



An inducible expression system in the alga *Nannochloropsis gaditana* controlled by the nitrate reductase promoter

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Abstract

The marine microalgal species *Nannochloropsis gaditana* has emerged as a model organism for research into algal-derived biofuels due to its ability to produce large amounts of lipids. In addition, this species has biotechnological potential owing to its ease of transformation and availability of a sequenced and annotated genome. The ability to control expression of transgenes in an inducible manner is an important requirement for many genetic engineering approaches and as such, inducible promoters are an important component of the molecular toolkit for a host organism. We designed an expression vector containing a ~1.3 kb region upstream of the endogenous nitrate reductase gene of *N. gaditana* and demonstrated this is capable of promoting the expression of a heterologous enhanced green fluorescent protein (eGFP) reporter. In the presence of ammonium, expression of the eGFP reporter was undetectable; however, in the presence of nitrate, eGFP expression is induced. In addition, we demonstrated how altering the nitrogen source of the growth media can be used to precisely control expression. We have shown that the nitrate reductase promoter can be used as a powerful molecular tool for heterologous protein expression in *N. gaditana*, further contributing to the development of this species as a candidate strain for biotechnology.

Keywords *Nannochloropsis* · Synthetic biology · Biofuels · Heterologous expression · Inducible promoter · Nitrate reductase

Introduction

Recent advances have seen the emergence of *Nannochloropsis* spp. as a platform for biotechnological application of marine microalgae. Reverse genetic approaches using random mutagenesis combined with high-throughput phenotypic screening have isolated *Nannochloropsis* strains with improved light-use efficiency and increased lipid accumulation (Doan and Obbard 2012; Beacham et al. 2015; Perin et al. 2015). Further,

Nannochloropsis spp. are haploids and transformation of the nuclear genome with integration constructs has been established and shown to occur by high-efficiency homologous recombination in *Nannochloropsis oceanica*, allowing the generation of knockout mutants (Kilian et al. 2011; Radakovits et al. 2012). Wang et al. (2016) reported the first use of a CRISPR/Cas9-based approach in a *Nannochloropsis* species, generating mutant strains of *N. oceanica* with precise five-base deletions in the nitrate reductase gene, demonstrating the potential for sophisticated gene editing techniques.

Within the genus, *Nannochloropsis gaditana* is a particularly promising organism for biofuel production systems due to its ability to accumulate high levels of lipids such as triacylglycerols (TAGs), which can exceed 60% of total biomass on a dry weight basis under nitrogen starvation (Rodolfi et al. 2009). A detailed understanding of key metabolic pathways in *N. gaditana* has been assisted by the draft assembly of the nuclear (~27.5 Mbp; 10,646 protein-coding genes) and organellar genomes (Radakovits et al. 2012; Jinkerson et al. 2013; Corteggiani Carpinelli et al. 2014). Codon utilization analysis of *N. gaditana* has shown no unused codons, an advantageous property for host systems for heterologous protein expression (Jinkerson et al. 2013). Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 has also

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been successfully implemented in *N. gaditana* to knock out 18 transcription factors involved in negative lipid regulation under nitrogen starvation (Ajjawi et al. 2017). Attenuation of one of the identified genes yielded strains with a twofold increase in lipid content and little effect on growth, which has previously been a problem of alteration to lipid metabolism (Ajjawi et al. 2017). These advances have resulted in the emergence of *N. gaditana* as a candidate platform species to enable further biotechnological development.

Endogenous promoters have been shown to be more effective for generating stable transformants and driving expression of heterologous genes (Walker et al. 2005). A limited number of endogenous promoters have been used to successfully drive expression of heterologous genes in *N. gaditana*, although this number is likely to increase (Radakovits et al. 2012; Ajjawi et al. 2017). The availability of a wide selection of endogenous promoters with well-characterized and novel expression traits is a key requirement for the development of a species from both a fundamental research and a biotechnological perspective (Doron et al. 2016). In particular, there is a requirement for inducible promoters that can be switched on and off, without negative effects on growth, to enable temporally controlled expression of toxic molecules such as Cas9 or for use in silencing systems such as RNAi (Jiang et al. 2014; Doron et al. 2016). Various inducible expression systems have been developed in algae; however, many rely on induction methods that may have limitations in a photoautotrophic species. For example, the *hsp70A* promoter has been used in green algae to regulate heat-induced expression; however, the use of heat as a method of induction may deem this promoter unsuitable as a molecular switch for *N. gaditana* due to the possibility of detrimental effects on growth caused by changing temperatures (Schroda et al. 2000; Jakobiak et al. 2004). Light-based induction systems have also been explored in algae, initially by use of the *CABII-I* promoter in *Chlamydomonas*, but pose similar concerns regarding the effects of the method of induction on growth (Blankenship and Kindle 1992; Park et al. 2013; Baek et al. 2016).

We therefore considered expression systems controlled by nutrients or chemicals that can be regulated in the growth media. Algae (including *Nannochloropsis* spp.) are able to utilise nitrate and subsequently convert it into ammonium through use of the enzyme nitrate reductase (Fernández et al. 1989; Berges 1997). Micromolar concentrations of nitrate elicit a rapid induction of expression of the nitrate reductase gene (Raven et al. 1992; Forde 2000; Galván and Fernández 2001; Llamas et al. 2002; Fernández and Galván 2008). The energy cost of direct ammonium assimilation is lower than that of nitrate (Bloom et al. 1992). Consequently, the presence of ammonium provides a negative feedback effect on nitrate assimilation and suppresses the nitrate reductase gene (Ohresser et al. 1997; Loppes et al. 1999; Llamas et al. 2002; Fernández and Galván 2008). Hence, expression of the nitrate reductase gene is induced in the presence of nitrate ions

and repressed in the presence of ammonium ions in a range of algae, including *Cylindrotheca fusiformis*, *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Dunaliella salina*, *Volvox carterii* and *Phaeodactylum triornutum* (Wang et al. 2004; Poulsen and Kröger 2005; Li et al. 2007; Schmollinger et al. 2010; Niu et al. 2012; von der Heyde et al. 2015). In addition, the promoter of the nitrate reductase gene has been used to regulate inducible expression of a reporter gene in several species of algae including *C. reinhardtii* (Schmollinger et al. 2010), *C. ellipsoidea* (Wang et al. 2004), *D. salina* (Li et al. 2008; Li et al. 2007) and *P. triornutum* (Niu et al. 2012).

