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Marker-free genetic engineering of the chloroplast in the green microalga *Chlamydomonas reinhardtii*

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Summary

The work applied a transgene expression method based on the replacement of an inactive *rbcL* gene as the selection marker in *Chlamydomonas reinhardtii* chloroplasts. The native *rbcL* gene in strain CC2653 has a point mutation that causes early translation termination, thus resulting in a photosynthesis mutant. Recovery of *rbcL* function for selection is offered along with the heterologous expression of the alcohol dehydrogenase *ADH1* gene from *Saccharomyces cerevisiae* in the *Chlamydomonas* chloroplast. The *CrCpADH1* gene was inserted via double homologous recombination in the *psaB-rbcL* chloroplast intergenic region of recipient strain CC2653, using the *psaB* and *rbcL* gene sequences for the double homologous recombination. This transformation conferred a functional *rbcL* gene and expression of the *CrCpADH1* transgene in the recipient strain. This method alleviated the need to use antibiotics for selection, resulting in a negligible number of false positives during screening, and attaining a transformation efficiency greater than 90%. The approach also ensured segregation of chloroplast DNA copies, so as to achieve homoplasmy of the transformant chloroplast DNA, with a concomitant elimination of recipient strain Cp DNA. High levels of steady-state *CrCpADH1* transcripts were detected in the homoplasmic transformants. However, CrCpADH1 protein levels were attenuated under continuous illumination growth conditions due to oxygen accumulation in the cells. Under conditions of low oxygen partial pressure, or anoxia, accumulation of CrCpADH1 protein in the cells and ethanol in the growth medium was observed. A metabolic pathway for ethanol production is proposed in *Chlamydomonas,* mediated by the chloroplast-localized CrCpADH1 transgenic enzyme.

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* has been used as a model organism for more than 50 years, serving to advance the fields of genetics, biochemistry and cell physiology. It is capable of photoautotrophic growth with inorganic carbon sources, such as CO_2 or bicarbonate, as well as heterotrophic growth with acetate as the sole carbon source. Ability to grow on acetate heterotrophically permits the organism to dispense with photosynthesis, greatly facilitating the analysis of photosynthesis mutants, thus contributing to gene discovery and elucidation of gene function. In addition to extensive use of *C. reinhardtii* as a model organism for basic research, there is interest to use this green microalga as a cellular factory for the commercial production of proteins, fuels and chemicals (for reviews see Griesbeck *et al.*, 2006; Walker *et al.*, 2005; Mayfield *et al.*, 2007; Specht *et al.*, 2010; Melis, 2012). Many such efforts focused on the use of the chloroplast as the site of transgene expression. Advantages of chloroplast versus nuclear transformation for transgene expression include lack of post-translational modification and gene-silencing mechanisms in the prokaryotic origin chloroplast; the technical ability to do targeted gene integration by homologous recombination, which is not currently possible for nuclear transformation; and the use of polycistronic expression constructs for simultaneous multiple gene expression. Through the expression of reporter or therapeutic proteins, a number of genetic elements have been identified that play important roles in regulating transgene expression in the chloroplast of *C. reinhardtii.* These include different chloroplast gene promoter and 5′ UTR sequences, gene coding sequences and nuclear factors that could further influence the level of transgene expression (Coragliotti *et al.*, 2011; Ishikura *et al.*, 1999; Kasai *et al.*, 2003; Mayfield *et al.*, 2007; Michelet *et al.*, 2011; Rasala *et al.*, 2010; Specht *et al.*, 2010). An inducible gene expression system in the chloroplast of *C. reinhardtii*, upon introduction of the *lac* regulation system from *Escherichia coli*, has been demonstrated (Kato *et al.*, 2007). Recent reviews provided comprehensive information on chloroplast transformation including that of *C. reinhardtii* (Day and Goldschmidt-Clermont, 2011; Fletcher *et al.*, 2007; Purton, 2007).

Chloroplast transformation in green microalgae commonly relies on the use of antibiotics as selection method for the isolation of transformants. For example, mutated ribosomal RNA genes conferring streptomycin or erythromycin resistance (Harris, 1989), or the aminoglycoside adenine transferase (*aadA*), conferring both spectinomycin and streptomycin resistance (Goldschmidt-Clermont, 1991), are used. However, isolation of transformants based on antibiotic resistance is subject to pitfalls: (i) method is subject to isolation of false positives due to the high frequency of rRNA mutation under antibiotic treatment; (ii) there is a low efficiency of transformation or co-transformation based on independent streptomycin and transgene transformations; (iii) it is challenging to attain transgenic chloroplast DNA copy segregation (homoplasmy) under antibiotic resistance pressure;

and (iv) there are environmental and regulatory issues associated with the use of antibiotics under mass culture and commercial production conditions.

In the present work, all of the above issues were eliminated with the application of a recovery of function selection for the isolation of transformant *C. reinhardtii* strains. The principle of such method was initially demonstrated by Boynton *et al.* (1988), who employed replacement of an inactive *atpB* gene as the selection marker in *Chlamydomonas reinhardtii* chloroplast transformations. Likewise, in this work, a more efficient replacement of an inactive *rbcL* gene was used as the selection marker in *Chlamydomonas* chloroplast transformations. Superior transformant recovery results were obtained with the *rbcL* gene replacement method, better than what has been observed with the streptomycin-based transformation of *C. reinhardtii* that is commonly used in the field. Case study of the recovery of function approach is offered in this work with the heterologous expression of the alcohol dehydrogenase *ADH1* gene from *Saccharomyces cerevisiae* in the chloroplast of *Chlamydomonas reinhardtii,* offering the possibility to assess the efficacy of *C. reinhardtii* in the production of ethanol. The work demonstrates an approach, different from that of other applications in the field, entailing expression of a transgene for product generation.

Results

Expression vector for *Chlamydomonas reinhardtii* chloroplast transformation

The alignment of the nucleotide sequence from the *Saccharomyces cerevisiae ADH1* gene *(ScADH1)* and the corresponding codon-optimized gene for *Chlamydomonas reinhardtii* chloroplast transformation (*CrCpADH1*) is shown in Figure S1. The *CrCpADH1* expression cassette includes 350 bp of the *rbcL* gene promoter-5′UTR region, plus the first 90 bp of the *rbcL* coding sequence (Figure 1, blue font), followed by the *CrCpADH1* sequence including a 6xHis-tag at the 3′ end (Figure 1, black font), and ending with 322 bp of the *rbcL* gene 3′UTR-terminator region, including the *rbcL* stop codon (Figure 1, red font). It was previously shown that the 5′ end of the *rbcL* coding sequence was essential not only for expression of the endogenous *rbcL* gene, but also for the expression of a reporter gene (Kasai *et al.*, 2003; Klein *et al.*, 1994). Inclusion of the first 90 bp of the *rbcL* coding region of this construct was thus designed to confer a greater efficiency of translation and improved stability of the *CrCpADH1* transgenic protein in the chloroplast. The entire *CrCpADH1* expression cassette was inserted into the intergenic region of *psaB* and *G(tRNAG)* genes in plasmid pGEMT67Sma (Experimental procedures) through the SmaI restriction site. The resulting expression vector, which also contains a wild-type copy of the entire *rbcL* coding sequence, was termed 'pCrCpADH1', a map of which is shown in Figure 2. Plasmid pCrCpADH1 was used to transform the chloroplast of the RuBisCO mutant CC2653 strain, through biolistic gene delivery system. Double homologous recombination in this case would take place between the entire *rbcL* and *psaB* genes of pCrCpADH1 and chloroplast DNA. The strategy in this case was to use a functional copy of the *rbcL* gene from the expression vector pCrCpADH1, in conjunction with *CrCpADH1* transgene, to simultaneously replace the mutated endogenous *rbcL* gene in the CC2653 recipient strain and to confer *ADH1* transgene expression, through the double homologous recombination. Replacement of the native mutated and nonfunctional *rbcL* gene in the recipient strain by a functional copy of the *rbcL* gene in the pCrCpADH1 expression vector conferred photoautotrophic growth properties to transformant *C. reinhardtii*. This recovery of function and recovery of photoautotrophic growth property served as the basis for the subsequent isolation of chloroplast transformants.

