conditions suitable for induction of hydrogen production. Cells of *cy6Nac2.49* were grown in TAP medium lacking copper to a concentration of 2×10^6 cells per ml. Copper was added, and the cells were transferred into a chamber connected to a mass spectrometer and illuminated with white light (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) [E (einstein) = mol of photons]. In this system, the bottom of the chamber is sealed with a polypropylene membrane, which allows dissolved gases to diffuse directly into the ion source of the mass spectrometer (20). In this way, the abundance of O_2 and H_2 could be measured at discrete time intervals. Because the chamber was closed and because copper repressed the synthesis of PSII, O_2 evolution diminished. Within a period of 200 min, the O_2 was consumed by respiration. An anaerobic state was reached, which led to the synthesis of active hydrogenase and H_2 production (Fig. 5*A*). The maximal rate of hydrogen production ranged between 1 and 3.1 mmol H_2 mol^{-1} Chl

Table 1. Aminoglycoside adenyl transferase activity in *Ind41_aadA-117* under inducing and repressing conditions

	Activity, cpm/μg of protein	
Strain	+Cu	−Cu
WT-*aadA*	207.0 ± 49.5 (3)	192.6 ± 51.4 (4)
Ind41_18	9.2 ± 4.1 (4)	12.2 ± 7.9 (4)
Ind41_117	24.2 ± 12.5 (4)	274.3 ± 90.6 (7)

Extracts from WT-*aadA*, *Ind41_18*, and *Ind41_aadA-117*strains were assayed for aadA activity and for total protein content as described in *Materials and Methods*. The activity is indicated as cpm incorporated per μg protein. The number of independent measurements is indicated in parentheses.

Fig. 5. Hydrogen production in the *cy6nac2-49* strain. (*A*) Cells of *cy6nac2-49* were grown in TAP medium lacking copper. The arrow indicates the time at which copper was added to the culture to inhibit *Nac2* expression and PSII synthesis. Measurements of O_2 and H_2 were performed in the liquid phase by diffusion of the gases through a polypropylene membrane into the ion source of a mass-spectrometer. The calculated accumulation of the gases (i.e., the amounts that have actually been produced by the cells) is shown in the *Upper* panels of *A* and *B*; it is derived from the concentration measurements by taking into account the slight consumption of gases by the mass spectrometer. During one cycle, 20 μmol H_2/liter was produced corresponding to a maximal rate of 1 mmol H_2 mol^{-1} Chl s^{-1}. These rates varied from one experiment to another and reached in some cases 3.1 mmol H_2 mol^{-1} Chl s^{-1}. Under conditions permissive for photosynthesis (Cu-deprived medium), the net rate of oxygen evolution was 23 mmol O_2 mol^{-1} Chl s^{-1} in *cy6Nac2.49* cells. The decline in O_2 because of respiration causes anaerobiosis, which leads to hydrogen production after 250 min. Because of anaerobiosis, the *Cyc6* promoter is turned on later, leading to PSII synthesis and oxygen evolution and inactivation of the hydrogenase. (*B*) Control: *cy6Nac2.49* cells without copper treatment. For oxygen in *A* and *B*, both the calculated accumulation (*Upper*) and the concentration in the medium (*Lower*) are given. (*Lower*) Indicates when anaerobiosis is attained.

s^{-1} (between 1 and 3.1 mmol of H_2 per mol of Chl per s), slightly lower than that obtained with sulfur-starved cells (12), and of much shorter duration (≈1.5 h vs. 3–4 days).

