Nuclear transformation of the marine pennate diatom *Nitzschia* sp. strain NIES-4635 by multi-pulse electroporation

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Abstract

Nitzschia is one of the largest genera of diatoms found in a range of aquatic environments, from freshwater to seawater. This genus contains evolutionarily and ecologically unique species, such as those that have lost photosynthetic capacity or those that live symbiotically in dinoflagellates. Several *Nitzschia* species have been used as indicators of water pollution. Recently, *Nitzschia* species have attracted considerable attention in the field of biotechnology. In this study, a transformation method for the marine pennate diatom *Nitzschia* sp. strain NIES-4635, isolated from the coastal Seto Inland Sea, was established. Plasmids containing the promoter/terminator of the fucoxanthin chlorophyll *a/c* binding protein gene (*fcp*, or *Lhcf*) derived from *Nitzschia palea* were constructed and introduced into cells by multi-pulse electroporation, resulting in 500 µg/mL nourseothricin-resistant transformants with transformation frequencies of up to 365 colonies per 10^8 cells. In addition, when transformation was performed using a new plasmid containing a promoter derived from a diatom-infecting virus upstream of the green fluorescent protein gene (*gfp)*, 44% of the nourseothricin-resistant clones exhibited GFP fluorescence. The integration of the genes introduced into the genomes of the transformants was confirmed by Southern blotting. The *Nitzschia* transformation method established in this study will enable the transformation this species, thus allowing the functional analysis of genes from the genus *Nitzschia*, which are important species for environmental and biotechnological development.

Keywords

Diatom, genetic transformation, *Nitzschia*, multi-pulse electroporation

Introduction

Diatoms are primary producers that are important for global carbon fixation (Nelson et al. 1995). Furthermore, diatoms have attracted considerable attention in the fields of materials engineering and nanotechnology owing to their ability to form species-specific, microstructured, and silica-based cell walls (Kröger and Brunner 2014). Diatoms are known to produce a variety of useful lipids, which have been used for a wide range of applications, including in the fields of energy, pharmaceuticals, and food (Yi et al. 2017; Ramachandra et al. 2009; Tanaka et al. 2015). The complex evolutionary history of diatoms has made them a prominent research topic (Bowler et al. 2008).

The genus *Nitzschia* comprises pennate diatoms that inhabit all aquatic environments, from freshwater to seawater, and includes various epipelic and planktonic species (Round et al. 1990; Mann et al. 2021). *Nitzschia* is one of the largest genera of diatoms (Mann 1986; Trobajo et al. 2006) and is abundant in the environment. Using metagenomic analysis, the estimation of the relative abundance of diatoms in the genus *Nitzschia* revealed that diatoms classified in this genus are dominant (Wyness et al. 2021). However, they are difficult to classify and do not cluster into a single clade in phylogenetic analyses (Mann et al. 2021). The genus *Nitzschia* contains species that are evolutionarily and ecologically unique. For example, previous studies identified *Nitzschia* diatoms that had completely lost their photosynthetic capacity were identified, and provided an excellent model to analyze the evolutionary transition from a photosynthetic to a heterotrophic lifestyle (Kamikawa et al. 2015, 2022). In addition, the dinoflagellate *Durinskia capensis* is known to symbiotically incorporate some marine species of the genus *Nitzschia* (Mann et al. 2023). Within the marine environment, symbiosis is an ecologically important phenomenon; indeed, the symbiosis described above has been studied as a unique model. The genus *Nitzschia* has been extensively studied as an indicator in environmental assessment. The freshwater species *Nitzschia palea* has been used as an indicator of anthropogenic eutrophication in lotic environments (Costa and Schneck 2022). *Nitzschia inconspicua* (Rovira et al. 2015), which is tolerant to salinity and organic or nutrient pollution, is

known to be a dominant species in highly polluted areas with high levels of ammonia, orthophosphate, and organic compounds (Fawzi et al. 2002). Recently, several useful *Nitzschia* have been reported in the field of biotechnology. Bioactive compounds, such as polysaccharides with antitumor activity (Sanniyasi et al. 2022) and phenolic and volatile compounds with antioxidant and radical scavenging activities (Lakshmegowda et al. 2020), have been identified in *N. palea* extracts. In addition, *N. palea* promotes the removal of organophosphorus insecticides (Wang et al. 2020). One of the marine species of the genus *Nitzschia* can also remove excess nitrogen and phosphorus from sea cucumber culture effluent, thereby reducing environmental impact (Xing et al. 2018). In addition, biosilica from the genus *Nitzschia* was explored as a potential drug carrier for chemotherapeutic applications (Javalkote et al. 2015).

In recent years, genetic information on the *Nitzschia* genus has begun to accumulate (Guillory et al. 2018; Oliver et al. 2021; Crowell et al. 2019; Nemoto et al. 2020; Kamikawa et al. 2022; Kamikawa et al. 2015). The use of a genetic transformation system is essential to analyze the genes of species of the genus *Nitzschia,* which includes evolutionarily and ecologically important species, and will improve the functionality of useful strains in the field of biotechnology.