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AATAAATATATTTATAAATTGATTGTTCTTAGAGCTAAAAGAGAAGAACAATGGGTTTAT
CTAATAAAGTCTACTATATTGGAGAGGAGTGAACAGTGGCCTCGCTTATCCCCGACAGGA
ATATACATGGTTTTAGTAAGTAAACTGCGTAAGACGACCGACATATACCTAAAGGCCCTT
TCTATGCTCGACTGATAAGACAAGTACATAAATTTGCTAGTTTACATTATTTTTTATTTC
TAAATATATAATATATTTAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTC
CGGACAGATTATTTTAGGATCGTCAAAAGAAGTTACATTTATTTATATAAATGGTTCCAC
AAACAGAAACTAAAGCAGGTGCTGGATTCAAAGCCGGTGTAAAAGACTACCTTTAACAT
ACTACACACCTGATTACGTAATGTCAATTCCTGAAACTCAAAAAGGTGTTATTTTCTATG
                    CrCpADHF1->
AATCTCACGGTAAATTAGAATACAAAGACATTCCAGTACCTAAACCAAAAGCTAATGAAT
TATTAATTAACGTTAAATACTCAGGTGTATGTCATACTGACTTACATGCTTGGCACGGTG
ATTGGCCTTTACCAGTAAAATTACCTTTAGTTGGTGGTCACGAAGGTGCAGGTGTTGTAG
TAGGTATGGGTGAAAATGTTAAAGGTTGGAAAATTGGTGATTATGCTGGTATTAAATGGT
TAAACGGTTCATGTATGGCATGTGAATATTGTGAATTAGGTAATGAATCTAACTGTCCTC
ATGCAGATTTATCAGGTTATACACACGATGGTTCTTTTCAACAATATGCAACTGCTGACG
CTGTTCAAGCTGCTCATATTCCTCAAGGTACAGACTTAGCACAAGTAGCACCAATTTTAT
CrCpADHF2->
GTGCTGGTATTACTGTATATAAAGCTTTAAAAAGTGCTAACTTAATGGCAGGTCATTGGG
TTGCTATTAGTGGTGCAGCTGGTGGTTTAGGTTCATTAGCTGTACAATATGCAAAAGCTA
TGGGTTATCGTGTATTAGGTATTGATGGTGGTGAAGGTAAAGAAGAATTATTTCGTTCTA
TTGGTGGTGAAGTTTTCATTGATTTTACAAAAGAAAAAGATATTGTAGGTGCTGTTTTAA
AAGCTACAGATGGTGGTGCACACGGTGTTATTAACGTATCAGTATCTGAAGCTGCTATTG
          <-CrCpADHR2
AAGCTTCTACTCGTTATGTTCGTGCTAACGGTACAACTGTTTTAGTTGGTATGCCTGCTG
GTGCTAAATGTTGTAGTGATGTTTTCAACCAAGTAGTAAAAAGTATTTCTATTGTTGGTT
CATACGTTGGTAATCGTGCAGATACTCGTGAAGCATTAGATTTTTTTGCTCGTGGTTTAG
TAAAATCACCTATTAAAGTAGTTGGTTTATCAACTTTACCAGAAATTTATGAAAAAATGG
AAAAAGGTCAAATTGTAGGTCGTTATGTTGTTGATACTAGTAAACATCACCATCACCATC
                <-CrCpADHR1
ACTAATTTTTATTTTTCATGATGTTTATGTGAATAGCATAAACATCGTTTTTATTTTTTA
TGGTGTTTAGGTTAAATACCTAAACATCATTTTACATTTTTAAAATTAAGTTCTAAAGTT
ATCTTTTGTTTAAATTTGCCTGTGCTTTATAAATTACGATGTGCCAGAAAAATAAAATCT
TAGCTTTTTATTATAGAATTTATCTTTATGTATTATATTTTATAAGTAATAAAAGAAATA
GTAACATACTAAAGCGGATGTAACTCAATCGGTAGAGTGCGATCCTTCCAAGTTCGAGGT
TGTGGGTTCGAGTCCCATCATCCGCTA
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Figure 1 Sequence of the *CrCpADH1* expression cassette for *Chlamydomonas reinhardtii* chloroplast transformation. The *rbcL* promoter employed, including the first 90 nucleotides of the coding sequence, is indicated in blue font in which the *rbcL* initiation codon is underlined; The *CrCpADH1* coding sequence is in black, and the initiation codons are in red font underlined; the *rbcL* terminator sequence used is in red. The primers used in RT-PCR experiments are underlined and named under each corresponding sequence.

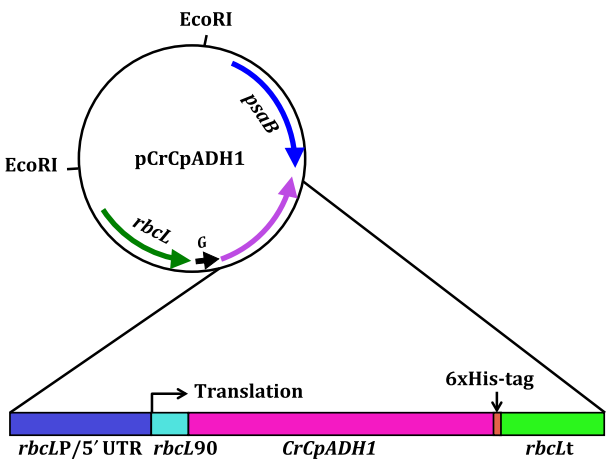

Figure 2 Map illustrating the structure of pCrCpADH1 vector. The CrCpADH1 expression cassette was cloned into the intergenic region of the chloroplast *PsaB-G*(*tRNAG*)*_rbcL* genes. The sequence of each element in the CrCpADH1 expression cassette can be found in Figure 1.

Analysis of chloroplast transformants

Application of the *rbcL* recovery of function method resulted in the generation of >30 transformants per plate, corresponding to a transformation efficiency of ~100 transformants/10 μg DNA, when 1 μm of gold particles was used for biolistic transformation. Higher transformation efficiencies were achieved upon using 0.6 μm of gold particles, in which case more than 100 transformants in average per plate could be obtained (Figure 3). Pretreatment of the culture with 5-fluoro-2′-deoxyuridine helped to attain higher transformation efficiencies. It was noted that such treatment slowed down CC2653 cell growth substantially and that it took 5–7 days to reach the mid-log growth phase (Experimental procedures). For the case study, in this work, seven independent transformant lines were randomly selected for further testing. These putative transformants were first tested via genomic DNA PCR analysis for the presence of the *CrCpADH1* transgene. Using *CrCpADH1* gene-specific primers (Experimental procedures) resulted in positive amplification of an anticipated 322-bp product in all randomly selected transformant lines (Figure 4a, lanes 1–7) but not in the mt *minus* strain CC2918 (Figure 4b, lane 1) or the mt *plus* CC2653 recipient strain (Figure. 4B, lane 2). To increase confidence that lack of *CrCpADH1* gene sequence amplification in the mt *minus* CC2918 control and CC2653 recipient strains is not due to the DNA preparation from these samples, mating type–specific primers were used in a separate PCR experiment to test the quality of these DNA samples. Both CC2918 and CC2653 strains showed the anticipated PCR products, that is, 600 bp for the mating type *minus* CC2918 (Figure 4b, lane 3) and 500 bp for the mating type *plus* CC2653 (Figure 4b, lane 4).

Chloroplast DNA homoplasmy in the *CrCpADH1* transformants was also tested by PCR analysis, following two consecutive transfers of the putative transformants onto fresh HS minimal medium agar plates with 2- to 3-week intervals. The position of forward *psaB*3′ and reverse *rbcL*3′ primers used to test homoplasmy of the chloroplast DNA in the transformants is shown in Figure 5a (see also Experimental procedures). With such primers, wild-type copies of the *C. reinhardtii* chloroplast DNA are expected to generate a 413-bp product. Transgenic copies of the *C. reinhardtii* chloroplast DNA are expected to generate a 2268-bp product instead. In addition, this set of primers would also permit the amplification of a 292-bp fragment with the transgenic chloroplast DNA because of the *rbcL* terminator in the transgene expression cassette. In our PCR experiments, only small DNA fragments such as the 413-bp or the 292-bp fragments could be amplified because of the short extension time used in the reaction settings.