An interesting feature of the *Cyc6* promoter is that it is also induced under anaerobic conditions even in the presence of copper (9). One would therefore expect that, once anaerobic conditions have been reached and hydrogenase is induced in *cy6Nac2.49* cells, Nac2 synthesis resumes, PSII is synthesized, and oxygen levels rise, thus inactivating hydrogenase and blocking hydrogen production. This process was indeed observed. PSII synthesis was switched on during the anaerobic hydrogen production phase with concomitant O_2 evolution and inactivation of hydrogenase so that the hydrogen levels remained constant (Fig. 5*A*). As control, the same cell culture was examined without addition of copper. Under these conditions, no hydrogen was produced (Fig. 5*B*) with a constant production of O_2 and a gradual decrease of CO_2 as observed in the copper-treated cells (data not shown). Because the *Cyc6* promoter is expected to

be switched off under aerobic conditions in copper-replete medium, a new cycle of hydrogen production would be expected. To test this possibility further, *cy6Nac2.49* cells were grown in TAP medium lacking copper in a sealed vessel for 50 h, and measurement of hydrogen and oxygen were performed by mass spectrometry. The results suggest that two successive phases of hydrogen and oxygen production occurred (SI Fig. 8). It remains to be seen whether the Cyc6-Nac2 system can be further improved for sustained cyclic hydrogen production in *Chlamydomonas*.

Discussion

Inducible Chloroplast Gene Expression System. In this work, we have developed an inducible chloroplast gene expression system in *Chlamydomonas*. Several attempts have been made in land plants for expressing specific nucleus-encoded chloroplast proteins with inducible promoters. The gene of a chloroplast omega-3 fatty acid desaturase was fused to a cold-inducible promoter and introduced into tobacco plants (21). The transgenic plants displayed enhanced cold tolerance. In another study, several glycine betaine-producing transgenic lines were produced in which a bacterial choline oxidase gene fused to a chloroplast targeting sequence was expressed under the control of an ABA-inducible promoter, which is induced under salt stress (22).

T7 RNA polymerase-dependent plastid gene transcription has been developed in land plants (3, 4). This system was used for expressing a single chain camel antibody fragment from a plastid transgene whose expression was driven by a plastid targeted T7 RNA polymerase under the control of a light-inducible promoter (23). However, expression of the plastid transgene occurred even under noninducible conditions, and the plants were pale-green and their growth was severely affected. One problem with this system seems to be the high specific activity of T7 RNA polymerase and its great stability in chloroplasts (5). Therefore, even a very low level of expression of T7 RNA polymerase can lead to significant accumulation of T7 RNA polymerase-dependent transcripts in chloroplasts.

The chloroplast-inducible gene expression system developed in this study takes advantage of the properties of the nucleus-encoded Nac2 factor of *C. reinhardtii*, whose expression is driven by the *Cyc6* promoter. This promoter is repressed by copper and induced by copper deprivation or in an anaerobic environment (8, 9). It is at present the tightest inducible promoter in *C. reinhardtii*. The Nac2 protein is specifically required for the accumulation of the *psbD* mRNA encoding the reaction center polypeptide D2 of PSII. In this system, PSII synthesis can be stopped in a reversible manner while maintaining all other photosynthetic complexes in the thylakoid membrane. Upon shut-off of the expression of *Nac2*, it takes 20 h to deplete cells of PSII (Fig. 3). This period depends on the half-lives of the Nac2 mRNA, of the Nac2 protein, and of PSII, which depends on the light conditions as high light is known to enhance D1 turnover. It is also possible to start with cells lacking PSII and to study its *de novo* synthesis and assembly in fully developed thylakoid membranes. However, in this case, the copper-containing medium needs to be replaced by a copper-free medium, and there is a lag of 24 h before the internal copper is exhausted.

The Cyc6-Nac2 System Can Act as a General Inducible Chloroplast Gene Expression System. The Nac2 factor is specifically required for the stable accumulation of the *psbD* RNA encoding the PSII reaction center subunit D2. Moreover, its target site is comprised within the *psbD* 5′ UTR because fusion of this leader sequence to any coding sequence renders the stability of this chimeric transcript dependent on Nac2. Because of this feature, the Cyc6-Nac2 system can be extended in principle to any chloroplast gene or transgene simply by fusing its coding sequence to the 230-nt *psbD* promoter-5′ UTR region. However, one limitation is that the strain used in this work will be PSII-deficient under noninducible conditions. To circumvent this problem, we have replaced the *psbD* 5′ UTR by the *petA* 5′ UTR in the strain *Ind41-18*, thus abolishing the dependence of *psbD* RNA accumulation on Nac2.

