High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp.

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Algae have reemerged as potential next-generation feedstocks for biofuels, but strain improvement and progress in algal biology research have been limited by the lack of advanced molecular tools for most eukaryotic microalgae. Here we describe the development of an efficient transformation method for *Nannochloropsis* sp., a fast-growing, unicellular alga capable of accumulating large amounts of oil. Moreover, we provide additional evidence that *Nannochloropsis* is haploid, and we demonstrate that insertion of transformation constructs into the nuclear genome can occur by high-efficiency homologous recombination. As examples, we generated knockouts of the genes encoding nitrate reductase and nitrite reductase, resulting in strains that were unable to grow on nitrate and nitrate/nitrite, respectively. The application of homologous recombination in this industrially relevant alga has the potential to rapidly advance algal functional genomics and biotechnology.

Research on eukaryotic algae has provided fundamental insights into many basic cellular processes, particularly photosynthesis. Recently, algae have reemerged as potential next-generation feedstocks for advanced biofuels, such as biodiesel and other hydrocarbons (1–4), and have attracted considerable interest from both the private and public sectors. Many algae have high photoautotrophic growth rates and can accumulate more than half their dry weight biomass as lipids (5), including triacylglycerol and a number of high-value pharmaceutical and nutraceutical products (6). Compared with agricultural plants, algae do not require arable land, and many species can be grown using wastewater or salt water. However, because unimproved algae are unlikely to possess all of the traits necessary for economic production of biofuels (1), robust molecular biology tools are required for strain optimization (7).

Eukaryotic algae comprise a diverse, polyphyletic group of organisms with members in four of the five eukaryotic supergroups (8). One supergroup, the Plantae, includes rhodophytes (red algae), glaucophytes, chlorophytes (green algae), and plants, which all contain plastids derived from primary endosymbiosis of a cyanobacterium (9). Photosynthetic plastids have been transferred to other eukaryotic supergroups through secondary endosymbiosis of green algae and red algae. For example, chromalveolate algae such as diatoms, brown algae, eustigmatophytes, and most dinoflagellates contain a plastid derived from a red alga (10).

The green alga *Chlamydomonas reinhardtii* is currently the eukaryotic model alga of choice, because of its genetic tractability (11), array of molecular tools (12, 13), and comprehensively annotated nuclear genome sequence (14). Genome sequences of several algae of ecological, economic, and/or phylogenetic significance have been determined recently (15). Transformation methods have been developed for several algal species, most notably the model diatom *Phaeodactylum tricornutum* (16, 17); however, the molecular genetic tools available for these species are generally quite limited in comparison with *Chlamydomonas*.

Although *Chlamydomonas* is often regarded as "the green yeast" (18, 19), the lack of efficient homologous recombination (HR) in the nuclear genome of this alga (20–22) has been a major limitation for algal biology research. By contrast, the implementation of HR in the yeast *Saccharomyces cerevisiae* has enabled precise genetic manipulation and systematic functional analysis of its genome (23) and has led to pioneering discoveries in eukaryotic biology.

Although it resembles green algae of the genus *Chlorella* (24), *Nannochloropsis* sp. is a eustigmatophyte that is related to diatoms and brown algae (25). Like *Chlorella* but unlike *Chlamydomonas*, *Nannochloropsis* is a robust industrial alga that has been extensively grown in outdoor ponds and photobioreactors for aquaculture (26, 27). Under nitrogen-starvation conditions, *Nannochloropsis* can accumulate oil exceeding 60% of its biomass on an ash-free dry weight basis (27), making it an excellent candidate for biofuel production. In addition, *Nannochloropsis* is a rich source of high-quality protein (28) and eicosapentaenoic acid (29), an omega-3 fatty acid with numerous health benefits (30).

Here we describe the development of a highly efficient transformation system for *Nannochloropsis*. Moreover, we demonstrate that *Nannochloropsis* sp. exhibits very efficient HR, allowing us to reliably and rapidly perform targeted genetic manipulation of this organism. These findings open the door for systematic functional genomics and biotechnological improvement of this industrial alga.

Results

Development of an Efficient Transformation System for *Nannochloropsis*. *Nannochloropsis* sp. (strain W2J3B) grows rapidly on solid or liquid media, with a doubling time of ~14 h under laboratory conditions (*Materials and Methods*). To establish a transformation system for *Nannochloropsis* sp., we used endogenous promoters and 3′ untranslated regions (3′ UTRs) from two unlinked violaxanthin/chlorophyll *a*-binding protein (VCP) genes, *VCP1* and *VCP2* (Fig. 1). The protein encoded by the *VCP1* gene is nearly identical to a previously characterized light-harvesting antenna protein from *Nannochloropsis* sp. (31). The *VCP2* locus consists of two identical copies of the *VCP2* gene driven by a bidirectional promoter (Fig. 1, *SI Materials and Methods*, and Fig. S1). Using transformation constructs containing the *Sh ble* selectable marker gene, which confers resistance to zeocin (32), we developed and optimized an efficient transformation method based on electroporation of intact *Nannochloropsis* sp. cells (*SI Materials and Methods* and Fig. S1). The highest transformation efficiency (~2,500 transformants/μg of DNA) was obtained using

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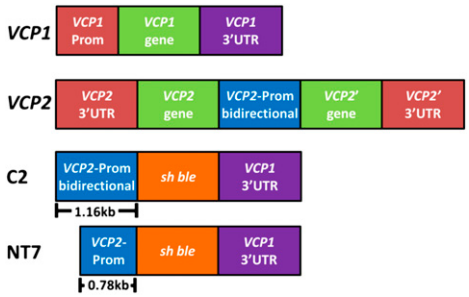