Generation of both 413-kb and 292-kb products would indicate the presence of both wild-type and transgenic DNAs in the chloroplast, with a copy ratio in direct proportion to the intensity of the respective signal. Eight transgenic lines were thus tested (Figure 5b). Four lines showed homoplasmy of their chloroplast DNA (Figure 5b, lanes 2, 4, 7 and 8) in which a single PCR product of 292 bp was generated. Two transformant lines (Figure 5b, lanes 5 and 6) showed amplification of

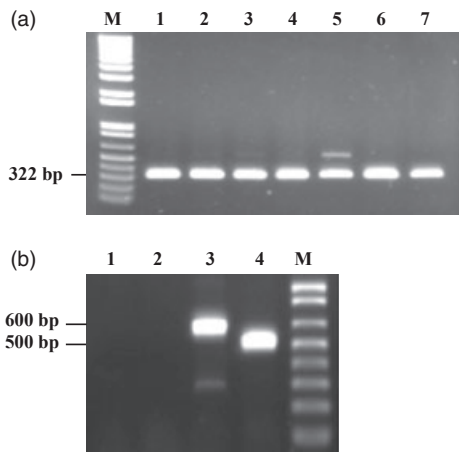

Figure 4 Analysis of seven transformant colonies that grew under selective (photoautotrophic) conditions. (a) Lanes 1 through 7 represent the genomic PCR results of seven independent transgenic lines. M: 1-kb plus ladder used as DNA size marker; (b) Lanes 1 and 2 show the negative PCR results of CC2918 and CC2653, respectively, when using *CrCpADH1*-specific primers. Lane 3: CC2918 mating type test by genomic PCR using mating type *minus* specific primers. Lane 4: CC2653 mating type test by PCR using mating type *plus* specific primers. *CrCpADH1*-specific primers used for this analysis are CrCpADHF2 and CrCpADHR2, and the corresponding sequences are given in Experimental procedures and also indicated in Figure 1.

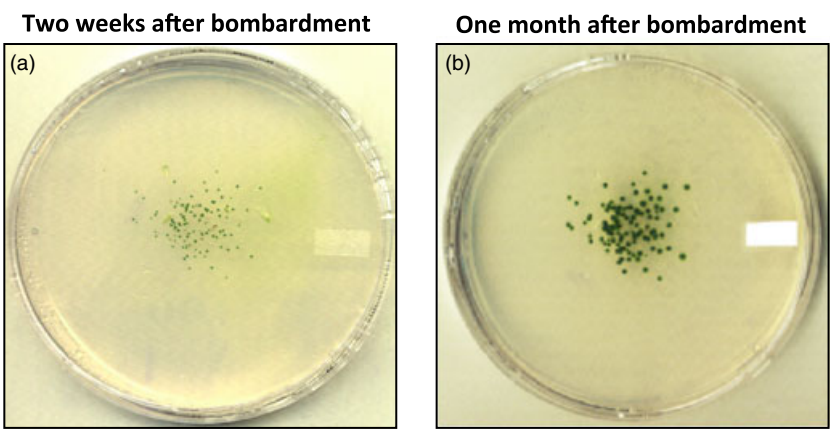

Figure 3 Formation of transgenic colonies on HS minimal media following the biolistic transformation of *Chlamydomonas reinhardtii* recipient strain CC2653. Untransformed CC2653 cells bleached under these conditions (note the cloudy green areas on the plates), but transformed cells grew into colonies upon restoration of autotrophic growth. Plates were photographed either 2 weeks (a) or 1 month (b) after bombardment.

the 413-bp products, derived from wild-type chloroplast DNA sequences. These lines were tested positive for the presence of the *CrCpADH1* transgene, confirmed using *CrCpADH* gene-specific primers in genomic PCR analysis. Thus, the predominant wild-type PCR fragments shown in Figure 5, lanes 5 and 6, indicate that wild-type copies of chloroplast genome were considerably more abundant than transgenic genome copies in the chloroplast of these cells. Lastly, two other lines (Figure 5b, lanes 1 and 3) contained a mixture of 413- and 292-kb products, apparently derived from chloroplasts that contained both wild-type and transgenic copies of chloroplast genomes. Control measurements were also conducted with the same set of primers showing that *Chlamydomonas reinhardtii* CC3269 wild-type (Figure 5c, lane c1) and the CC2653 recipient strains (Figure 5c, lane c2) generated only the 413-bp fragment, whereas transformant lines # 4 and # 7 (Figure 5c, lane 4 and 7, same as Figure 5b, lane 4 and 7) generated only the 292-bp fragment. Similarly, the pCrC-pADH1 plasmid control also generated the 292-bp fragment only (Figure 5c, lane 5).

Further rigorous molecular and biochemical screening of homoplasmic *CrCpADH1* transformant strains was undertaken. Lines 4 and 7 (from Figure 5b,c) were selected and henceforth termed 'chloroplast transformant line 4 (CpT-4)' and 'chloroplast transformant line 7 (CpT-7)'. Southern blot analysis was employed to examine the integration of the transgenic expression cassette in the chloroplast genome of transgenic lines CpT-4 and CpT-7. Wild-type strain CC3269 and the recipient strain CC2653 were also included in this analysis as controls. Total genomic DNA was digested with EcoRI and BamHI restriction enzymes. These are expected to generate an EcoRI-BamHI 3.9-kb fragment in the wild type and 5.8-kb fragment in the transformants (Figure 6a). Conversely, a BamHI-BamHI fragment would be sized at 1.1 kb for both control and transformant lines. Four probes were used independently in four different hybridization experiments, targeting the following regions of DNA: probe # 1 targeting the *rbcL* gene; probe # 2 targeting the *rbcL*90-*CrCpADH1* domain; probe # 3 targeting the *psaB gene*; and probe # 4 targeting the *CrCpADH1* gene (Figure 6a).

As shown in Figure 6b-e, the #1 *rbcL* probe hybridized to 3.9-kb DNA fragments in the CC3269 wild-type and CC2653 recipient strains, whereas it hybridized to 5.8-kb fragments in both the CpT-4 and CpT-7 transformants (Figure 6b). A similar result was observed with the #2 rbcL90-ADH1 fusion probe (Figure 6c). Noted in this case was the much stronger 5.8-kb hybridization signal from the transformants than the 3.9-kb signal from the wild-type and recipient strains, reflecting the greater portion of the *CrCpADH1* sequence in the probe than the 5'UTR of the *rbcL* gene. The #3 *psaB* probe hybridized to a 1.1-kb fragment in all samples, as would be expected on the basis of restriction fragment analysis (Figure 6d). An extra faint band at 5.3 kb was also noted in this analysis, attributed to sequence similarity between a partial sequence of the *psaB* probe used here and the nucleotide sequence of the *PsaA* exon 3, located within an EcoRI-BamHI fragment of 5.3 kb of the chloroplast genome. The #4 *CrCpADH1* probe hybridized to 5.8-kb fragments in the transformants only, consistent with the presence of the *CrC-pADH1* transgene in the CpT-4 and CpT-7 transformants only (Figure 6e).

Expression analysis of *CrCpADH1* in *Chlamydomonas reinhardtii* chloroplasts

Steady-state levels of *CrCpADH1* transcripts were measured by RT-PCR in lines CpT-4 and CpT-7 grown under continuous illumination and oxygen evolution conditions. Using *CrCpADH1* gene-specific primers, evidence of expression of the *CrCpADH1* gene was obtained from the presence of a 720-bp product in the transformants (Figure 7a). As an internal control of endogenous chloroplast gene expression, Figure 7b shows the RT-PCR analysis result with *rbcL* gene-specific primers, where a 495 bp was generated in both strains. These results proved that transcription of the *CrCpADH1* transgene is not impaired in the light.