Using an enhanced green fluorescent protein (eGFP) as a fluorescent reporter and for immunoblot analysis, we investigated the ability of the endogenous *N. gaditana* nitrate reductase promoter to robustly drive inducible expression of heterologous protein in transgenic cell lines. In addition, the temporal regulation characteristics of the nitrate reductase promoter over a range of nitrogen conditions were explored at the protein level to help define the biotechnological potential of this inducible promoter.

Methods

Strains and culture conditions

Nannochloropsis gaditana CCMP526 was obtained from the National Center for Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, Maine, USA) and grown in F2N, a modified version of F/2 media for *Nannochloropsis* (Kilian et al. 2011), containing 5 mM ammonium (NH_4Cl) as a nitrogen source. Media were prepared with 30 g L^{-1} Tropic Marin artificial sea salts (Tropical Marine Centre, Rickmansworth, UK). Where mentioned, nitrogen sources were replaced with sodium nitrate (NaNO_3 ; 5 mM), urea ($\text{CH}_4\text{N}_2\text{O}$; 5 mM), or in some cases, no nitrogen was present. F2N agar plates were prepared with 1.5% (*w/v*) bacteriological agar. Liquid cultures were maintained at a photon flux density of 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30 °C in 25 cm^2 vented corning flasks (Thermo Scientific, UK) with gentle shaking. Agar plates were maintained under continuous light at the same irradiance and temperature. All chemicals were reagent-grade and were obtained from Sigma-Aldrich (UK) or Melford Laboratories Ltd. (UK) unless otherwise mentioned.

For the P_{NR} nitrogen source regulation experiments, liquid starter cultures were grown to the exponential phase, harvested, washed in nitrogen-deplete medium ($\times 3$), and subsequently inoculated at an $A_{540 \text{ nm}}$ of 0.02.

$A_{540 \text{ nm}}$ to cells per millilitre calibration curve

A series of dilutions of *N. gaditana* wild-type cells was prepared. The absorbance at 540 nm ($A_{540 \text{ nm}}$) was measured for

all dilutions using a Jenway 7315 spectrophotometer (Keison Products, UK). Cell concentrations in cells per millilitre were determined for all dilutions using a Beckman Multisizer 3 Coulter Counter (Beckman Coulter Life Sciences, USA).

Molecular cloning

All primers used in this study are listed in Online Resource 1. PCRs for amplification of vector elements were performed using the manufacturers cycling parameters and Q5 polymerase (New England Biolabs, USA). Wild-type *N. gaditana* genomic DNA was purified with the Wizard genomic DNA purification kit (Promega, USA) and used as a template to amplify genomic sequences.

Vectors were generated by in vivo homologous recombination in yeast (Oldenburg et al. 1997). To obtain the native promoter sequence of the nitrate reductase gene, a region of ~1.3 kb upstream of the gene (Naga_100699g1) was amplified using primers P_{NR} fw XX ext. and P_{NR} rv eGFP ext. A region of ~1.1 kb upstream of the native lipocalin protein coding gene (Naga_100131g14) was amplified using primers P_{LCP} fw XX ext. and P_{LCP} rv eGFP ext. The eGFP sequence was amplified from pGAP- α -GFP (unpublished) using the eGFP primer pair. The native ATP synthase α subunit terminator was generated using primers $T_{ATP\alpha}$ fw eGFP ext. and $T_{ATP\alpha}$ rv CC ext. The ubiquitin extension protein promoter (UEP) promoter (Radakovits et al. 2012) was amplified using primers P_{UEP} fw CC ext. and P_{UEP} rv hph ext. The hygromycin B selection marker was amplified from pZC1 using the *hph* primer pair. The NOS terminator was amplified from the pGWB402 vector (Addgene) using primers T_{NOS} fw *hph* ext. and T_{NOS} rv YY ext. The amplicons generated have 30 bp extensions at the 5' and 3' ends that direct recombination with adjacent amplicons upon co-transformation into yeast. Amplicons were co-transformed along with the acceptor vector pRS426 (Fungal Genetics Stock Centre, School of Biological Sciences, Missouri, USA) following linearization by PCR with primer pair YY fw PmeI ext. and XX rv PmeI ext., for assembly via the endogenous recombination system of yeast (Oldenburg et al. 1997). Yeast transformations were carried out using the lithium acetate/PEG method (Schiestl and Gietz 1989) and grown in 20 mL of SC-uracil for selection. The assembled plasmid was transferred from yeast to *Escherichia coli* and, following restriction digest screening, the cassette was released from the backbone by digestion with *PmeI* before transformation into *N. gaditana*. Plasmid DNA was purified with mini-prep kits from Zymo Research (USA). FastDigest restriction enzymes were purchased from Thermo Scientific (UK).

Transformation of *N. gaditana*

Nannochloropsis gaditana was transformed largely in accordance with the methods described previously (Kilian et al.

2011; Radakovits et al. 2012). Two hundred-millilitre cultures were grown in F2N media to the exponential phase ($\sim 2 \times 10^6$ cells mL⁻¹). Then, 5×10^8 cells were harvested per reaction and washed ($\times 3$) in 375 mM sorbitol before resuspension in 100 μ L of 375 mM sorbitol with addition of 2–5 μ g of linearized plasmid DNA. Centrifugation was performed at $3000 \times g$ for 10 min (4 °C). Electroporation was carried out at 12 kV cm⁻¹ in pre-chilled 2-mm cuvettes using a BIO-RAD MicroPulser electroporator. Cells were then immediately recovered in 10 mL of F2N medium and incubated overnight in 70 μ mol photons m⁻² s⁻¹ fluorescent white light at 30 °C. The following day cells were plated at 5×10^7 cells plate⁻¹ F2N agar plates (1.5%) containing 300 μ g mL⁻¹ hygromycin B; artificial sea salts and NH₄Cl were lowered to 15 g L⁻¹ and 2 mM, respectively. Selection plates were incubated under 70 μ mol photons m⁻² s⁻¹ fluorescent white light at 30 °C until transformants appeared (~4 weeks) and were ready for further processing (~5–6 weeks). Recovered transformants were sub-cultured once in 3 mL F2N medium containing 150 μ g mL⁻¹ Hygromycin B and subsequently maintained in F2N medium containing no antibiotic.