Fig. 1. Composition of transformation constructs. The *VCP1* gene from *Nannochloropsis* sp. consists of a promoter (Prom), the coding region, and a 3′ untranslated region. The *VCP2* locus consists of two identical coding sequences (dubbed *VCP2* and *VCP2′*) driven by a central, bidirectional promoter element (*VCP2*-Prom bidirectional) and each followed by a 3′ UTR. In the C2 transformation construct, the *Sh ble* gene conferring resistance to zeocin is fused to the bidirectional promoter from *VCP2* and the 3′ UTR from *VCP1*. In the NT7 cassette, the bidirectional promoter is truncated.

a very high electric field strength within the electroporation cuvette (~11,000 V/cm field strength, 50 μF capacitance, and 500 Ohm shunt resistance; *Materials and Methods*, *SI Materials and Methods*, and Fig. S2). The number of transformants obtained was approximately linear with respect to the amount of DNA used, over a range from 200 ng to 3 μg (*SI Materials and Methods* and Fig. S3). Transformation of a modified version of the C2 transformation construct (Fig. 1) in which the promoter had been flipped (construct C1; Fig. S1) generated a similar number of transformants as the C2 construct, showing that the *VCP2* promoter indeed drives transcription in both directions (*SI Materials and Methods* and Fig. S4). When we tried to transform a plasmid containing the C2 cassette without prior linearization, we obtained only a single zeocin-resistant colony in five independent transformation experiments, indicating that efficient stable transformation of *Nannochloropsis* sp. requires linear DNA fragments (*SI Materials and Methods*).

We also constructed transformation cassettes based on selectable markers conferring resistance to hygromycin B and blasticidin S (Fig. S1). To determine the frequency of cotransformation, we performed experiments in which a zeocin-resistance cassette was transformed together with an excess of the hygromycin B- and blasticidin S-resistance constructs, and transformants were initially selected by plating the cells on zeocin only (*SI Materials and Methods*). Subsequent replating of zeocin-resistant colonies on hygromycin B- or blasticidin S-selective media revealed total cotransformation frequencies of 50 and 55% for at least one unselected marker and 23 and 31% for both unselected markers in two independent experiments (Table S1).

Gene Knockouts by Homologous Recombination. To test for the occurrence and frequency of HR in *Nannochloropsis* sp., we designed gene knockout (KO) constructs based on a modified zeocin-resistance cassette (NT7) with a truncated *VCP2* promoter (Fig. 1). *Nannochloropsis* sp. is able to grow on medium containing nitrate, nitrite, or ammonium as the sole nitrogen source, and we chose the nitrate reductase (NR) and nitrite reductase (NiR) genes as targets for HR, because successful disruption of these genes can easily be detected by growth on different nitrogen sources. Within the algal cell, nitrate is first reduced to nitrite by the action of NR and then further reduced to ammonia by NiR. Thus, cells lacking NR can grow on nitrite but not nitrate, whereas cells without NiR are not able to use nitrate or nitrite. Ammonia assimilation does not require NR or NiR, and thus cells with a defective NR or NiR gene can be grown on ammonium as a nitrogen source.

We performed transformation with KO constructs containing ~1-kb flanking sequences that target the NR (Fig. 2*A*) and NiR (Fig. 2*B*) genes. KO constructs were designed to replace 242 bp of the NR gene encoding a part of the molybdenum cofactor-binding domain (cd02112) or 793 bp of the NiR gene encoding the terminal nitrite/sulfite reductase ferredoxin-like half-domain (pfam03460) with the NT7 selection marker cassette. In both cases, we obtained zeocin-resistant transformants on medium containing ammonium as the sole nitrogen source. Replating of these colonies on media containing either nitrate or ammonium as the sole nitrogen source revealed that 25–94% of the NR-KO and 11–22% of the NiR-KO transformants bleached on nitrate, whereas all transformants grew on ammonium (Fig. 3 and Table S2).

PCR analysis of the genomic DNA isolated from NR-KO and NiR-KO transformants using one or both primers outside of the homologous genomic DNA-flanking regions in the KO constructs (Fig. 2) revealed that the NT7 cassette had successfully inserted into the genome and replaced part of the target genes in the transformants that bleached on nitrate (Fig. 3). We obtained single PCR products for the NR-KO and NiR-KO transformants and, as expected, these products were longer in the cases of successful HR than those obtained for transformants that did not exhibit bleaching on nitrate (Fig. 3). DNA sequencing of the longer PCR products confirmed a perfect exchange of the targeted genomic DNA with the NT7 cassette. The presence of a single PCR product and the absence of the shorter wild-type allele in the KO strains strongly suggest that *Nannochloropsis* sp. W2J3B is haploid. This was further supported by PCR analysis of two nontargeted insertion sites obtained with transformation constructs lacking additional flanking sequences (*SI Materials and Methods*). In both cases, we also obtained a single PCR product, indicating the presence of a single allele (Fig. S5).