For negative controls, RNA samples were used without subjecting them to the reverse transcription reaction. When

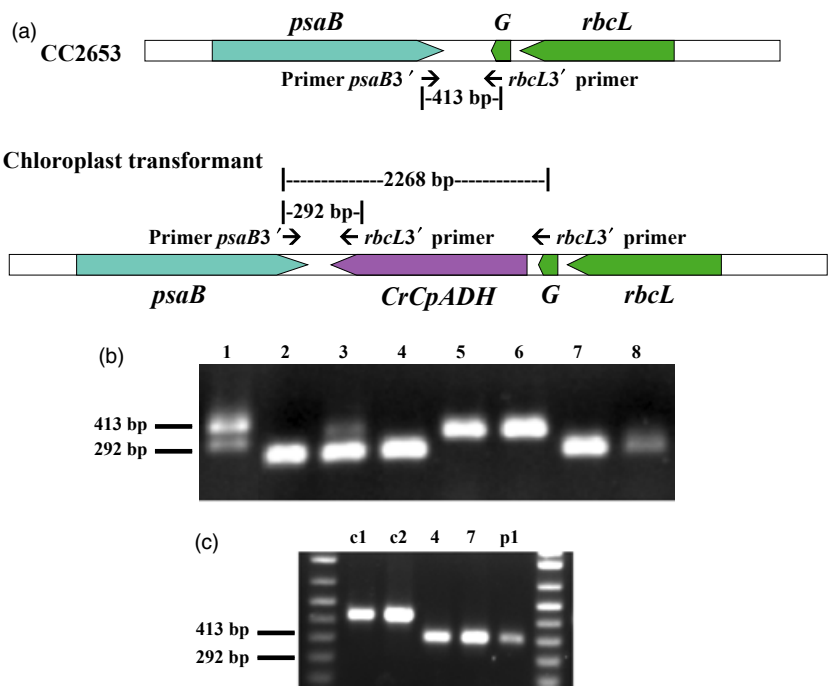

Figure 5 (a) Illustration of the endogenous chloroplast DNA EcoRI 14 fragment and the transgenic DNA fragment used for chloroplast transformation. Locations of the primers, *psaB*3′ and *rbcL*3′, used for homoplasmy test are indicated, as well as the predicted sizes of the PCR fragments. (b) Homoplasmy test of the eight transformants by PCR using specific primers as indicated in (a). (c) PCR analysis using specific primers as indicated in (b) above. Lane c1: CC3269 *Chlamydomonas reinhardtii* wild type; Lane c2: CC2653 *Chlamydomonas reinhardtii* RBCL-less mutant; Lane 4: transformant line # 4 also shown in (b) above. Lane 7: transformant line # 7 also shown in (b) above. Lane p1: pCrCpADH1 plasmid control.

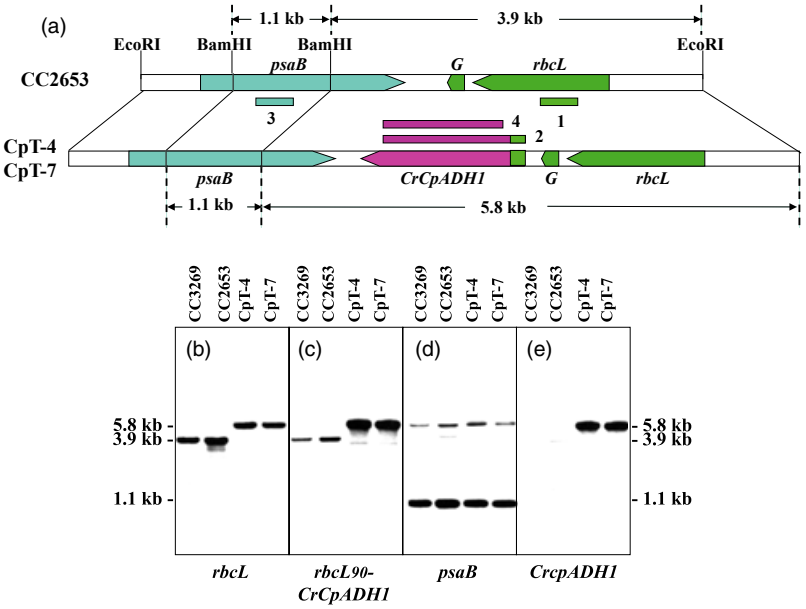

Figure 6 (a) Restriction map of the chloroplast genome regions in the recipient CC2653 and chloroplast transformant CpT-4 and CpT-7 strains, as well as the location of probes used in Southern blot analysis. Four probes were used 1. *rbcL* gene-specific probe. 2. *rbcL*90*CrCpADH1* probe that includes 90 bp of *rbcL* gene and 1065 bp of *CrCpADH1* gene sequence; 3. *psaB* gene-specific probe. 4. *CrCpADH1* gene-specific probe. (b-e). Southern blot analysis of *Chlamydomonas reinhardtii* wild-type CC3269, recipient strain CC2653 and chloroplast transformants CpT-4 and CpT-7. Genomic DNA was digested with EcoRI and BamHI. Four probes were used: *rbcL* gene-specific probe (b), *rbcL*90*CrCpADH1* probe that includes 90 bp of *rbcL* gene and 1065 bp of *CrCpADH1* gene sequence (c), *psaB* gene-specific probe (d), *CrCpADH1* gene-specific probe (e). Strains CC2653 and CC3269 were used as controls.

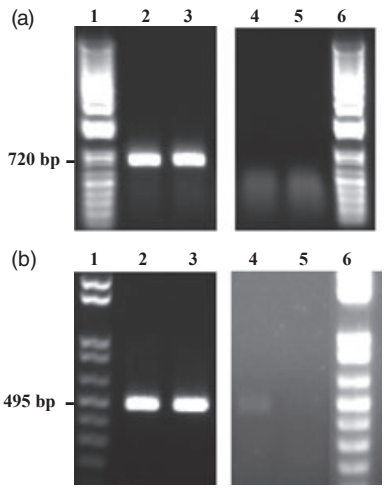

Figure 7 RT-PCR analysis of gene expression in CpT-4 and CpT-7 chloroplast transformants. (a) RT-PCR results using *CrCpADH1* gene-specific primers. Lanes 1 and 6: DNA size markers; Lane 2: CpT-4 cDNA was used as a template; Lane 3: CpT-7 cDNA as template; Lane 4: CpT-4 total RNA was used as a template; Lane 5: CpT-7 total RNA as template. (b) RT-PCR results using *rbcL* gene-specific primers. Lanes as marked in (a). Cells were grown under continuous illumination conditions.

these were used as templates for the PCR, no amplification could be observed (Figure 7, lanes 4 and 5).

Following the positive detection of transgene transcripts, it was important to test for transgenic protein accumulation in the CpT-4 and CpT-7 lines. Specific polyclonal antibodies were generated against the full-length CrCpADH1 apoprotein from which the RuBisCO N-terminal sequence of 30 amino acids was excluded (Figure S2). The specific polyclonal antibodies thus generated were used for the investigation of CrCpADH1 protein expression in the CpT transformants. When cells were grown under 12-h light–12-h dark cycles, significant levels of the CrCpADH1 protein accumulated in both CpT-4 and CpT-7 transformants (Figure 8a). A cross-reaction with a ~43-kD protein was shown specifically in the transformants that was absent in both a wild-type CC3269 (Figure 8a, WT) and the recipient CC2653 strains (Figure 8a, R). It is noted that the level of CrCpADH1 in CpT-4 was lower than that of the CpT-7, probably due to variations in the metabolic status of the culture upon which the cells were harvested for analysis**.** The size of the cross-reacting protein corresponds to the theoretical molecular mass of a protein that includes the CrCpADH1 coding sequence plus the first 30 amino acids of the RBCL. These results indicated that translation is initiated at the ATG codon of the *rbcL*, leading to the synthesis of the rbcL30-CrCpADH1 fusion protein. Translation initiation from the 'native' ATG of the *CrCpADH1* gene (e.g. see Figure 1) was not observed in this work. As a control, Figure 8a also shows cross-reactions of proteins with specific polyclonal antibodies raised against the RBCL. In this case, positive cross-reaction with about equal signal intensity was observed in samples from the wild-type CC3269 (WT) and the CpT-4 and CpT-7 lines. Absence of RuBisCO from the recipient CC2653 was also noted (Figure 8a, R), as expected.