Microplate reader assays and calculation of fluorescence levels

The fluorescence of live cells was analysed using a FLUOstar OPTIMA microplate reader (BMG Labtech Ltd) in black-bottomed 96-well plates (Thermo Scientific) in triplicate. One hundred microlitre samples were used for the expression level analysis; for all other experiments, 200 μ L samples were used. eGFP fluorescence was measured using filter settings appropriate to the maximal emission and excitation wavelengths for eGFP, 488 and 509 nm, respectively. F2N medium was used as a blank for all samples; average blank measurements ($3n$) were subtracted from all fluorescence measurements. Fluorescence measurements for each replicate were then normalised to its absorbance at a wavelength of 540 nm ($A_{540 \text{ nm}}$). Finally, average normalised wild-type fluorescence values ($3n$) were subtracted from all normalised fluorescence values to determine eGFP fluorescence or divided into normalised fluorescence values to determine fold changes.

For quantification of fluorescence as nanogram eGFP per 10^7 cells, normalised wild-type fluorescence-subtracted fluorescence measurements were converted to eGFP concentrations in nanograms per microlitre using an eGFP calibration curve. For the eGFP calibration curve (Online Resource 2), 100 μ L eGFP standards were prepared in dH₂O at the following concentrations (ng μ L⁻¹): 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10. The $A_{540 \text{ nm}}$ to cell mL⁻¹ calibration curve was used to calculate the cell concentration in cells per microlitre at an $A_{540 \text{ nm}}$ of 1, eGFP concentrations in nanograms per microlitre for each replicate were then divided by this value to determine the nanogram of eGFP per unit cell.

Immunoblotting and quantification of eGFP

Forty-millilitre cultures of *N. gaditana* were used to prepare whole cell protein extracts. Cells were harvested by centrifugation at 3000×g for 10 min at room temperature. For homogenization, approximately 100 mg of 0.1 mm zirconia beads (Biospec Products, USA) were added to the pelleted cells in addition to an equal volume (~150 µL) of SDS lysis buffer (200 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, 200 mM Tris-Cl, pH 8.5). Cells were then lysed in a TissueLyser II tissue lyser (QIAGEN, Germany) for 2 × 30 s cycles at a frequency of 30 oscillations per second. Samples were heated to 95 °C in a heat block for 10 min, cooled briefly on ice, and then pelleted by centrifugation at 17,000×g for 10 min at 4 °C. The total protein content of the lysate was quantified using a Pierce bicinchoninic acid (BCA) protein assay (Thermo Scientific) with a bovine serum albumin (BSA) standard. Recombinant green fluorescence protein (rGFP; Sigma-Aldrich) was used to prepare protein standards for quantification of immunoblots. Ten micrograms of each protein sample was added to LDS loading buffer (Invitrogen) containing 50 mM DTT before heating to 70 °C for 10 min. Electrophoresis was carried out on 4–12% (w/v) gradient Bis-tris NuPAGE gels in MES buffer (Invitrogen) in a Novex XCell SureLock Mini Cell (Invitrogen) for 35 min at 200 V. An rGFP standard was loaded at 20, 40, 80, 160 and 320 ng per well.

Blotting was performed as described previously (Berepiki et al. 2016). Membranes were incubated first with an anti-GFP antibody (N-terminal antibody produced in rabbit; Sigma-Aldrich) diluted to 1:1000 in blocking solution and then with goat anti-rabbit IgG (Agriseria) diluted to 1:1000 in blocking solution. Blots were then incubated with SuperSignal West Dura reagents (Thermo Scientific) and imaged using a C-DiGit Imaging system (LI-COR Biosciences, USA). For quantification of immunoblots, images were analysed using ImageJ software (National Institute of Health, Maryland, USA).

Results

Construction of the nitrate reductase expression vector and generation of transgenic cell lines

The efficiency of gene expression driven by the nitrate reductase promoter sequence (P_{NR}) of *N. gaditana* was analysed using the enhanced green fluorescent protein (eGFP) expression vector pNR (Fig. 1; see Online Resource 3 for complete annotated vector map). The promoter was defined as the region ~1.3 kb upstream of the nitrate reductase gene (Naga_100699g1) from the *N. gaditana* B31 genome assembly (assembly accession GCA_000569095.1; Corteggiani Carpinelli et al. 2014). A length of ~1.3 kb was chosen in order to ensure all of the regulatory elements within the promoter sequence were included.

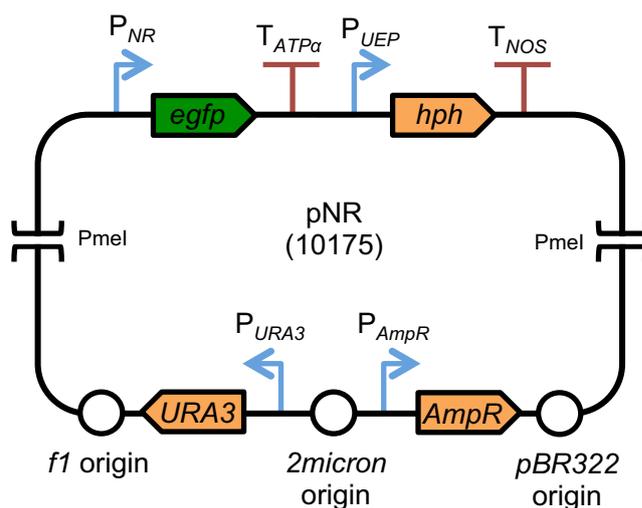


Fig. 1 Vector used for characterization of the NR promoter. P_{NR} = nitrate reductase promoter, *egfp* = enhanced green fluorescent protein gene, $T_{ATP\alpha}$ = ATP synthase α subunit terminator, *hph* = hygromycin phosphotransferase gene, T_{NOS} = *Agrobacterium tumefaciens* nopaline synthase terminator. Vector was linearized with PmeI before transformation of *N. gaditana* to improve transformation efficiency and remove accessory elements for vector preparation in *S. cerevisiae* and *E. coli*. Figure was drawn to SBOL standards (Galdzicki et al. 2014)