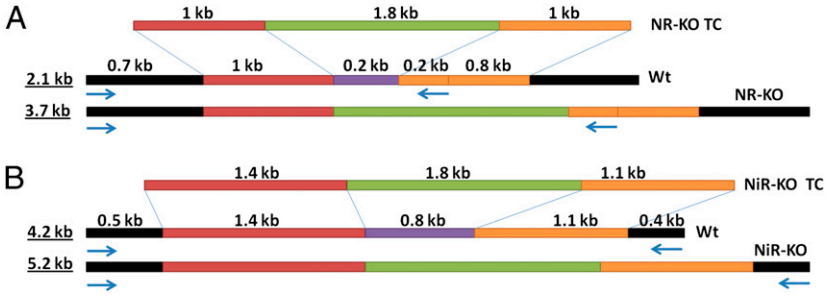

Fig. 2. KO of nitrate reductase and nitrite reductase genes by HR in *Nannochloropsis* sp. W2J3B. Structures of NR-KO (*A*) and NiR-KO (*B*) transformation constructs (TC), wild-type (Wt) genes, and HR products. Each KO construct consists of a left flank (red) and a right flank (orange) separated by the NT7 selection marker cassette (green). A target region (purple) of each gene is replaced by the NT7 cassette when HR occurs. Primer positions (arrows) for PCR analysis and expected PCR product sizes for Wt and KO mutants are indicated.

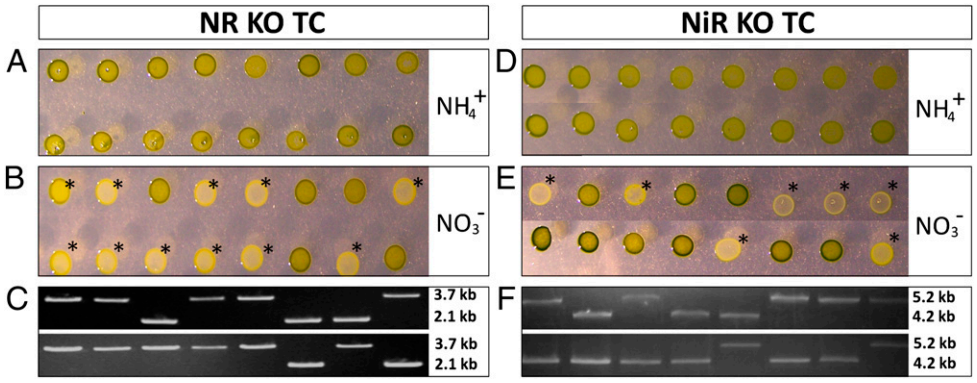

Fig. 3. Analysis of transformants obtained with NR-KO and NiR-KO transformation constructs. (*A*, *B*, *D*, and *E*) Sixteen randomly selected zeocin-resistant transformants obtained with each TC were resuspended in medium lacking a nitrogen source and spotted on plates containing ammonium (*A* and *D*) or nitrate (*B* and *E*) as a sole nitrogen source. Putative NR-KO and NiR-KO mutants bleach, because they cannot use nitrate as a nitrogen source. (*C* and *F*) PCR analysis of the transformants shown in *B* and *E*. Genomic DNA was amplified by PCR with the primer pairs indicated in Fig. 2. Transformants shown by PCR analysis to have undergone HR at the NR or NiR locus are marked with an asterisk in *B* and *E*, respectively.

Liquid growth analysis of two NR-KO and two NiR-KO transformants that bleached on nitrate demonstrated that NR-KO transformants could not use nitrate for growth, but were able to grow on nitrite as a nitrogen source (Fig. 4). NiR-KO transformants could not grow on either nitrate or nitrite as a nitrogen source (Fig. 4).

Discussion

We have established a highly efficient transformation protocol for *Nannochloropsis* sp., a photoautotrophic alga of high importance for the production of animal feed, biofuels, and nutraceuticals. Transient transformation of *Nannochloropsis* sp. using *Agrobacterium* has been described (33). Transformation of *N. oculata* after enzymatic digestion of the cell wall has also been reported (34), but the lack of a selectable marker gene in the transformation construct necessitated PCR screening of hundreds of colonies to identify a few stable transformants. In contrast, our protocol for the genetic transformation of *Nannochloropsis* sp. is simpler and can be directly applied to wild-type cells without prior removal of the cell wall, while yielding high numbers of transformants. Successful transformation of *Nannochloropsis* sp. via electroporation requires unusually high electric field strengths within the electroporation cuvette, which might be explained by the very robust cell wall of this alga (35).

Efficient transformation of *Nannochloropsis* sp. enables research on this alga using a wide range of approaches that are commonly used with *Chlamydomonas* (12, 13). For example, the C2 transformation construct might be useful for protein expression by fusing a gene of interest to the other end of the bidirectional *VCP2* promoter (Fig. 1). Additional, unselected expression constructs or other DNA elements can be introduced by cotransformation, which occurs efficiently (Table S1). Because the C2 and NT7 transformation constructs use a 5′ promoter and 3′ UTR from different genes, integration of these cassettes (without additional flanking sequences) into the nuclear genome of *Nannochloropsis* sp. can occur by nonhomologous recombination (Fig. S5), resulting in insertional mutagenesis that could be exploited for forward genetics. The C2 construct with the bidirectional *VCP2* promoter could also potentially be used for activation tagging (36).

Based on our results, we conclude that *Nannochloropsis* sp. is a haploid alga that is amenable to targeted gene manipulation by HR. Addition of flanking DNA sequences to the NT7 selectable marker cassette (Fig. 2) allowed for targeted integration of the cassette into the nuclear genome of *Nannochloropsis* sp. by HR, and we demonstrated the occurrence of efficient HR by knocking out the NR and NiR genes (Figs. 3 and 4 and Table S2). In the NR-KO and NiR-KO strains, the NT7 cassette had integrated into the NR and NiR genes, respectively, and the corresponding wild-type allele was undetectable by PCR (Fig. 3). Successful KO of NR or NiR in a single transformation step suggests that *Nannochloropsis* sp. is haploid. Diploid organisms that integrate transforming DNA by HR generally require successive rounds of transformation to obtain homozygous KO strains (37). The conclusion that *Nannochloropsis* sp. is haploid is further supported by the results of mutant isolation experiments (38) and the recent genome sequencing of a *N. oceanica* strain (39).