To test whether the Cyc6-Nac2 system can be indeed used for any chloroplast gene fused to the *psbD* 5′ UTR, we have tested this property by inserting a chimeric *psbD* 5′ UTR-*aadA* construct into the chloroplast genome of the *Ind41-18* strain. As expected, the transformants express *aadA* and are resistant to spectinomycin in the absence of copper. In copper-replete medium, *aadA* is no longer expressed, and the cells are sensitive to spectinomycin. In this respect, it is noticeable that the inducible system works with AadA, which is known to have a high specific activity. Thus, there is only a small amount of leaky expression under noninducing conditions. This feature may be very important for expression of toxic foreign proteins in the chloroplast. This system thus provides a tool for probing the function of plastid genes and for expressing new proteins in the chloroplast of *Chlamydomonas*.

A similar strategy might be possible for establishing an inducible gene expression system in land plants. Indeed, nucleus-encoded plastid factors that act similarly to Nac2 have been identified in *Arabidopsis*. One of these proteins, HCF107, is specifically required for the intercistronic processing of the *psbH* 5′ UTR or the stabilization of 5′ processed *psbH* transcripts arising from the *psbB-psbT-psbH-petB-petD* operon. HCF107 seems to be the orthologue of Mbb1, a chloroplast protein of *C. reinhardtii*, which is also specifically involved in the processing and stability of the *psbB-psbH* transcripts, and the target site of this protein is within the *psbB* 5′ UTR (24). It is interesting that Nac2, Mbb1, and HCF107 contain many TPR (tetratricopeptide)-like repeats. Because chloroplast transformation is not yet possible in *Arabidopsis*, the target site of HCF107 is not known. Once this system is established, the same strategy as in *Chlamydomonas* could be used for *Arabidopsis*.

Inducible Hydrogen Production in *Chlamydomonas*. An interesting property of the Cyc6-Nac2 system is that it allows one to arrest PSII synthesis by adding copper to the growth medium. Loss of PSII activity leads to the decrease of oxygen evolution below the rate of oxygen consumption by respiratory processes, allowing the culture to reach anoxia in the light. Further, as a result of the tight interplay between photosynthesis and respiration, and as long as PSII activity remains lower than respiration, all of the oxygen that might be produced by residual PSII is consumed through mitochondrial activity, thus leading to anoxic conditions, which are required for hydrogenase function (15). This property is at the basis of this study and of other experimental designs for sustainable evolution of hydrogen by *Chlamydomonas*. One advantage of our system is that anaerobiosis can be achieved simply by adding copper to the culture medium and the cells remain healthy. This procedure thus differs from the classical method in which PSII is inactivated through sulfur depletion, a condition that leads to impairment of cell growth and eventually to cell death. The maximal rates of hydrogen production obtained with the *cy6Nac2.49* strain are slightly lower than those achieved through sulfur deprivation (Fig. 5 and *SI Materials and Methods*). However, this production is transient because the *Cyc6* promoter is induced by anaerobiosis and as a consequence PSII recovers and reinitiates an aerobic phase.

This apparent limitation may turn out to be an advantage, as it opens the possibility of establishing a cycling hydrogen producing system, based on alternate expression and repression of Nac2, without the need for removing copper from the medium. Such a system would accumulate carbon reserves during the oxygenic phases, a part of which would be converted to hydrogen during the anaerobic phases before PSII activity has been restored to a level that is sufficient for inhibiting hydrogenase. A system of this type would be particularly interesting in the context of hydrogen production because it would avoid changes of growth media, which consume energy, and it could operate in the absence of micronutrient starvation, which may limit cell viability.

Further improvements of the system need to take into account

that hydrogen production depends on other factors besides the turn-off of PSII activity, namely starch accumulation, which feeds electrons downstream of PSII into the photosynthetic electron transport chain and sustains respiration required for consuming the remaining O_2 produced by the residual activity of PSII (13–15). Additionally, there is competition of hydrogen production with other electron sinks such as the Calvin–Benson cycle and cyclic electron flow. It is in principle possible to modify genetically *Chlamydomonas* to circumvent these limitations.