The work further tested different growth conditions for the level of expression of the *CrCpADH1* transgene. In the conduct of such experiments, it became clear that levels of the CrCpADH1 protein were lower under photoautotrophic and photoheterotrophic conditions, than when cells were grown heterotrophically in the dark. Lower levels of CrCpADH1 in the light could not be attributed to the level of transcripts, as similar levels of *CrCpADH1* transcripts were detected in cells that were grown under illumination or in the dark. To better understand the cause of such difference, light-shift experiments were conducted (Figure 8b). Western blot analysis of protein samples from cells grown in the dark showed the presence of the CrCpADH1 protein in the CpT-4 and CpT-7 lines but not in the wild-type CC3269 (Figure 8b, D→D). Growth in the dark, followed by a shift of the sample to illumination for 24 h, however, resulted in substantially lower levels of the CrCpADH1 protein in the cells (Figure 8b, D→L). These results suggested that photosynthesis in the light negatively affected CrCpADH1 protein accumulation and/or stability.

Levels of O_2 in the growth media were found to be substantially different under heterotrophic conditions in the dark versus

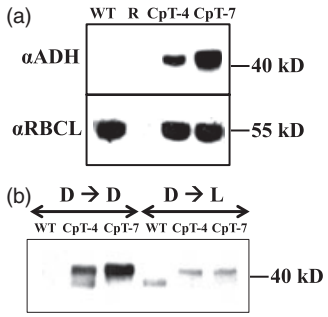

Figure 8 Western blot analysis of CrCpADH1 protein accumulation. (a) Upper panel shows the immunodetection using CrCpADH1-specific polyclonal antibodies: WT: CC3269 wild type, R: recipient CC2653, and Cp-T4 and CpT-7: chloroplast transformant strains. Lower panel shows the immunodetection using RBCL-specific antibodies with protein extracts from the same samples as in upper panel. Specific cross-reactions with a protein of 43 kD were observed only in the CpT transformants. (b) Light-to-dark culture shift assay. (D→D) Dark-to-dark: dark-grown cultures followed by incubation in the dark for 24 h; (D→L) Dark-to-light shift: dark-grown cultures followed by incubation under illumination for 24 h.

autotrophic or heterotrophic conditions in the light. Photoautotrophic or photoheterotrophic growth produces saturating amounts of O_2 in the growth media, and it could be O_2 that causes instability in the CrCpADH1 protein. To test this hypothesis and to delineate the effect of O_2 from a direct effect of irradiance on the protein, we assayed the effect of dichlorophenyl-dimethylurea (DCMU), a powerful photosynthetic electron transport and O_2 evolution inhibitor, on the accumulation of the CrCpADH1 protein. In such experiments, cells were cultivated in the dark or under illumination, with or without DCMU. Rates of cell growth and levels of CrCpADH1 protein accumulation were measured. Figure 9a shows the growth curves of CC3269 WT, CC2653 recipient strain, CpT-4 and CpT-7 transformant strains under continuous light conditions. Similar growth rates were observed for CC3269 WT, CpT-4 and CpT-7 strains, whilst CC2653 recipient strain could not grow under such conditions and so those cells were bleached after 24 h. Figure 9b-d shows the growth curves of CC3269 WT (Figure 9b), CpT-4 (Figure 9c) and CpT-7 (Figure 9d), under three different growth conditions. About the same rates of cell growth were noted in the dark in the absence of DCMU for all three lines (Figure 9b-d, diamonds). Conversely, similar rates of growth were determined for all three strains in the presence of DCMU, irrespective of whether cells were incubated in the light (Figure 9b-d, circles) or in the dark (Figure 9b-d, squares). These results are consistent with the notion that DCMU suppresses photoautotrophic growth of the cultures through the inhibition of photosynthesis, but exerts no toxic effect(s) via which to impede heterotrophic growth.

Western blot analysis with anti-CrCpADH1 antibodies showed lack of CrCpADH1 from wild-type cell extracts, regardless of growth in the presence of DCMU under illumination (Figure 9e WT, lane 1), in the presence of DCMU in the dark (Figure 9b WT, lane 2) or in the absence of DCMU in the dark (Figure 9e WT, lane 3). In contrast, similar levels of CrCpADH1 were detected in CpT-4 and CpT-7 samples grown in the presence of DCMU, regardless of whether they were incubated under illumination (Figure 9e CpT, lanes #1) or in the dark (Figure 9e CpT, lanes #2). This amount of the CrCpADH1 protein present was about the same as that detected from samples grown in the dark in the absence of DCMU (Figure 9e CpT, lanes #3). Taken together, results in Figure 8b and 9e showed that illumination *per se* did not cause loss of the CrCpADH1 protein from the transformant cells, but rather, lower amounts of CrCpADH1 under illumination conditions are probably a consequence of active oxygen production and accumulation in the medium through photosynthesis. This notion was supported further by measurements with photoheterotrophic cultures that were maintained with continuous bubbling with air. Under such conditions, a lower oxygen partial pressure was maintained, and levels of CrCpADH1 were enhanced, often approaching those measured from the dark-grown cells (results not shown). The above analysis revealed a negative impact of oxygenic photosynthesis on CrCpADH1 protein accumulation, one that could be largely alleviated by externally lowering the oxygen partial pressure of the culture.

Ethanol production analysis

Ethanol production analysis (see Supplementary Data S1, Figure S3) was carried out with wild-type and CpT-4 and CpT-7 transformant strains. Absolute yields of ethanol varied significantly among experiments. Factors such as cultivation conditions and the growth phase of the culture upon which cells were collected for ethanol production assays affected the absolute yield of ethanol measured. Nevertheless, substantial and reproducible differences existed in ethanol production between the CpT-4 and CpT-7 transformant and wild-type lines, under all conditions employed. Example is offered in Figure 10, where the ethanol concentration in the culture liquid media is presented as a function of growth time. Ethanol production increased linearly for the first 72 h in the CpT-4 (Figure 10, diamonds) and CpT-7 (Figure 10, squares) transformants, followed by a gradual levelling off. Substantially lower levels of ethanol accumulation were detected in the CC3269 wild type (Figure 10, circles). The recipient strain CC2653 was not used for ethanol production experiment because the strain is highly sensitive to irradiance.

Discussion

Metabolic engineering of the Chlamydomonas chloroplast for the production of fuels and chemicals

A paradigm of the potential of *Chlamydomonas* to serve in the production of fuels and chemicals is offered in this work. The venue tested the functionality of a *Saccharomyces cerevisiae* ADH1-type transgene incorporated in the *C. reinhardtii* chloroplast genome. When aiming at transgene expression in the chloroplast of *Chlamydomonas*, two approaches could be applied: one is through the nuclear transformation process, integrating a transgene the coding sequence of which includes a chloroplast transit peptide in addition to the protein of interest. In this case, the transgenic protein is synthesized in the cytoplasm and then targeted to the chloroplast through the function of a transit peptide. A different approach is via direct chloroplast transformation, which allows integration of the transgene into the chloroplast genome, allowing synthesis of the transgenic protein in the chloroplast, thus bypassing the protein import process.