Although HR is routine in several cyanobacterial species (40) and in the chloroplast genome of *Chlamydomonas* (41), the dominant route of integration of transforming DNA into the nuclear genome of most photosynthetic eukaryotes is by nonhomologous recombination. DNA integration by HR is a relatively rare event in most model plants (42) and algae (7), including *Arabidopsis*, maize, rice, *Chlamydomonas*, and diatoms. Indeed, efficient targeted insertion of DNA constructs into the nuclear genome has been demonstrated in very few photosynthetic

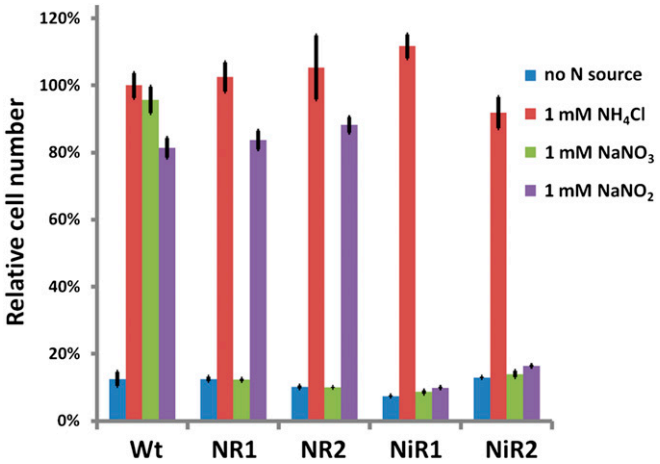

Fig. 4. Growth of wild-type, NR-KO (NR1 and NR2), and NiR-KO (NiR1 and NiR2) strains with different nitrogen sources. Cells in mid-log phase were washed in nitrogen-free medium, resuspended in media with the indicated nitrogen sources, and allowed to grow to early stationary phase. Results are expressed relative to the wild type in 1 mM NH_4Cl, and SDs for three independent cultures of each strain are indicated.

eukaryotes. One well-established example is the moss *Physcomitrella patens*, in which the use of HR has helped to establish this organism as a powerful model organism for studying the evolution of land plants (43). The only eukaryotic alga previously demonstrated to exhibit efficient HR is the red alga *Cyanidioschyzon merolae* (44, 45); however, this organism is a rather divergent red alga and an extremophile that is not generally viewed as a suitable model species for investigating biofuel production by algae. In contrast, *Nannochloropsis* sp. is a robust industrial alga that is considered to be a candidate feedstock species for the generation of biomass, biofuels, and valuable bioproducts.

The identification of a photoautotrophic alga that (*i*) is haploid, (*ii*) can be easily transformed with high efficiency, (*iii*) is amenable to high-frequency HR, and (*iv*) can be readily grown in mass culture opens the door to new opportunities for basic research and biotechnological applications using algae. The systematic generation and characterization of targeted KOs and promoter replacements by HR are likely to greatly accelerate gene function analysis and lead to rapid strain improvements for algal biotechnology. In summary, we believe that *Nannochloropsis* sp. W2J3B and the molecular tools that we have established for this organism will provide the scientific community with a new "green yeast" that has the potential to rapidly advance algal biology.

Materials and Methods

Growth Conditions. *Nannochloropsis* sp. W2J3B was grown photoautotrophically in F2N medium, which is identical to F/2 medium (46) except that trace metals, vitamins, and phosphate solutions were added in fivefold higher concentrations, 10 mM Tris·HCl (pH 7.6) was added to maintain the pH, and 5 mM NH_4Cl was included as a nitrogen source. All chemicals were obtained from Sigma as reagent-grade. Agar plates were prepared with 0.8% Bacto agar (Difco) in F/2 medium (46) with 50% artificial seawater, except that 2 mM NH_4Cl was used as a nitrogen source. Zeocin, blasticidin S, or hygromycin B, if needed, was added to a final concentration of 2 μg/mL, 50 μg/mL, or 300 μg/mL, respectively. Liquid cultures were generally maintained in F2N medium at a photon flux density of 85 μmol photons·m^{-2}·s^{-1} and bubbled with CO_2-enriched air (3% CO_2) at 28 °C. Agar plates were maintained at the same light intensity at 26 °C.

