Although diatoms are important primary producers in the marine ecosystem, currently it is difficult to genetically transform diatoms due to the technical limitations of existing methods. The promoter/terminator of the nitrate reductase gene of the model diatom *Phaeodactylum tricornutum* was cloned and used to drive chloramphenicol acetyltransferase (CAT) reporter gene expression. The construct was transferred by electroporation into *P. tricornutum* grown in medium lacking silicon. CAT expression was induced in transformed diatoms in the presence of nitrate, enabling growth in selective medium, and was repressed when ammonium was the only nitrogen source. Expression of CAT transcript and protein were demonstrated by RT-PCR and Western blot analysis, respectively. Our study is the first to report a successful genetic transformation of diatom by electroporation in an economical and efficient manner and provides a tightly regulated inducible gene expression system for diatom.

**Keywords:** diatom; electroporation; nitrate reductase promoter; inducible expression.

**Method summary:**
The nitrate reductase promoter/terminator of the model diatom *Phaeodactylum tricornutum* was cloned and used to control the expression of the chloramphenicol acetyltransferase (CAT) reporter gene. The construct was electroporated into *P. tricornutum* grown in f/2 medium minus silicon. Expression of CAT in diatom conferred resistance to chloramphenicol and the expression of CAT transcript and protein was demonstrated by reverse transcription PCR and Western blotting, respectively. CAT expression was induced by nitrate and repressed when ammonium was the sole nitrogen source.
Cylindrotheca fusiformis is inducible and can be used for the controllable expression of foreign genes (7,8). Since nitrogen is a component of culture medium, a nitrate-inducible promoter could be ideal for foreign gene expression. The NR promoter from P. tricornutum has been tested in the green microalga C. vulgaris (5) and is tested in this work for diatom-inducible expression.

P. tricornutum Bohnlin was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, China (no. FACHB-863). Seawater from the Gulf of Dayawan was filter-sterilized and supplemented with f/2 nutrients as medium. Diatom cells were grown as batch cultures in flasks containing f/2-Si medium (prepared as for f/2 medium, but omitting Na$_2$SiO$_3$·9H$_2$O), thus minimizing the capability of creating silicon-based extra-cellular skeleton that could be a barrier to electroporation. Cultures were grown at 23°C ± 1°C in an artificial climate incubator. Cool-white fluorescent tubes provided an irradiance of 200 µmol photons m$^{-2}$ s$^{-1}$ under long-day light conditions (15 h/9 h light/dark regime). For nitrate induction, cells were inoculated in media containing different nitrogen sources: either 1.5 mM NH$_4$Cl or 2.5 mM NaNO$_3$.

To generate transgenic P. tricornutum, plasmid pHY11 (Figure 1A) containing the CAT reporter gene controlled by the NR promoter/terminator of P. tricornutum was used for electroporation with a GenePulser Xcell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Diatom cells in exponential phase (0.5 × 10$^6$ cells) were collected by centrifugation at 1350×g for 10 min, resuspended with 150 µL 1.0 M NaCl, and then mixed with 150 µL 0.1 M mannitol and kept on ice for 30 min. The salt concentration used here is higher than that used in other microalgae, such as the 10 mM CaCl$_2$ used for C. reinhardtii (9), which contributes to the permeability of the diatom cells during the pulse phase. A suspension aliquot of 0.4 mL was mixed with 0.4 µg plasmid and then transferred into an electroporation cuvette (Gene Pulser/MicroPulser Cuvette, 0.4 cm gap; Bio-Rad Laboratories). By observing electroporated cells under microscope to examine their mortality ratio, electroporation parameters were set as 1.5 kV, 25 µF, and 400 Ohm, to keep the ratio of normal cells to damaged cells at about 3:1.

To achieve high transformation efficiency, the pulsed diatoms were kept in nonselective medium at 23°C for 24 h (12L:12D) under a photon flux density of 200 mol m$^{-2}$ s$^{-1}$. Cells were then collected by centrifugation at 1500×g for 5 min and resuspended in 1 mL medium. Transformed cells were selected on selection plates with nitrate as the nitrogen source. The sensitivity of the NR promoter to chloramphenicol was tested in transgenic cells (lane 1), but was absent in wild-type (lane 2); middle panel: actin 16 is present in both wild-type (lane 1) and transgenic cells (lane 2); right panel: ethidium bromide staining of total RNAs indicates the relative amounts of total RNA in wild-type (lane 1) and transgenic cells (lane 2). (D) Western blot analysis, equal amount of each protein from the wild-type and transgenic cells were loaded; the cross-reacting CAT band (arrowhead) was detected in transgenic cells (lane 2) while not in wild-type (lane 1). (E) Inducer treatment, wild-type and transgenic cells were streaked on the chloramphenicol-containing medium composed of different nitrogen sources of either NaNO$_3$ or NH$_4$Cl; (a) wild-type (left side) and transgenic (right side) cells streaked on the plate containing NaNO$_3$; (b) wild-type (left side) and transgenic cells (right side) streaked on the plate containing NH$_4$Cl.