Promoter analysis with TSSPlant (Shahmuradov et al. 2017) suggested a putative transcription start site at –633 bp relative to the *NR* start codon (although this has not been confirmed experimentally) with a TATA box at –681 bp. Analysis with PLACE and PlantCARE (Higo et al. 1999; Lescot 2002) identified several putative CAAT box motifs upstream of the TATA box (see Online Resource 4 for an annotated sequence of the *NR* promoter). Further motif analysis of this promoter region of the *N. gaditana* nitrate reductase gene was performed against *NR* genes from *C. reinhardtii* (XP_001696697.1), *Thalassiosira pseudonana* (XP_002294410.1), *Micromonas pusilla* (XP_003058321.1), *Chondrus crispus* (XP_005714489.1) and *Chlorella variabilis* (XP_005844793.1) using the MEME search tool (Multiple EM for Motif Elicitation; Bailey et al. 2009). No significantly conserved motifs between the promoter sequences were detected. This is not surprising given the low identity between the *NR* genes of these respective species (40–50%). Algal *NR* genes are diverse in sequence and have a larger numbers of introns than higher plants (Song and Ward 2004). The promoter of the *NR* gene in *N. oceanica* is located between a divergent gene pair and may therefore be bidirectional; however, this is not the case in *N. gaditana* (Poliner et al. 2018). This analysis highlights the diversity in the promoter regions of *NR* genes, which warrants further investigation.

This *N. gaditana* nitrate reductase promoter sequence was incorporated into the pNR vector at the 5' end of an eGFP coding sequence (*egfp*) that was terminated by a ~0.5 kb terminator region ($T_{ATP\alpha}$) of the endogenous ATP synthase α subunit (Naga_100099g7). The hygromycin phosphotransferase resistance gene (*hph*) from *E. coli*, conferring resistance to

hygromycin B was used as a selectable marker for transformation. From 5' to 3', the resistance cassette of the pNR vector consisted of an endogenous ubiquitin extension protein promoter (P_{UEP}) (Radakovits et al. 2012), the *hph* gene, and an *Agrobacterium tumefaciens* nopaline synthase terminator (T_{NOS}). Two PmeI restriction sites flanking the expression cassette were used to release the cassette from the vector backbone and linearize the DNA for transformation of *N. gaditana*. *Nannochloropsis gaditana* was grown to the exponential phase in liquid media before being harvested for transformation with the pNR vector by electroporation. After selection on hygromycin B, 20 transformants were recovered (NgNR1–20). These cell lines were sub-cultured once in liquid media containing hygromycin B to confirm antibiotic resistance and to remove any wild-type cells. (Fig. 2a). They were subsequently maintained in standard media and on agar plates without antibiotic selection. Integration of the expression cassette into the *N. gaditana* genome in the NgNR cell lines was shown by PCR confirmation of the presence of the *hph* gene (Fig. 2b; see Online Resource 1 for primer sequences).

eGFP expression level analysis of transgenic cell lines

The integration sites of chimeric DNA following electroporation-based transformation are largely randomly distributed within the genome and positional effects can affect transgene expression levels (Thompson and Gasson 2001; Zhang et al. 2014; Chen and Zhang 2016). Accordingly, the eGFP expression levels of all 20 of the pNR transformants were analysed to determine the average and the range of expression from the nitrate reductase promoter. NgNR1–20 and *N. gaditana* wild-type cultures were grown to the exponential phase in media containing either 5 mM sodium nitrate or 5 mM ammonium chloride as the sole nitrogen source to induce or repress expression, respectively.

eGFP fluorescence was analysed as described previously (Rasala et al. 2013). Fluorescence from eGFP was measured

in a series of recombinant eGFP standards, the *N. gaditana* wild type and NgNR1–20 enabling estimation of eGFP content volumetrically and per cell. Qualitative eGFP fluorescence measurements were made quantitative using calibration curves as in Richards et al. (2003). A calibration curve for the eGFP standard (fluorescence vs ng μL^{-1}) was generated in order to convert the fluorescence measurements of the samples from relative fluorescence to nanogram eGFP per microlitre (Online Resource 2). Additionally, a calibration curve of $A_{540\text{ nm}}$ to cell concentration in cells per millilitre (Online Resource 2) was used to calculate the cell concentration and subsequently an estimate of the concentration of eGFP 10^7 cells^{-1} .

The eGFP expression levels varied across the 20 NgNR strains (Fig. 3a). Average eGFP expression across all the NgNR strains in the presence of nitrate was 443.5 ± 330.1 SD ng eGFP per 10^7 cells. In the ammonium-grown cultures the average eGFP expression level was undetectable above wild-type auto-fluorescence, indicating strong suppression. Fold change in fluorescence over wild-type auto-fluorescence was also calculated for each NgNR strain in both nitrate and ammonium (Fig. 3b). The average fold change in fluorescence across all NgNR strains was 4.2 ± 2.2 SD in the presence of nitrate and 1.0 ± 0.4 SD (i.e., no change) in the presence of ammonium. Having demonstrated that the regulation of expression is consistent in 20 independent strains, NgNR3, which displayed an average expression level and had low background fluorescence levels in the suppressed state, was selected for further analysis.