Nucleic Acids Used for Transformation. For PCR we used the Takara LA Taq polymerase. Two overlapping PCR products containing the *Sh ble* gene were amplified from pTEF1/Zeo (Invitrogen) via primer pair 5′-ATGGCCAAGTTGACCAGTGCCGT-3′ and 5′-TTAGTCCTGCTCCTCGGCCACGAA-3′ and primer pair 5′-ATGGCCAAGTTGACCAGTGCCGT-3′ and 5′-ACAGAAGCTTAGTCCTGCTCCTCGGCCACGAA-3′ (phosphorylated). The resulting products with different lengths were gel-purified (QiaEx II; Qiagen), mixed in equimolar amounts, denatured, and allowed to anneal at room temperature. Similarly, two overlapping products containing the 3′ UTR of the *VCP1* gene (GenBank accession no. JF957601) were amplified from genomic DNA of *Nannochloropsis* sp. W2J3B with primer pair 5′-CTGATCTTGTCCATCTCGTGTGCC-3′ and 5′-GCTTCTGTGGAAGAGCCAGTGGTAG-3′ and primer pair 5′-CTGATCTTGTCCATCTCGTGTGCC-3′ and 5′-GGAAGAGCCAGTGGTAGTAGCAGT-3′. These products were also gel-purified, mixed in equimolar amounts, denatured, and allowed to anneal at room temperature. The products of the two annealing reactions were ligated for 1 h with T4 ligase (Fermentas) to generate the product ble^{UTR}, which was then gel-purified and amplified with primers 5′-ATGGCCAAGTTGACCAGTGCCGTTCC-3′ (phosphorylated) and 5′-CTGATCTTGTCCATCTCGTGTGCC-3′ and gel-purified. Primers 5′-ACTTAAGAAGTGGTGGTGGTGGTGC-3′ and 5′-ACTTGAGAGAGTGGTGGAGTTGACT-3′ were used to amplify the bidirectional *VCP2* promoter ($VCP2^{Prom}$; GenBank accession no. JF946490). The $VCP2^{Prom}$ and ble^{UTR} products were blunt-ligated, gel-purified, cloned into the pJet1 vector (Fermentas), and transformed into *Escherichia coli* DH5α cells. After reisolation of plasmids and sequencing, we obtained vectors pJet-C1 and pJet-C2, driving expression of the *Sh ble* gene from one side or the other of the bidirectional *VCP2* promoter. The selection marker cassettes C2 or NT7 were amplified from pJet-C2 with primer pair 5′-ACTTAAGAAGTGGTGGTGGTGGTGC-3′and 5′-CTGATCTTGTCCATCTCGTGTGCC-3′ or 5′-AAGCAAGACGGAACAAGATGGCAC-3′ and 5′-CTGATCTTGTCCATCTCGTGTGCC-3′, respectively. The difference between NT7 and C2 is that C2 contains the entire bidirectional promoter, whereas NT7 contains only the part driving expression of the *Sh ble* gene.

For the nitrate reductase (GenBank accession no. JF946488) KO construct, we amplified two ∼1-kb parts of the NR gene separated by 242 bp within the genome as recombination flanks with the primers 5′-AGTCGTAGCAGCAGGAATCGACAA-3′ and 5′-GGCACACGAGATGGACAAGATCAGTGGAATAATGAGGCGGACAGGGAA-3′ (NR left flank), and 5′-GTGCCATCTTGTTCCGTCTTGCTTGCGCAAGCCTGAGTACATCATCAA-3′ and 5′-ATGACGGACAAATCCTTACGCTGC-3′ (NR right flank). Flanks were constructed for the nitrite reductase (GenBank accession no. JF946489) gene by amplifying left and right flanks (separated by 793 bp within the genome) with the primers 5′-TGACATGGACCAGCGGCTTAAGTA-3′ and 5′-GTGCCATCTTGTTCCGTCTTGCTTGCCGTATTTGGCATTGGTCTGCAT-3′ (NiR left flank), and 5′-GGCACACGAGATGGACAAGATCAGAGGCCGCATATGACATTCCTCAGA-3′ and 5′-ACGGTGGAAGAGATGGTGAGAGAA-3′ (NiR right flank). Flanks derived from the NR or NiR gene were fused to the NT7 transformation cassette by fusion PCR using the primers 5′-AGTCGTAGCAGCAGGAATCGACAA-3′ and 5′-ATGACGGACAAATCCTTACGCTGC-3′ or 5′-TAACGGGCTACTCACATCCAAGCA-3′ and 5′-AGTATCGCGTGGCAATGGGACATA-3′, respectively. The resulting PCR products (NR-KO and NiR-KO, respectively) were gel-purified before transformation.

Nuclear Transformation of *Nannochloropsis* sp. W2J3B. Cells were grown in F2N medium to mid-log phase (∼2×10^6 cells/mL) and washed four times in 384 mM D-sorbitol. Cell concentration was adjusted to 10^{10} cells/mL in 384 mM D-sorbitol, and 100 μL cells and 0.1–1 μg DNA were used for each electroporation within an hour. Electroporation was performed with a Bio-Rad Gene Pulser I electroporator in 2-mm cuvettes. The electroporator was adjusted to exponential decay, 2,200 V field strength, 50 μF capacitance, and 500 Ohm shunt resistance. After electroporation, cells were immediately transferred to 15-mL conical Falcon tubes containing 10 mL F/2 medium and incubated in low light overnight. Cells (5×10^8) were plated the next day on F/2 square agar plates (500-cm^2) containing 2 μg/mL zeocin. Colonies appeared after 2 wk and could be further processed after 3 wk.

Screening and Analysis of Knockout Mutants. ***Initial screen.*** Zeocin-resistant colonies obtained by transformation with either NiR-KO or NR-KO were resuspended in 50 μL F/2 medium lacking any nitrogen source, and 6 μL was spotted on agar plates containing 1 mM KNO_3 or 1 mM NH_4Cl as a sole nitrogen source. Many clones started to bleach on plates containing nitrate, indicating starvation for a nutrient, whereas no signs of starvation were visible on plates containing NH_4Cl (Fig. 3).

PCR screen. Genomic DNA was isolated, and PCR with primers 5′-AGTAGGCGTAGCCTTGGAGTTTGT-3′ and 5′-TCTGAAGCACAAGCGAAGCACT-3′ on NR-KO mutants or with primers 5′-ACGGTGGAAGAGATGGTGAGAGAA-3′ and 5′-AAGCTTAAGAAGGACGGCTCGGTA-3′ on NiR-KO mutants was used to amplify the genomic DNA around the NR or NiR gene, respectively. PCR on genomic DNA isolated from the wild type was used as a control.