Materials and Methods

Strains and Media. The *nac2-26* mutant strain has been described (6, 7). The *cyc6Nac2.49* strain contains a trans-gene consisting of the *Cyc6* promoter fused to the *Nac2* midi-gene inserted into the nuclear genome of the *nac2-26* mutant (Δ*nac2*::*cy6proNac2*). *Ind41* was derived from *cy6Nac2.49* by replacing the *psbD* promoter and 5′ UTR with a 675 fragment containing the *petA* promoter and 5′ UTR (25) (Δ*nac2*::*cy6proNac2*::*5′petA-psbD*). *Ind41-18* is related to the *Ind41* strain, except that the *aadA* cassette in the *Ind41-18* strain has been completely excised from the chloroplast DNA and the strain is therefore sensitive to spectinomycin (Δ*nac2*::*cy6proNac2*::*5′petA-psbD*[Spc^S]). *Ind_aadA_117* was derived from *Ind41-18* and contains the *aadA* cassette driven by the *psbD* promoter and 5′ UTR inserted downstream of the *atpB* gene (Δ*nac2*::*cy6proNac2*::*5′petA-psbD*::*5′psbD-aadA*).

All strains were maintained on TAP medium supplemented with 1.5% Bacto-agar (26) at 25°C under dim light. In experiments where copper-supplemented or copper-deficient ($-Cu^{+2}$) solid agar and liquid TAP and HSM medium was used, medium was prepared according to Quinn and Merchant (27). TAP and TAP-Cu^{+2} media were supplemented with 100 μg/ml spectinomycin (Sigma–Aldrich) or 20 μg/ml paromomycin (Sigma–Aldrich) where necessary. In experiments where cells were deprived of oxygen, liquid cultures where bubbled with N_2 gas with 150 rpm/min agitation and constant light illumination (20 $\mu E \cdot m^{-2} \cdot s^{-1}$). Cell density was determined by using a hemacytometer.

Plasmid Construction. Standard techniques were used to manipulate and analyze all plasmid constructs (28). Sequencing of constructs was carried out by using BigDye terminator sequencing kit (Applied Biosystems, La Jolla, CA) and an ABI Prism 377 automated sequencing machine. The bacterial host used for cloning in *Escherichia coli* was DH10B (GE Healthcare, Oelfingen, Switzerland). All oligonucleotides were ordered from Microsynth GmbH (Balgach, Switzerland).

Transformation of *Chlamydomonas* Cells. Nuclear transformation of *C. reinhardtii* strains *nac2-26* was performed by electroporation essentially as described (29). For details, see *SI Materials and Methods*.

Chlorophyll and Oxygen Evolution Rate Measurements. Oxygen evolution and respiration rates were determined by using a Clark-type oxygen electrode attached to an X-type light source at 25°C (Hansatech Instruments, Norfolk, U.K.).

Hydrogen Measurements and Calculations. All liquid phase and gas phase measurements of N_2, O_2, H_2, and CO_2 were performed as follows. Continuous monitoring of dissolved gases was made by using a sealable, thermo-stated Clark-type vessel as described (30) where gases were fed into the ion source of a mass spectrophotometer (model MM 880; VG Instruments, Cheshire, U.K.) through a polypropylene membrane under continuous agitation and constant illumination using a fiber-optic illuminator (model KL 1500; Schott, Mainz, Germany) (30). In experiments where transcription of the *Cy6Nac2* transgene was repressed, 12 μM copper was added to the growth medium within the vessel (TAP-Cu^{+2} to TAP). Calibration of the mass spectrometer before all gas phase time points was achieved through injection of air and pure hydrogen gas samples.

Assay for *aadA* Activity. Assays for *aadA* activity were carried out on WT, Ind41-18, and Ind_aadA_117 strains essentially as described (31), except that ^{32}P-labeled dATP was used in place of the radiolabeled rATP used in the original experiments.

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