To confirm that the changes in eGFP fluorescence in the NgNR strains were due to regulation of the nitrate reductase promoter and not to other physiological responses due to the alteration of the nitrogen source from ammonium to nitrate, a control experiment was performed. An expression vector was constructed (pLCP) that was identical to pNR except that the nitrate reductase promoter was switched for an endogenous constitutive promoter (PLCP; Naga_100131g14) coding for a

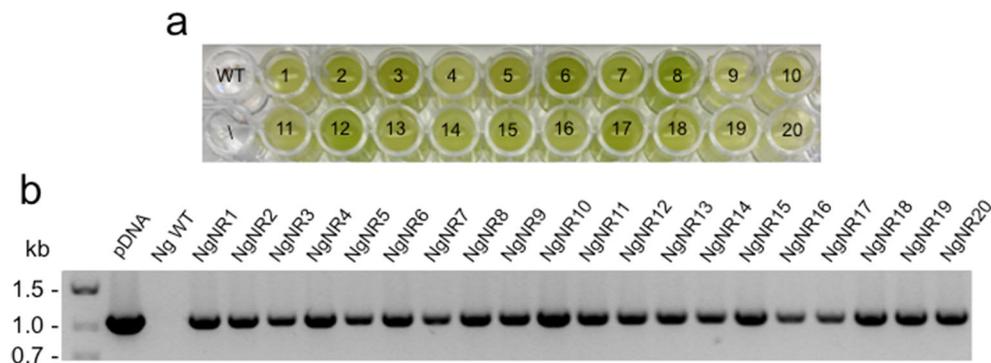


Fig. 2 Confirmation of vector integration into 20 transgenic *N. gaditana* cell lines. **a** The recovered transformants (NgNR1–20) were sub-cultured in liquid media containing hygromycin B once to confirm resistance. WT = *N. gaditana* wild type; NgNR strains are numbered from 1 to 20. **b**

Genotyping by PCR using primers binding to the 5' and 3' end of the *hph* gene, producing a product of 1029 bp in length. Template DNA: DNA ladder, lane 1; pNR vector (positive control), lane 2; *N. gaditana* wild type (negative control), lane 3; NgNR strains 1 to 20, lanes 3 to 23

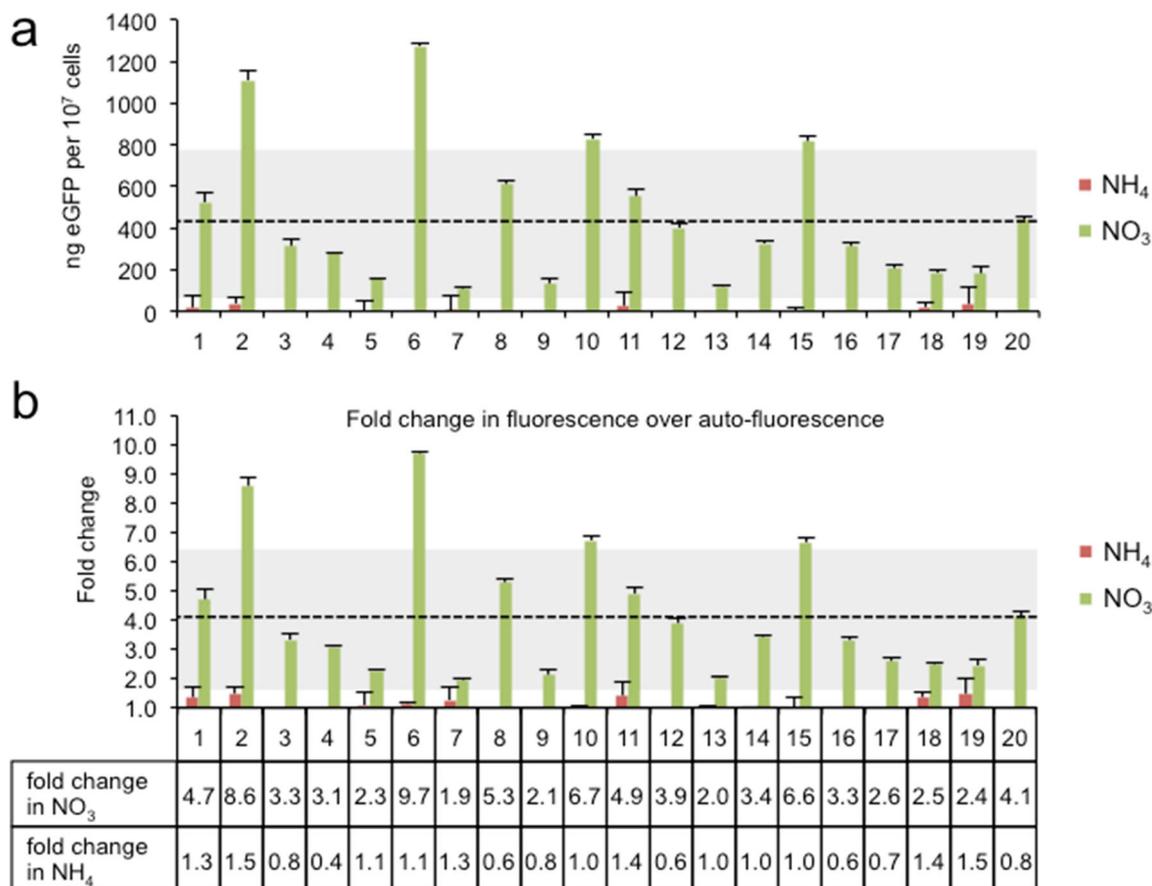


Fig. 3 Analysis of eGFP expression levels in 20 NgNR strains. **a** Analysis of eGFP expression in NgNR1–20. Nanogram eGFP per 10^7 cells was calculated using calibration curves (see Online Resource 2). The average eGFP expression level across the 20 NgNR strains was 443.5 ng eGFP per 10^7 cells in the nitrate-grown cultures (green bars); in the ammonium-grown cultures (red bars) the average eGFP was undetectable above wild-type auto-fluorescence. Dotted line and shaded

area denote mean \pm SD across all strains. Values are means \pm SD of triplicate measurements. **b** The fold change in fluorescence over wild-type auto-fluorescence across all strains. The average fold increase across the NgNR strains was 4.2 in the nitrate-grown cultures (green bars) and 1.0 (i.e. no change) in the ammonium-grown cultures (red bars). Dotted line and shaded area denote mean \pm SD across all strains. Values are means \pm SD of triplicate measurements

lipocalin protein. Transcriptomic data obtained in *N. gaditana* showed that native transcript levels of the lipocalin protein were unchanged under nitrogen deprivation: 464.18 reads per kilobase of transcript per million mapped reads (RPKM) on day 3 of growth in sodium nitrate and 436.61 RPKM on day 3 under nitrogen depletion (Corteggiani Carpinelli et al. 2014). Three transformants generated with the pLCP vector (NgLCP1–3) were grown to the exponential phase in 5 mM sodium nitrate and 5 mM ammonium chloride and analysed for eGFP fluorescence as described for the NgNR cultures (Online Resource 5). The wild-type subtracted normalised fluorescence measurements in NgLCP1–3 were variable between replicates as was observed for the NgNR strains. However, there was no significant difference between eGFP fluorescence in each replicate when the nitrogen source was altered ($p = 0.44$, Student's *t* test). Hence, the control analysis with the NgLCP strains showed no significant effect on eGFP fluorescence levels when the nitrogen source was switched from ammonium to nitrate, confirming that the changes

observed in the NgNR strains were due to regulation of the nitrate reductase promoter.