Growth test. Wild type and two clones each of confirmed NR- and NiR-KO mutants (NR1, NR2, NiR1, and NiR2) were grown to mid-log phase in F2N medium containing 1 mM NH_4Cl. Cells were washed three times with 50% artificial seawater by centrifugation (5 min, 3,000 × g) and subsequent resuspension of the cells. Beakers with a clear lid containing 100 mL of F2N medium with no nitrogen source, 1 mM KNO_3, 1 mM $NaNO_2$, or 1 mM NH_4Cl were inoculated in triplicate with washed cells to a concentration of 4×10^5 cells/mL and allowed to grow under 3% CO_2 atmosphere at 200 μmol photons·m^{-2}·s^{-1} for 4 d under constant shaking (80 rpm). At this time, wild-type cultures supplemented with 1 mM NH_4Cl reached stationary phase after exhausting the nitrogen source. Cells were counted with an Accuri C6 flow cytometer equipped with an Accuri C6 sampler in duplicate. Growth was estimated as % cells compared with wild-type cultures grown in F2N medium containing 1 mM NH_4Cl.

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Supporting Information

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SI Materials and Methods

Determination of the Electric Field Strength Required for Successful Electroporation. The transformation construct PL90 (1 μg) was transformed into *Nannochloropsis* sp. W2J3B cells with a Bio-Rad Gene Pulser I electroporator in 2-mm cuvettes. Preparation of cells and electroporation were carried out as described in *Materials and Methods*, except that the field strength was varied. After 3 wk, the number of zeocin-resistant colonies obtained in single electroporation experiments was counted. The percentage of colonies obtained, normalized to the experiment that yielded the highest number of transformants, was plotted as a function of the field strength (Fig. S2). No transformants were obtained at field strengths of 7,500 V/cm or less. In subsequent experiments performed in triplicate, we aimed to resolve the optimal field strength (Fig. S2, *Inset*). As a result of these experiments, we decided to use 11,000 V/cm for the electroporation of *Nannochloropsis* sp. W2J3B.

Number of Transformants Obtained with Different Amounts of DNA. *Nannochloropsis* sp. W2J3B was grown and prepared for electroporation as described in *Materials and Methods*. Varying amounts of NT7 DNA were transformed in single reactions (using 10^9 cells in 100 μL), and the total number of resulting zeocin-resistant colonies was counted. Fig. S2 shows a nearly linear relationship between the amount of DNA used and the number of transformants obtained. More than 8,000 transformants were obtained in a single electroporation experiment when 3 μg of NT7 was used. The lowest amount of DNA used (200 ng) yielded 70 transformants.

Determination of Cotransformation Efficiency. We designed selection markers with different resistance genes based on the *Nannochloropsis* sp. W2J3B violaxanthin/chlorophyll *a*-binding protein (VCP) gene *VCP1* by replacing the coding region (Fig. S1) with the *Sh ble* gene (selection marker PL90; conferring resistance against zeocin), the hygromycin B phosphotransferase gene *HygR* (selection marker H8; conferring resistance against hygromycin B), or the blasticidin S deaminase gene *Bsr* (selection marker B9; conferring resistance against blasticidin S). Wild-type cells were not able to survive on agar plates containing zeocin, blasticidin S, or hygromycin B added to a final concentration of 2 μg/mL, 50 μg/mL, or 300 μg/mL, respectively. In each electroporation experiment, 100 μL of a cell suspension (10^{10} cells/mL in 384 mM D-sorbitol) was mixed with 500 ng PL90, 2 μg H8, and 2 μg B9 and electroporated as described in *Materials and Methods*. Controls were transformed with 500 ng PL90 DNA only. Cells were treated as described in *Materials and Methods* and 5×10^8 cells were plated the next day on media containing 2 μg/mL zeocin.

The resulting zeocin-resistant colonies were resuspended in 50% artificial seawater, and 5 μL was spotted on plates containing zeocin, blasticidin S, or hygromycin B. The experiment was performed twice. The results shown in Table S1 indicate an overall cotransformation efficiency of 50–55% for at least one unselected marker and 23–31% for both unselected markers.

Transformation of *Nannochloropsis* sp. with Circular or Linear DNA. Transformation constructs conferring zeocin resistance were cloned into the plasmid pJet1 and used to transform *Nannochloropsis*. In five independent experiments using 500 ng to 2 μg circular plasmid DNA we obtained only one single transformant, whereas we routinely obtained hundreds to thousands of transformants per experiment in which we used either linearized plasmid DNA or a selection marker cassette amplified from a vector via PCR (Fig. S3).

Additional Evidence That *Nannochloropsis* W2J3B Is Haploid. We isolated flanking genomic DNA from several nontargeted insertion sites of transformation constructs via SiteFinding-PCR (1) and selected two insertion mutants, IM1 and IM2, in which the transformation construct had inserted into the genome without any DNA deletion, for further analysis (Fig. S5). We then performed PCR with primers A13 (5′-AAGGTACCATTGTGACAGGTCGCT-3′) and A14 (5′-TGTGCCTTTCAGGGCTTCTGTACT-3′), or A15 (5′-AAACTTTGAAGCCCTGCTGTCCAC-3′) and A16 (5′-ACGACATGGGAATACGCGTAAGGT-3′) designed based on regions upstream and downstream of the insertion sites. If *Nannochloropsis* W2J3B were diploid, two PCR products (one for the wild-type allele and one for the allele carrying the insertion construct) would be expected, whereas only the larger PCR product (the one carrying the insertion construct) would be expected if *Nannochloropsis* W2J3B is haploid. We obtained only a single PCR product from both insertion mutants, consistent with the conclusion that *Nannochloropsis* W2J3B is haploid.