**Figure 1. Molecular analysis of transgenic P. tricornutum by genomic PCR, RT-PCR and Western blot analysis, and inducer treatment.** (A) Map of pHY11. Pnr/Tnr indicates the promoter/terminator of P. tricornutum NR, respectively. Unique restriction sites for cloning of target gene are indicated. (B) Genomic DNA PCR. Lane M, 125-bp DNA ladder marker; lane 1, untransformed control; lane 2, transgenic cell line showing a 0.7-kb CAT band. (C) RT-PCR analysis, left panel: CAT transcript was detected in transgenic cells (lane 1), but was absent in wild-type (lane 2); middle panel: actin 16 is present in both wild-type (lane 1) and transgenic cells (lane 2); right panel: ethidium bromide staining of total RNAs indicates the relative amounts of total RNA in wild-type (lane 1) and transgenic cells (lane 2). (D) Western blot analysis, equal amount of each protein from the wild-type and transgenic cells were loaded; the cross-reacting CAT band (arrowhead) was detected in transgenic cells (lane 2) while not in wild-type (lane 1). (E) Inducer treatment, wild-type and transgenic cells were streaked on the chloramphenicol-containing medium composed of different nitrogen sources of either NaNO$_3$ or NH$_4$Cl; (a) wild-type (left side) and transgenic (right side) cells streaked on the plate containing NaNO$_3$; (b) wild-type (left side) and transgenic cells (right side) streaked on the plate containing NH$_4$Cl.
CAT transcription was shown by RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and finally treated with RNase-free DNase I (New England BioLabs, Ipswich, MA, USA) for 30 min at 37°C. First-strand synthesis was carried out using the SuperScript First-Strand Synthesis system (Invitrogen). The CAT transcript was amplified using primers CATF and CATR. Actin16 was used as an internal control, and primers were designed according to Siaut et al. (11) to amplify a 156-bp fragment. The 0.7-kb CAT transcript was detected in transgenic cells (Figure 1C, left, lane 1) but not in wild-type cells (Figure 1C, left, lane 2). A 156-bp actin16 band was present in both wild-type and transgenic cells (Figure 1C, middle).

For Western blot analysis, total protein was extracted, and the protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Protein (20 µg/well) was separated by SDS-PAGE and electrophoretically transferred to PVDF membrane (Pall, Pensacola, FL, USA). The blot was blocked in Tris-buffered saline with Tween (TBST) containing 5% BSA for 2 h and incubated for an additional 2 h with rabbit anti-chloramphenicol antibodies (Bluegene Biotech Ltd, Shanghai, China) at 1:300 dilution. The blot was washed three times with TTBS and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5000) (Beijing ComWin Biotech Ltd., Beijing, China) for 1 h. The BeyoECL Plus (Beyotime, Jiangsu, China) kit was used to detect cross-reacting bands. Although the antibodies showed several nonspecific bands, the cross-reacting 29-kDa CAT band (arrowhead) was detected in transgenic cells (Figure 1D, lane 2) while absent in wild-type (Figure 1D, lane 1).

For nitrogen induction, cells were tested for growth up to 3 weeks in chloramphenicol-containing media with either nitrate or ammonium as the nitrogen source. Transgenic cells streaked on the plate containing NaNO₃ as the sole nitrogen source could grow well, while no growth occurred on the plate containing NH₄Cl as the sole nitrogen source even after a 3-week incubation (Figure 1E). Wild-type cells could not grow in both cases (Figure 1E). These results indicated that CAT expression under the NR promoter control was repressed in the presence of ammonium (NH₄Cl) and induced by nitrate (NaNO₃).

In addition to pHY11, we have successfully transformed constructs incorporating an additional cassette containing either enhanced GFP (eGFP, 6.3-kb construct; Supplementary Figure S1) or phospholipid:diacylglycerol acyltransferase gene (6.9-kb construct, unpublished) driven by the fcpC promoter/fcpA terminator of Phaeodactylum tricornutum. In conclusion, we have demonstrated that electroporation is a convenient method for the transformation of the diatom Phaeodactylum tricornutum and have also presented a tight gene regulatory system useful for conditional gene expression in Phaeodactylum tricornutum.

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Competing interests
The authors declare no competing interests.

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