To confirm that eGFP was expressed, NgNR3 was further examined by immunoblot analysis (Online Resource 6). Protein samples were extracted from wild-type cells and NgNR3 grown to the exponential phase in media containing either 5 mM ammonium or 5 mM nitrate as the sole nitrogen source. eGFP was detected as a single band at 27 kDa in the nitrate-grown NgNR3 culture but was undetectable in both the *N. gaditana* wild-type and NgNR3 grown in ammonium. Quantification of the protein band in the nitrate-grown NgNR3 culture using the recombinant eGFP standards calibration curve determined a concentration of 115 ng of eGFP per 10 μ g of total protein (approx. 1.15% of total protein).

Regulation of P_{NR} in different nitrogen conditions

The ability to induce expression of the nitrate reductase promoter in cells activity growing on other nitrogen sources is a

potentially useful feature for heterologous protein expression in *N. gaditana*. eGFP expression and cell density in NgNR3 cultures pre-grown in different nitrogen conditions is shown in Fig. 4 (see Online Resource 2 for a cell density calibration curve for conversion of $A_{540\text{ nm}}$ to cells per millilitre). NgNR3 and wild-type cells were grown to exponential phase in standard media, washed in nitrogen-deplete media three times, and then used to inoculate cultures containing ammonium, urea (each at 5 mM) or no nitrogen. Cells were treated with 5 mM nitrate on day 5 to induce expression. Cell growth and eGFP expression in the NgNR3 cultures was measured every 2 days (see “Methods”). eGFP fluorescence was undetectable in NgNR3 for the first 4 days of growth on ammonium. After the addition of nitrate to the ammonium-grown cultures on day 5, eGFP fluorescence was detected the following day, showing that induction of P_{NR} is possible in the presence of both ammonium and nitrate. Fluorescence continued to increase and peaked on day 10 (Fig. 4a). However, the eGFP fluorescence level in NgNR3 on day 10 was $\sim 13.9\%$ of that achieved when NgNR3 was grown in media containing

nitrate as the only source of nitrogen (Fig. 4c). The growth of the ammonium-grown wild-type and NgNR3 cultures was not statistically significantly different over the 12 days ($p = 0.38$, Student's t test; Fig. 4d).

When NgNR3 was grown on urea, eGFP fluorescence was observed before the addition of nitrate on day 5, indicating that the presence of ammonium is required for full suppression of P_{NR} in these conditions. The level of eGFP expression after the addition of nitrate to the urea-grown cells was similar to the ammonium-grown cells (equivalent to $\sim 14.1\%$ of the expression achieved in the presence of nitrate alone; Fig. 4b). As in the ammonium-grown cultures, there was no statistically significant difference between growth of the wild type and NgNR3 over the 12 days ($p = 0.37$, Student's t test; Fig. 4e).

eGFP fluorescence in the nitrogen-depleted cells peaked on day 4, before the addition of nitrate on day 5, and was equivalent to 81.1% of the expression achieved in the presence of nitrate alone (Fig. 4c). This is the highest fluorescence level observed in this series of experiments, further indicating that the presence of ammonium is required for full suppression of