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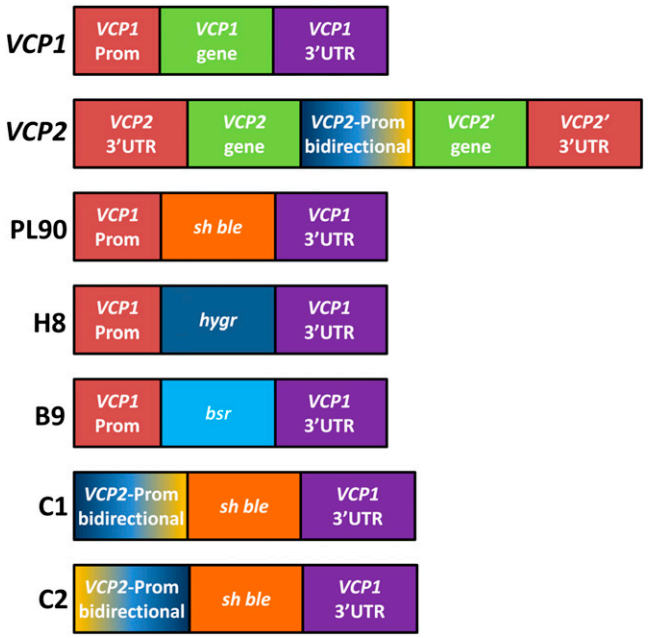

Fig. S1. Genes and additional transformation constructs used in this work. The *VCP1* gene of *Nannochloropsis sp*. W2J3B was used to design a series of transformation constructs in which the VCP1 coding sequence was replaced by various selectable marker genes, resulting in vectors conferring resistance against zeocin (PL90), hygromycin B (H8), and blasticidin S (B9). Additionally, the *VCP2* gene cluster from *Nannochloropsis* sp. W2J3B is illustrated. The replacement of the *VCP1* promoter with the bidirectional promoter (*VCP2*-Prom bidirectional) from the *VCP2* gene cluster resulted in two constructs, C1 and C2. The only difference between C1 and C2 is the orientation of the bidirectional promoter, as indicated by a color gradient.

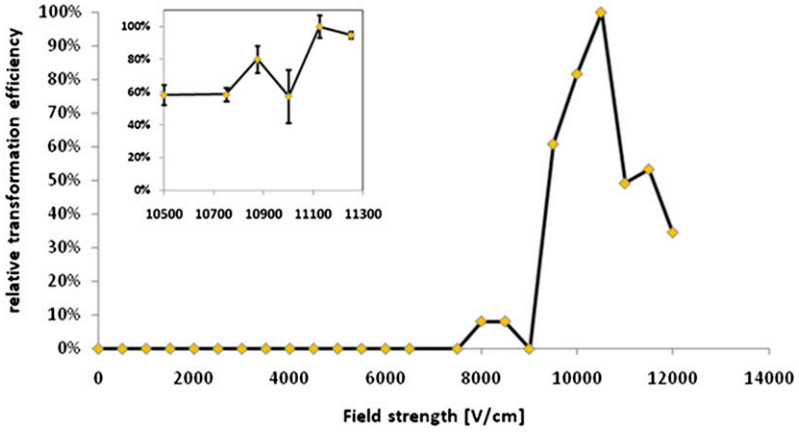

Fig. S2. Transformation efficiency as a function of field strength used during the electroporation of *Nannochloropsis* sp. W2J3B with 1 μg of the transformation construct PL90. In the initial experiment, field strengths higher than 9,000 V/cm resulted in the highest numbers of transformants. In subsequent experiments (*Inset*), the transformation efficiency obtained at field strengths between 10,500 and 11,250 V/cm was determined. Error bars denote SD, $n = 3$.

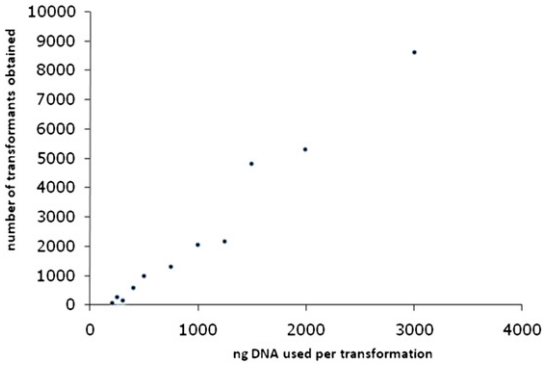

Fig. S3. Number of transformants obtained per electroporation experiment with varying amounts of NT7 DNA. The lowest number of transformants obtained was 70 when 200 ng of NT7 DNA was used.