The mode of integration of transgenes in the *Chlamydomonas* nuclear genome is at random, naturally leading to significant variations in the levels of transgene expression (Mussgnug *et al.*, 2007). In consequence, screening of a large population of transformants is usually necessary to obtain lines with maximal transgene expression levels. One of the reasons for such variation

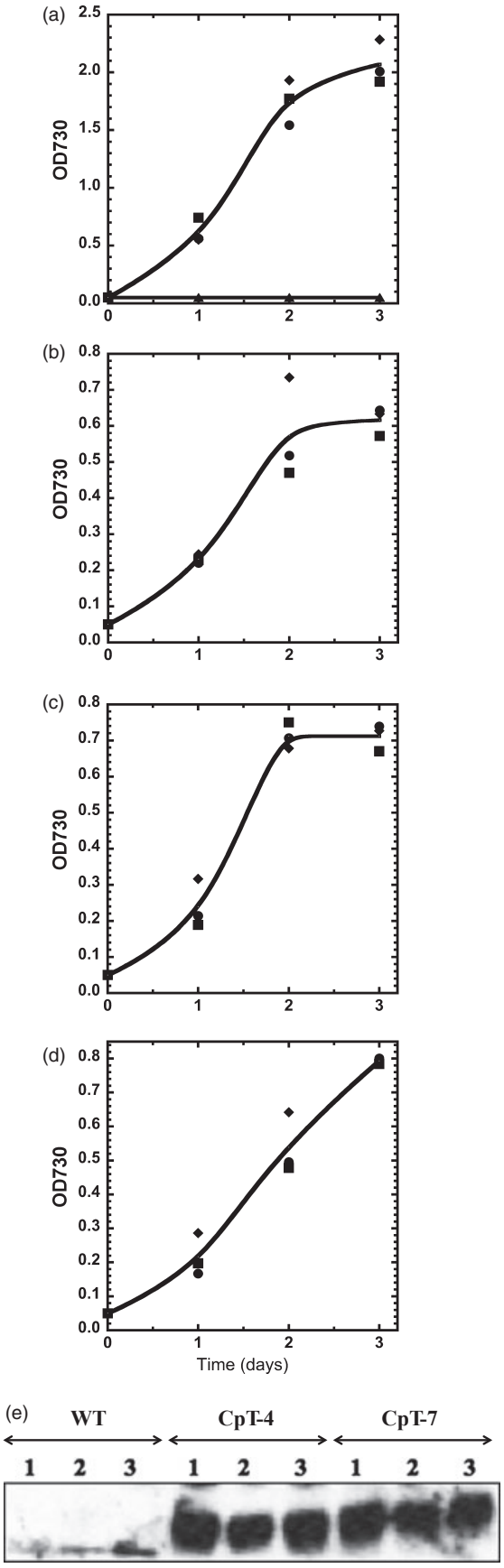

Figure 9 (a) Growth curves of CC3269 WT (circles), CpT-4 (squares), CpT-7 (diamonds) and CC2653 RBCL-less mutant (triangles) of *Chlamydomonas reinhardtii* under continuous illumination conditions. (b-d). Growth curves of CC3269 WT (b), CpT-4 (c), CpT-7 (d) in the presence DCMU (2 μM) in the light (circles), DCMU in the dark (squares) or in the absence of DCMU in the dark (diamonds). (e) Western blot analysis of protein extracts from WT, and CpT-4 and CpT-7 transformant strains with CrCpADH1-specific polyclonal antibodies. Lanes #1: Samples grown in the light in the presence of DCMU. Lanes #2: samples grown in the dark in the presence of DCMU. Lanes #3: samples grown in the dark in the absence of DCMU. The standard deviation was ± 17%.

in the level of nuclear transgene expression is that epigenetic silencing mechanisms that operate in the eukaryotic genome may suppress gene expression. Moreover, there are regions of naturally slow transcriptional activity in the nuclear genome, impeding the expression of transgenes, when the latter are therein incorporated.

In contrast, integration of transgenes into specific sites of the chloroplast DNA is mediated through homologous recombination. Therefore, one can aim the insertion site in a region where high rates of transgene expression could be expected. With this caveat, this work targeted the *CrCpADH1* transgene downstream of the *rbcL* gene, resulting in the successful expression of the *CrCpADH1* gene, as evidenced by the accumulation of transcripts in all transformants tested. Moreover, chloroplasts contain up to 100 copies of circular DNA. The recovery of function pressure exerted through the RuBisCO activity, as applied in this work, causes a gradual elimination of the endogenous copies of the chloroplast DNA and their replacement with transgenic copies, until homoplasmy is reached. The high copy number of the transgene in the chloroplast genome could potentially leads to higher levels of expression. Other advantages of chloroplast expression of transgenes are the lack of post-translational modification as well as lack of epigenetic interference mechanisms in the chloroplast, all of which offer a less stringent environment for the overexpression of foreign proteins.

To further increase chloroplast transformation efficiency, we used a marker-free selection method that is based on the requirement of photoautotrophic growth by the cell. This method is superior to that of using an antibiotic resistance selection, because it (i) substantially minimizes the recovery of false-positive lines, (ii) alleviates the need to continuously apply and effectively deliver antibiotics to chloroplasts in all transformant cells in order to achieve chloroplast DNA homoplasmy, and (iii) alleviates concerns emanating from the observation that the mutation rate of 16S rRNA under antibiotic selection is similar to that of the transformation efficiency in *Chlamydomonas*, which would lead to a high proportion of recovery of false positives (Harris, 1989). In this work, we reported greater than 90% efficiency in the isolation of positive transformants. The much higher efficiency in this study could be attributed to the marker-free recovery of function based on *rbcL* gene activity. High levels of transgene expression could be a result of the integration site and/or the nature of the genetic elements used such as the promoter, terminator and expression cassette employed in this work.

Ethanol production in *Chlamydomonas reinhardtii*

For a photosynthetic eukaryote, *Chlamydomonas reinhardtii* and other green microalgae possess a rather unusual repertoire of

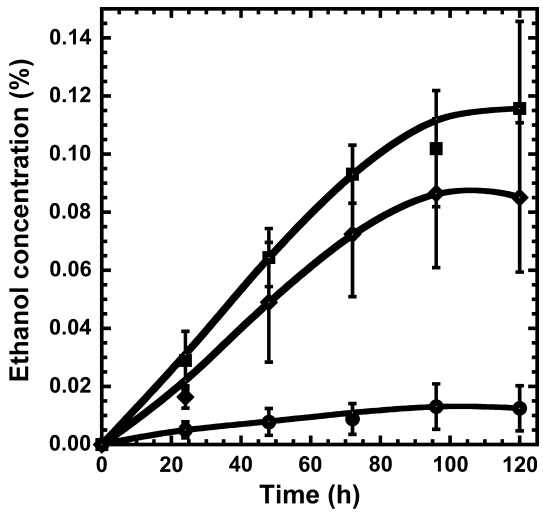

Figure 10 Ethanol accumulation is plotted as a function of *C. reinhardtii* incubation time over the course of 120 h in the dark for CpT-7 (squares), CpT-4 (diamonds) and CC3269 WT (circles). Cells were grown to the mid exponential growth phase (about 4–5 × 10^6 cells/ml) under 12-h light–12-h dark cycles. Cultures were then centrifuged and cell pellet resuspended in TAP-S medium at a Chl concentration of ~50 μg/ml. The concentrated cultures were sealed followed by dark incubation.

fermentative pathways that include hydrogen evolution (Melis, 2007; Melis and Happe, 2001) and various pathways that lead to the breakdown of sugar with the attendant formation of organic acids, alcohols and CO_2. Four fermentative pathways were identified in *Chlamydomonas,* with pyruvate serving as a common substrate that leads to the formation of formate, acetate, ethanol, lactate, malate and hydrogen (Grossman *et al.*, 2011).