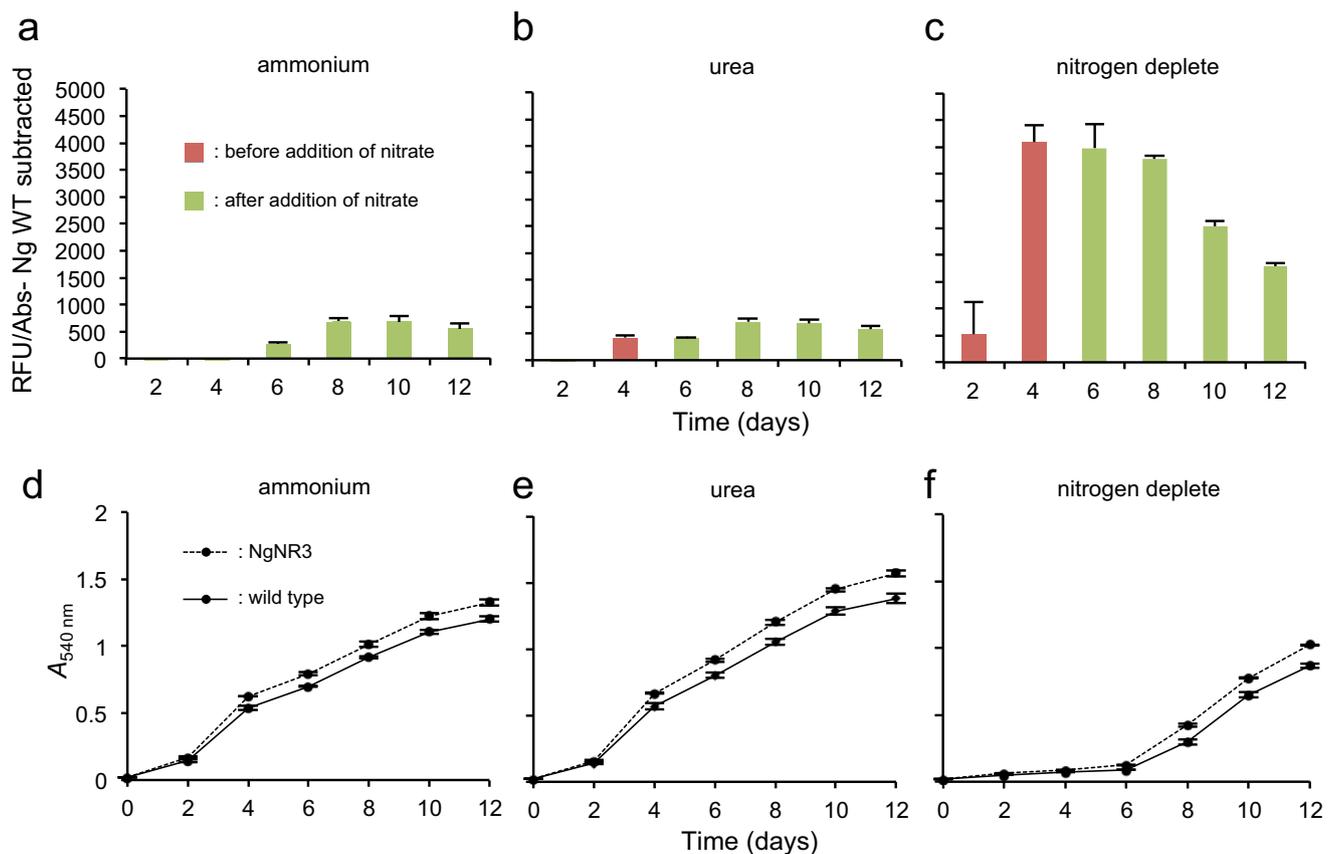


Fig. 4 Regulation of P_{NR} with different nitrogen sources. *N. gaditana* wild-type and NgNR3 cultures were grown on the following nitrogen sources before addition of 5 mM sodium nitrate to cultures on day 5: 5 mM ammonium chloride (a), 5 mM urea (b), and nitrogen-depleted media (c). P_{NR} -driven eGFP expression in NgNR3. eGFP fluorescence is expressed as normalized fluorescence with wild type auto-fluorescence subtracted. Red bars indicate expression before addition of nitrate on day

5; green bars indicate expression after addition of nitrate. Growth curves for NgNR3 grown on ammonium chloride (d), urea (e), and nitrogen-deplete media (f). Dashed lines indicate NgNR3 and solid lines the *N. gaditana* wild type. Growth in each condition was not significantly different between NgNR3 and the wild-type ($p = 0.37$ – 0.38 , Student's t test). Data shown as mean \pm S.D. of triplicate measurements

expression from P_{NR} under these conditions. The absence of any nitrogen source until the addition of nitrate on day 5 resulted in low levels of growth up until this point. However, in these conditions, growth of NgNR3 was not statistically significantly different from the wild type ($p = 0.37$, Student's t test; Fig. 4f). Furthermore, a comparison of the growth after the addition of nitrate on day 5 to that in the ammonium (nitrogen source in standard F2N medium; Kilian et al. 2011) grown cultures showed no significant difference for both the wild type and NgNR3 ($p = 0.31$ and 0.26 , respectively, Student's t test; Fig. 4d, f).

Long-term stability of NgNR3

The ability of transgenic biotechnological strains to withstand the effects of silencing of transgene expression is of particular importance. To confirm the long-term stability of the pNR cassette, NgNR3 was sub-cultured for 20 months in liquid culture without selective pressure before being re-tested for resistance to hygromycin B. Figure 5a shows that this line still retained resistance under these conditions. The presence of the *hph* gene was confirmed by PCR (Fig. 5b) and eGFP expression was confirmed by fluorescence, as described previously (Fig. 5c).

Discussion

Systems for inducible expression are of great importance, allowing for the temporal expression of toxic proteins and potentially for the control of heterologous protein expression in large-scale commercial growth operations. The potential of the promoter of the endogenous nitrate reductase gene of *N. gaditana* to meet these demands was explored in this study.

The eGFP fluorescence-based analysis of expression levels over 20 strains transformed with the pNR constructed (NgNR1–20) allowed calculation of an average eGFP expression level of 443.5 ± 330.1 SD ng eGFP per 10^7 cells in the presence of nitrate and suppression of expression to undetectable levels in ammonium (Fig. 3a). Assuming a cellular protein content of approximately 3 pg protein cell⁻¹ (Fábregas et al. 2002), the average level of eGFP expression in the presence of nitrate in picogram per cell (4.435×10^{-2} ; Fig. 3a) is equivalent to approximately $1.48 \pm 1.10\%$ SD of total protein. This is comparable to expression systems established in the model organism *C. reinhardtii* in which heterologous protein production in the chloroplast typically ranges from 1 to 5% of total protein (Manuell et al. 2007; Rasala et al. 2011; Almaraz-Delgado et al. 2014). Using the nitrate reductase promoter to drive expression of eGFP, an average fold change in fluorescence of 4.2 was achieved (Fig. 3b). Many factors influence the ability to detect fluorescent proteins in a cellular context, such as background auto-fluorescence, the strength of the