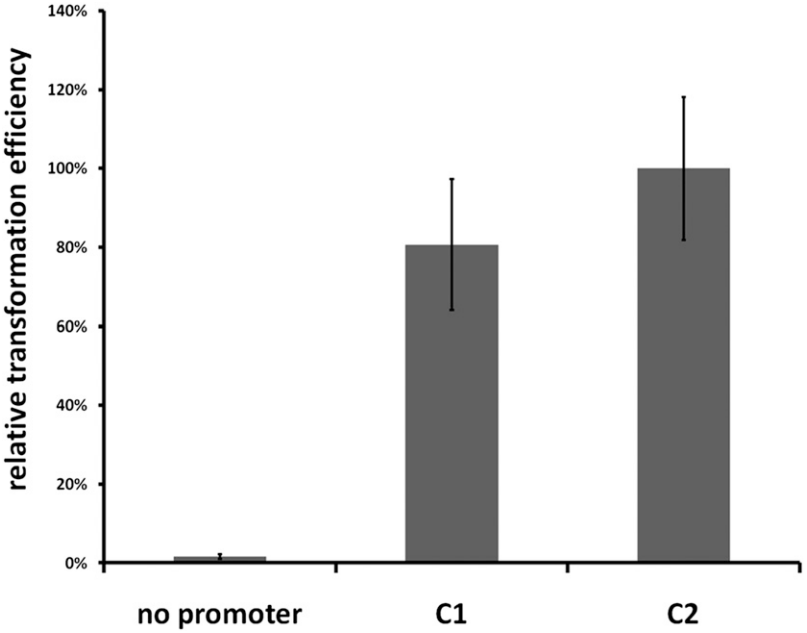

Fig. S4. Relative transformation efficiency for different transformation constructs containing the *VCP2* promoter. The C1 and C2 transformation constructs differ in having the *VCP2* promoter fragment inverted. The "no promoter" construct lacks the entire *VCP2* 5′ promoter except for 36 bp. Transformation frequencies were normalized to the frequency obtained with the C2 transformation construct. Error bars denote SD, $n = 4$.

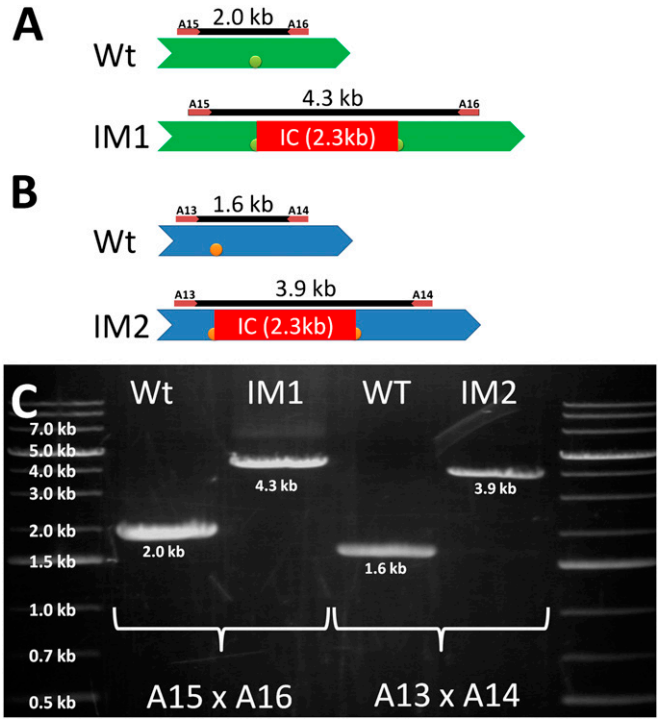

Fig. S5. Additional evidence for haploidy of *Nannochloropsis* sp. W2J3B from analysis of insertional mutants (IM) obtained by nontargeted integration of an insertion construct (IC). (*A*) Diagram of insertion site in IM1. (*B*) Diagram of insertion site in IM2. Green and orange dots denote the insertion sites in IM1 and IM2, respectively. (*C*) PCR analysis of genomic DNA obtained from wild type (Wt), IM1, and IM2 with the primer pairs A15/A16 and A13/A14 indicated in *A* and *B*. The presence of a single PCR band in reactions performed with IM1 and IM2 DNA indicates the presence of a single allele. Furthermore, the PCR product is 2.3 kb larger, indicating that the single allele carries the insertion construct.

Table S1. Results of cotransformation experiments

	Experiment 1		Experiment 2	
DNA used	Control	Mix	Control	Mix
Zeo^r transformants analyzed	14	16	8	88
Only Zeo^r	14	8	8	40
Zeo^r $Blast^r$	0	0 (0%)	0	12 (14%)
Zeo^r Hyg^r	0	3 (19%)	0	16 (18%)
Zeo^r $Blast^r$ Hyg^r	0	5 (31%)	0	20 (23%)
% CT $Blast^r$ and/or Hyg^r	NA	50%	NA	55%

In two independent experiments, 500 ng PL90 DNA (control) or a DNA mixture consisting of 500 ng PL90, 2 μg H8, and 2 μg B9 (mix) was transformed into *Nannochloropsis* sp. W2J3B. Colonies obtained after zeocin selection were resuspended and spotted on media containing zeocin, blasticidin S, or hygromycin B. The number of transformants growing on zeocin (Zeo^r), zeocin and blasticidin S (Zeo^r $Blast^r$), zeocin and hygromycin B (Zeo^r Hyg^r), or all three antibiotics (Zeo^r $Blast^r$ Hyg^r) is indicated. As expected, none of the control transformants obtained with PL90 DNA alone was capable of growing on blasticidin S or hygromycin B. Calculated percentages of cotransformation (%CT) of each or both of the unselected markers H8 or B9 are indicated. NA, not applicable.

Table S2. Results of homologous recombination (HR) experiments

Transformation construct	HR1	HR2	HR3	HR4
NR-KO	90/96 (94%)	53/96 (55%)	27/96 (28%)	21/84 (25%)
NiR-KO	—	21/96 (22%)	11/96 (11%)	11/72 (15%)

Zeocin-resistant colonies resulting from four or three independent transformation experiments (HR1–HR4) with the nitrate reductase (NR)-KO and nitrite reductase (NiR)-KO transformation constructs (Fig. 2), respectively, were picked and spotted on plates containing either ammonium or nitrate as a sole nitrogen source. The number of putative KO mutants obtained, based on obvious bleaching of the cells on nitrate (as shown in Fig. 3), is shown along with the number of zeocin-resistant transformants analyzed. The calculated HR frequency is shown in parentheses.