Several studies showed that formate, acetate and ethanol are the main fermentation products in the dark under anaerobic incubation, whereas only trace amounts of H_2, glycerol, D-lactate and CO_2 formed. The ratio of these products differed, however, in different reports from 2:1:1 for formate/acetate/ethanol (Gfeller and Gibbs, 1984; Kreuzberg, 1984) to 2:2:1 (Mus *et al.*, 2007; Ohta *et al.*, 1987) produced under dark-anaerobic conditions. Illumination under anaerobic conditions caused a lowering in the yield of formate by 50%, no acetate could be detected, and ethanol production was lowered to 10%. In contrast, the levels of H_2 and CO_2 were increased drastically by several folds (Gfeller and Gibbs, 1984). Through nutrient deprivation, it is also possible to trigger anaerobiosis in the light. High levels of H_2 production in *Chlamydomonas reinhardtii* upon sulphate deprivation in the light were reported (Melis *et al.*, 2000; Zhang *et al.*, 2002). Following this discovery, a number of studies showed that sulphur deprivation (TAP-S medium) in the light also permits the occurrence of fermentation that leads to accumulation of formate and ethanol as major fermentation by-products, in addition to H_2 and CO_2 (Hemschemeier *et al.*, 2008a,b; Kosourov *et al.*, 2003; Tsygankov *et al.*, 2002). In a sealed culture, TAP-S causes anaerobiosis for prolonged periods of time, without loss of cell viability, irrespective of whether cells are incubated in the light or dark. With the above caveat (TAP-S conditions), ethanol production with the CpT-7 transformant yielded about 16 μg ethanol per μg Chl over 100-h incubation (Figure 10), compared with 1.9 μg ethanol per μg Chl in the wild type (Winkler *et al.*, 2002).

It was proposed that ethanol production in *Chlamydomonas* is coupled to PFL1 (pyruvate formate lyase, accession XM_001689667)/PRF1 (pyruvate-ferredoxin oxidoreductase, accession XM_0 0001701156) pathway from acetyl-CoA, through the function of ADHE (ADH1, accession XM_001703533), which is an acetaldehyde dehydrogenase–alcohol dehydrogenase bifunctional two-domain protein. Evidence was provided for the presence of PFL in both the mitochondria and chloroplasts, and yet, there is only a single copy of the *PFL* gene, and only one type of *PFL* transcript was detected. Therefore, a hypothesis of dual targeting of the protein was suggested (Atteia *et al.*, 2006). In this study, it was also proposed that ADHE is targeted into mitochondria based on transit peptide analysis. Nevertheless, expression of ADHE at the transcript level was not found under all experimental conditions employed, including light versus dark and aerobic versus anaerobic incubation (Atteia *et al.*, 2006).

Although two additional genes in Chlamydomonas have been designated as *ADH2* (JGI version 4.0 protein identifier 121409) and *ADH3* (JGI version 4.0 protein identifier 8202) (Magneschi *et al.*, 2012), there is no experimental evidence for the functionality of these genes. Opposite to a lack of functional information about these two genes, analysis of *adh1,* an ADH1 knockout *Chlamydomonas* mutant, revealed a total inhibition of cellular ethanol production (Magneschi *et al.*, 2012), suggesting minimal or no contribution to ethanol by the *ADH2* and *ADH3* genes. Interestingly, other reports showed that ethanol production is enhanced in PFL1 knockout mutants (Philipps *et al.*, 2011). In a *pfl1-1* allele, Catalanotti *et al.* (2012) proposed that, while oxidation of pyruvate by PFR was lowered, pyruvate decarboxylation was higher, thereby explaining the higher levels of ethanol accumulation. These results indicate that the pathway involving PFL1/PFR1 to generate acetyl-CoA and subsequent reduction by ADH1 may not be the sole or main pathway for ethanol generation, but instead, the PDC-ADH1 pathway could function and sustain higher levels of ethanol production in the cell. Based on the current information, the only *PDC* gene that has been identified in *C. reinhardtii* is the *PDC3*, the expression of which has been shown to increase under anaerobic conditions (Catalanotti *et al.*, 2012; Hemschemeier *et al.*, 2008b; Magneschi *et al.*, 2012; Mus *et al.*, 2007). Interestingly, localization of the PDC3 protein was suggested to be in the cytoplasm (Terashima *et al.*, 2010).

The above analysis raises the possibility that a cytosolically localized PDC could function together with a chloroplast-localized ADH1 for ethanol synthesis in *C. reinhardtii*. This hypothesis is consistent with results in this work, as expression of a CrCpADH1 in the chloroplast of *C. reinhardtii* resulted in sustained and higher levels of ethanol production when compared to wild-type controls. It is thus plausible that acetaldehyde generated by PDC3 in the cytosol diffuses to the chloroplast, where it is converted into ethanol. This hypothesis is consistent with the small size, lack of charge and high volatility of the acetaldehyde molecule, making it easy to diffuse thought membrane lipid bilayers. Thus, acetaldehyde synthesis by the endogenous PDC3 gene would serve as a substrate for both the endogenous ADH1 and the CrCpADH1 in the transformants. Additional acetaldehyde could also be generated as intermediate from the acetyl-CoA reduction by the bifunctional aldehyde–alcohol dehydrogenase ADH1 of *Chlamydomonas* in the chloroplast. These considerations could explain the higher ethanol production shown by the CrCpADH1 transformants, especially considering that the ADH1 alcohol dehydrogenase of *Saccharomyces cerevisiae* exhibits higher

catalytic activity using acetaldehyde as a substrate, when compared to that of a bifunctional aldehyde–alcohol dehydrogenase. Taken together, the results support a model whereby acetaldehyde generated by PDC3 in the cytosol could diffuse through the chloroplast envelope and, thus, become available to the transgenic CrCpADH1 in the chloroplast. It is through this pathway that the transgenic CrCpADH1 exerts its function, in addition to its reduction in acetaldehyde generated by the native ADH1 in the chloroplast, leading to sustained ethanol production in the transformants (Figure 11).

Experimental procedures

Strains, culture media and growth conditions

Chlamydomonas reinhardtii strain CC2653 (Spreitzer *et al.*, 1985), obtained from the Chlamydomonas Center (http://www.chlamy.org), was employed as the recipient strain for chloroplast transformation in this work. Strain CC2653 is a chloroplast mutant that contains a point mutation in the 5′ end region of the *rbcL* gene coding sequence, causing an early termination of the RBCL protein synthesis (Spreitzer *et al.*, 1985). In consequence, lack of the large RBCL subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC4.1.1.39) leads to the absence of both the chloroplast-encoded RBCL, large subunit, and the nuclear-encoded RBCS, small subunit of RuBisCO.

Construction of expression vectors for Chlamydomonas chloroplast transformation

Plasmid P-67 containing the *C. reinhardtii* chloroplast DNA EcoRI 14 fragment (5.8 kb) was acquired from the Chlamydomonas Center (www.chlamy.org) and used as the starting material for the construction of the expression vector employed in this work. The EcoRI 14 fragment (5.8 kb) includes wild-type copies of the *psaB*, *tRNAG* and *rbcL* genes, as well as the 5′ end of the *atpA* gene. To build an expression vector for chloroplast transformation, the EcoRI 5.8-kb insert was first cloned from the original P-67 into the pGEMT-easy vector (Promega, Corporation, Madison, WI). Then, a unique *Sma*I restriction site was introduced in the intergenic region of *psaB* and *tRNAG* genes through site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit; Agilent Technologies, Inc., Santa Clara, CA), generating plasmid pGEMT67Sma. Plasmid pGEMT67Sma was then used as the backbone vector for the cloning of the CrCpADH1 expression cassette.

The *CrCpADH1* gene sequence for the transformation of the *Chlamydomonas* chloroplast was designed on the basis of the *ADH1* gene from *Saccharomyces cerevisiae* (YOL086C, EC 1.1.1.190). For this purpose, the ADH1 protein sequence was back translated and codon optimized to match the frequency of the codon usage in the chloroplast of *C. reinhardtii* so as to maximize the expression of the yeast ADH1 protein in *C. reinhardtii*. Codon use optimization was based on the codon usage table from the Kazusa DNA Research Institute, Japan (http://www.kazusa.or.jp/codon/). We applied a low-frequency codon usage cut-off thread of 20% in this optimization process. In addition to the *ADH1* gene sequence, a sequence encoding the 6xHis-tag was included at the 3′ end of the *CrCpADH1*. Expression of the *CrCpADH1* gene in the chloroplast of *Chlamydomonas reinhardtii* was placed under the control of the promoter of the native *rbcL* gene (accession number J01399). The *rbcL* sequence used for driving the expression of *CrCpADH1* included the sequence from nucleotide −350 to +90 (whereby nucleotide 'A' of the initiation codon ATG of the *rbcL* gene is considered as +1). The combined nucleotide sequence, including the *rbcL* −350 to +90 promoter region, the *CrCpADH1* codon-optimized DNA sequence, including a 6xHis-tag at the 3′ end of the coding sequence, and the *rbcL* 3′UTR were custom synthesized by DNA 2.0 (Menlo Park, CA).