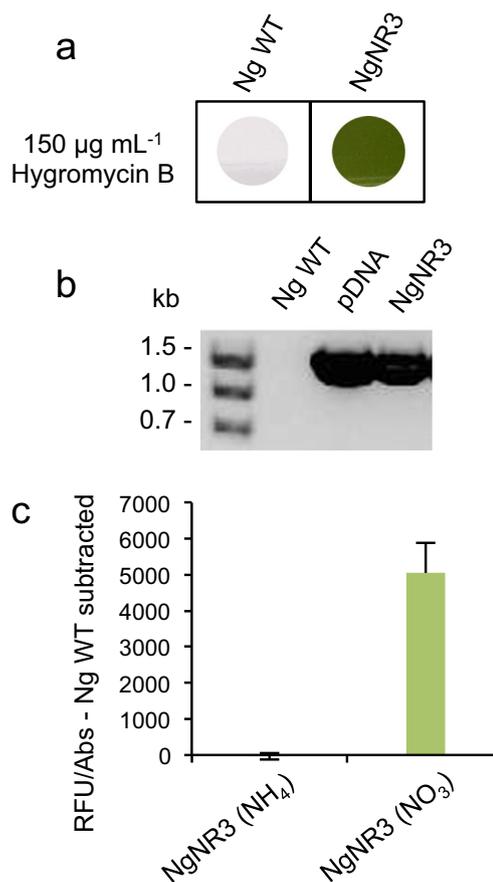


Fig. 5 Long-term stability of NgNR3 strain. After 20 months of sub-culturing in liquid media the following analyses were repeated to assess the long term-stability of the NgNR3 strain. **a** Resistance of NgNR to $150 \mu\text{g mL}^{-1}$ of hygromycin B in liquid culture. **b** The presence of the *hph* resistance gene was detected via PCR using the same primers as previously described. Template DNA: DNA ladder; lane 1, *N. gaditana* wild type (negative control); lane 2, pNR vector (positive control); lane 3, NgNR3; lane 4 **c** eGFP fluorescence expressed as A540 nm normalized fluorescence with wild-type auto-fluorescence subtracted in ammonium and nitrate-grown NgNR3 cultures. Values are means \pm SD of triplicate measurements

promoter used to drive expression, and rates of mis-folding and successful maturation of the fluorescent protein. In a previous study, expression of a widely used *Chlamydomonas* codon-optimized GFP (CrGFP) in *C. reinhardtii* under the control of a robust promoter (*hsp70/rbcS2*) yielded a comparable fold change in fluorescence of 2.8 (Rasala et al. 2013). Although the pattern of suppression and induction was conserved across the 20 NgNR strains, there was a large degree of variability in expression levels in the P_{NR} -induced conditions (nitrate-grown). This variability is likely to be the result of random integration of the expression cassette and positional effects within the genome on expression, which is commonly observed in microbes transformed with random integration-based transformation procedures such as electroporation (Thompson and Gasson 2001; Zhang et al. 2014; Chen and Zhang 2016). The fluorescence-based approach of quantifying

expression using calibration curves presented here (Fig. 3) provides a quantitative method for rapidly analysing multiple strains with relative ease, enabling cell lines with the desired level of expression to be identified.

The series of experiments exploring nitrogen regulation (Fig. 4) shed light on the ability of the nitrate reductase promoter to regulate expression under a variety of nitrogen conditions. Transcript levels of the *NR* gene of the eukaryotic alga *Dunaliella tertiolecta* have been shown to be suppressed in the presence of ammonium and induced in the presence of nitrate; however, in the presence of both ammonium and nitrate the transcript was fully suppressed (Song and Ward 2004). This observation at the transcript level in *D. tertiolecta* was not observed at the protein level in NgNR3 as eGFP expression was seen in the combined presence of ammonium and nitrate (Fig. 4a). Therefore, removal of residual ammonium before induction of P_{NR} , which could be costly and impractical on a large scale, may not be necessary for exploitation of this system in a biotechnological context. However, expression was lower than when nitrate was the sole source of nitrogen. When grown on urea, expression of eGFP was observed before the addition of nitrate, suggesting the strong effects of ammonium on expression may be required if total suppression is required (Fig. 4b) (Forde 2000; Galván and Fernández 2001; Llamas et al. 2002; Fernández and Galván 2008). The strong expression of eGFP in the nitrogen-deplete cultures before addition of nitrate suggests that P_{NR} is also induced by nitrogen deprivation in *N. gaditana*. Transcript levels of genes involved in nitrogen uptake, scavenging mechanisms and assimilation, including nitrate reductase, have been reported to increase under nitrogen deprivation in other algae as a scavenging mechanism (Alipanah et al. 2015). However, expression of NR is not induced by nitrogen deprivation in *D. tertiolecta*, which highlights the diversity in the regulation of the *NR* gene in eukaryotic algae. Expression from P_{NR} under nitrogen-depletion could offer another means of induction for industrial application whereby the nitrogen concentration of the growth media could be set such that cells grow to a desired density before depletion of nitrogen and concurrent onset of expression from P_{NR} .

Several key studies in *Nannochloropsis* spp. in recent years have identified the genus as one of the best candidate biofuel feedstock strains for advanced genome editing techniques (Kilian et al. 2011; Radakovits et al. 2012; Jinkerson et al. 2013; Ajjawi et al. 2017). This was illustrated most clearly by the work of Ajjawi et al. (2017), in which the lipid content of *N. gaditana* was doubled through the combined use of a range of the available technologies; namely, RNA seq, the use of annotated genomic sequences, CRISPR technology and RNAi. These advances highlight *Nannochloropsis* spp. as worthy species for continued biotechnological development and further expansion of the available genetic tools. Interestingly, genes involved in nitrogen assimilation in this

high-lipid mutant (including a nitrate transporter) were down-regulated. This is distinct from the wild-type *N. gaditana* response to nitrogen deprivation, in which an increased accumulation of lipid is concurrent with an upregulation of genes involved in nitrogen assimilation (Ajjawi et al. 2017).

As has been shown in other algae, the endogenous promoter of the nitrate reductase gene was able to efficiently regulate expression of heterologous protein in *N. gaditana* (Wang et al. 2004; Poulsen and Kröger 2005; Li et al. 2007; Schmollinger et al. 2010; Niu et al. 2012; von der Heyde et al. 2015). Strong expression and suppression was possible in the sole presence of nitrate and ammonium, respectively. Furthermore, the ability to fully suppress expression from the *NR* promoter through continuous growth on ammonium chloride and then induce protein production through addition of sodium nitrate, without the removal of ammonium, is a useful feature that will enable future applications requiring precise temporal induction of protein expression at a specific point in the growth phase while also maintaining growth rates. Induction and suppression of P_{NR} -driven eGFP expression was greatly affected by the nitrogen source of the growth media, presenting a variety of ways to control expression; accordingly, nitrogen sources should be user-defined on the basis of the intended use of the promoter. The establishment and validation of the in situ eGFP reporter system and ability to temporally control transgene expression with the endogenous nitrate reductase promoter are valuable additions to the advancement of sophisticated genetic engineering technologies that will enable further development of the oleaginous alga *N. gaditana* as a model organism for biotechnology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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