Chloroplast transformation and selection

Chlamydomonas reinhardtii strain CC2653 was grown under dim light (~5 μmol photons/m^2/s) in TAP medium to a cell density of 2–3 × 10^6 cells/ml and then diluted to a cell density of 5 × 10^4 cells/ml. Filter-sterilized solution of 5-fluoro-2′-deoxyuridine (FdUrd obtained from Sigma-Aldrich) was added to this diluted cell culture at a final concentration of 0.5 mM, as prescribed for chloroplast genome copy number reduction (Harris, 1989). Incubation of the culture was continued for 5–7 days to reach a cell density of 3–5 × 10^6 cells/ml. Cells were then collected by centrifugation and resuspended in fresh HS minimal medium at a density of approximately 1–2 × 10^7 cells/ml. Cells were spread as a thin liquid layer on Petri plates (85 mm diameter) containing HS minimal media in 1.5% agar. The

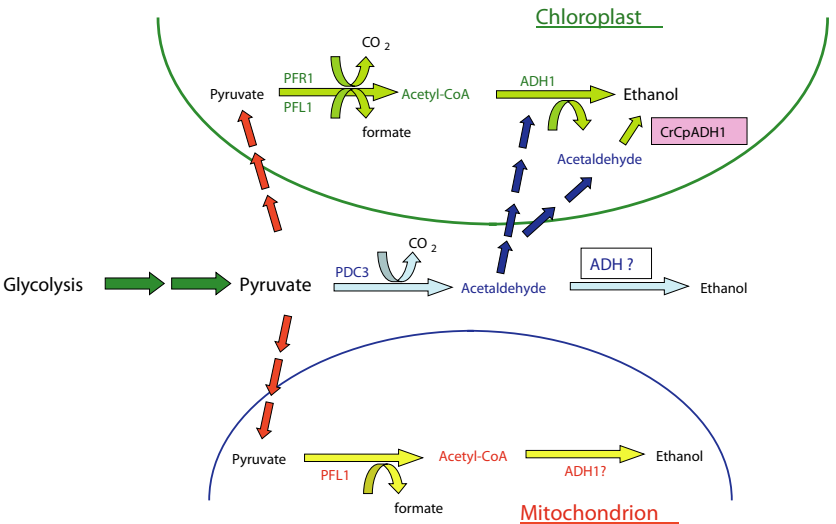

Figure 11 Schematic of hypothetical ethanol fermentation pathways in *Chlamydomonas reinhardtii*. The blue arrows indicate the proposed diffusion of acetaldehyde, generated by the *C. reinhardtii* PDC3 enzyme in the cytosol, to the chloroplast where the CrCpADH1 converts it into ethanol. Acetaldehyde could also be generated from the native 'ADH1' enzyme, as intermediate of a process that serves to provide acetaldehyde substrate for the CrCpADH1 enzyme. PFR1 pyruvate-ferredoxin oxidoreductase: accession #: XM_00001701156, PFL1-pyruvate formate lyase 1: accession #: XM_001689667, PDC3: accession #: XM_001703478; ADH1: accession #: XM_001703533, CrCpADH1: transgenic *Saccharomyces cerevisiae* CrCpADH1.

CC2653 strain was transformed upon coating either 1 μm or 0.6 μm of gold particles with pCrCpADH1 plasmid DNA and upon delivering these particles to cells through a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Hercules, CA) operated at 1100 psi. Approximately 10 μg of plasmid DNA was used to coat 0.6 mg of gold particles, and such DNA-coated particles were used for the bombardment of three different plates per experiment. The distance between the rupture disc and Mylar macrocarrier was set at 8-10 mm and 1 cm between Mylar macrocarrier and the stopping screen. Petri plates containing a thin layer of *Chlamydomonas* cells were placed in the Biolistic PDS-1000He device approximately 6 cm below the stopping screen. Following the bombardment, plates were incubated at 24 °C under continuous illumination at about 50 μmol photons/m^2/s. After two-week incubation, transformant colonies became visible to the eye, indicating ability of autotrophic growth by the cells. Such individual colonies were transferred onto fresh HS minimal media on agar plates. Once photoautotrophic strains established themselves (about 2 weeks following transfer of the transformants), individual colonies were then cultured in liquid HS minimal media for 2 days and replated onto HS agar to obtain single cell lines. The resulting independent transformant lines were then tested for the presence of the *CrCpADH1* gene in the chloroplast DNA. Homoplasmic lines were selected for further molecular, biochemical and physiological analyses.

Presence of the *CrCpADH1* transgene in the transformants was tested upon PCR analysis using *CrCpADH1*-specific primers CrCpADH1F2 5′CTGTTCAAGCTGCTCATATT3′ and CrCpADH1R2 5′TACAGATGGTGGTGCACA3′, anticipating a fragment size of 322 bp. Homoplasmy of the chloroplast DNA in the transformants was also tested by PCR analysis using a pair of primers, one located in the 3′ UTR region of the *psaB* gene with the sequence 5′CTACCTCATCAGATCAGC3′ (Figure 5a, primer *psaB3′)* and the other located at the 3′ end of *rbcL* gene with the sequence 5′ GGATGTAACTCAATCGGTAGAG3′ (Figure 5a, *rbcL*3′ primer).

Primers used for the PCR analysis of mating type *plus* and *minus* strains were as follows: mt- F (forward): 5′ATGGCCTGTTTCTTAGC3′; mt- R (reverse): 5′CTACATGTGTTTCTTGACG3′; mt+ F: 5′ATGCCTATCTTTCTCATTCT3′; mt+ R: 5′ GCAAAATACACGTCTGGAAG3′.

Expression analysis by RT-PCR

Total RNA was extracted with TRIzol (Life Technologies) following the manufacturer's instructions. Amplification of *CrCpADH1* transcripts was performed as follows: 0.1 μg of total RNA was used for the reverse transcription with an *CrCpADH1* gene-specific primer located at the 3′ end of the gene, termed CrCpADH1R1: 5′CATAACGACCTACAATTTGACC3′ (Figure 1), using Superscript III from Life Technologies, and by following the manufacturer's instructions. The reaction mix was then diluted 3x, and 1-μl aliquot was employed for the subsequent PCR using the primers: CrCpADH1F1 5′ATGTCAATTCCTGAAACTC3′ and CrCpADH1R2 5′TGTGCACCACCATCTGTAGC3′ (Figure 1) anticipating a product size of 720 bp. For the RT-PCR amplification of the *rbcL* gene transcripts, encoding the RuBisCO large subunit (accession number J01399), 0.1 μg of total RNA was used for reverse transcription with random hexamer primers. The reaction mix was then diluted 3x, and 1 μl was used for the subsequent PCR using primers: *rbcL* forward 5′ATTCGTAGGTCCTCCACACG3′ and *rbcL* reverse 5′ CGGAAGTGAATACCGTGGTT3′, anticipating a PCR product of 495 bp.

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Conflict of interest

The authors have no conflict of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Sequence alignment of *ADH1* gene from *Saccharomyces cerevisiae* (*ScADH1*) and the *Chlamydomonas reinhardtii* chloroplast codon optimized *ADH1* gene (*CrCpADH1*).

Figure S2 Expression of rbcL90-ADH1 and ADH1 proteins in *E. coli*.

Figure S3 Ethanol production measurements.

Data S1 Supplementary experimental procedures for Strains, culture media, and growth conditions; Extraction of genomic DNA; Expression vector for *Chlamydomonas reinhardtii* chloroplast transformation; Generation of CrCpADH1 specific polyclonal antibodies; Protein analysis by SDS-PAGE and Western blotting; Ethanol production measurements.