In this study, a transformation system for the marine pennate diatom *Nitzschia* sp. strain NIES-4635, isolated from seawater collected from the Setouchi region of Japan, was established. Transformation vectors containing the promoter/terminator sequences of the fucoxanthin chlorophyll *a/c* binding protein gene (*fcp*), which encodes a putative light-harvesting complex protein (Lhcf) in *N. palea*, were constructed and introduced into the NIES-4635 strain. Multi-pulse electroporation was used for gene transfer into the NIES-4635 strain. This method has been used to transform *Phaeodactylum tricornutum*, *Chaetoceros gracilis*, and *Fistulifera solaris*, with a high transformation frequency reported (Miyahara et al. 2013; Ifuku et al. 2015; Naser et al. 2022). The genetic transformation system of the NIES-4635 strain established in this study is expected to provide fundamental technology with further applications in *Nitzschia* species.

Materials and methods

Diatom strains and culture conditions

N. palea (NIES-487), *Cylindrotheca fusiformis* (NIES-2351), and *Nitzschia* sp. (NIES-3877) were obtained from the National Institute for Environmental Studies, Tsukuba, Japan (NIES). *P. tricornutum* (UTEX-646) and *P. tricornutum* (UTEX-642) were obtained from the University of Texas Culture Collection. The *Nitzschia* sp. strain NIES-4635 (the original name of this strain is NOH-41) was isolated from New Okayama Port, Okayama, Japan (34°36'17"N, 133°59'09"E) as a marine pennate diatom. *N. palea* was cultured in CSi medium (Watanabe et al. 1988). *C. fusiformis* and *Nitzschia* sp. (NIES-3877) were cultured in f/2 medium (Guillard and Ryther 1962). The *Nitzschia* sp. strain NIES-4635 was cultured in f/2 medium (Guillard and Ryther 1962) or Daigo's IMK medium (Nihon Pharmaceutical, Osaka, Japan) supplemented with artificial seawater Marine Art SF-1 (Tomita Pharmaceutical, Tokyo, Japan) and 0.2 mM Na_2SiO_3. All cultures were maintained at 20–23°C and provided with continuous illumination of 20 μmol photons/m^2/s. For all plate cultures, agar (Nacalai Tesque Inc., Kyoto, Japan) was added to the liquid medium at a final concentration of 1% (w/v).

Growth curve analysis

Cells from the *Nitzschia* sp. strain NIES-4635 were pre-cultured in IMK medium to the stationary phase, and inoculated into fresh IMK medium at 1 in 100 by volume and cultured under light illumination (20 μmol photons/m^2/s) for 9 days at 23°C with shaking (115 rpm) (Double Shaker NR-30, Taitec, Koshigaya, Japan). Cells were collected daily until day 9 and counted using a bacterial counter (#A161, SLGC, Japan) under a microscope (Nikon Eclipse E100, Nikon, Tokyo, Japan). The specific growth rate of NIES-4635 was calculated using a graphical method. The value of the natural logarithm of the number of cells during the logarithmic

growth phase was plotted against time, and a regression line was obtained using the least squares method. The slope of the obtained regression line was used as the specific growth rate (μ). The doubling time was calculated by dividing the natural logarithm of 2 by the specific growth rate (μ).

Extraction of genomic DNA from diatoms

Diatom cells in the logarithmic growth phase were harvested by centrifugation from 300 mL of cell culture medium and flash frozen in liquid nitrogen. An aliquot of 40 mg of frozen cells was weighed and ground with a mortar and pestle. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) in accordance with the protocol for plant tissue. Genomic DNA extracts were stored at 4°C until use.

Isolation of the putative *fcp* promoter and terminator sequences from *N. palea*

The *fcpB* gene of *F. solaris* (AB642241.1), the *fcp* gene of *C. fusiformis* (AY125594.1), the *fcp* gene of *Thalassiosira pseudonana* (XM_002294707.1), the *fcp* gene of *Cyclotella cryptica* (AJ000670.1), and the *fcpG* (U66179.1) and *fcpF* (U66177.1) genes of *Skeletonema costatum* were used to design the degenerate primers for the *fcp* genes in diatoms (Table S1). Degenerate primers were used for PCR amplification of the putative *fcp* gene from the *N. palea* genome. PCR was performed using TaKaRa Ex *Taq* (Takara Bio, Shiga, Japan) under the following conditions: 1 min at 94°C followed by 30 cycles of 10 s at 98°C, 30 s at 55°C, 1 min at 72°C, and 10 min at 72°C. The putative *fcp* gene sequence amplified by PCR was sequenced and the primers for amplifying the unknown region were designed based on the *fcp* gene sequence. The upstream and downstream sequences of the putative *fcp* gene in *N. palea* were identified by genome sequencing using a Straight-Walk kit (Tsuchiya et al. 2009). The promoter and terminator regions of the *fcp* gene were predicted to be approximately 500 bp upstream from the start codon and 300 bp downstream from the stop codon in

the putative *N. palea fcp* gene, respectively. The sequences of the putative *fcp* promoter and terminator regions were deposited in the GenBank/EMBL/DDBJ database under accession numbers LC776935 and LC776936.

Plasmid construction

The plasmid pUC19 (Yanisch-Perron et al. 1985) was used as the backbone of the transformation vector. The template for amplifying the green fluorescent protein gene (*gfp*) and Zeocin resistance gene (*Sh ble*) was pLenti CMV GFP Zeo (Addgene plasmid #17449). The *fcpA* promoter and terminator of *P. tricornutum* (Zaslavskaia et al. 2000) and the nourseothricin (NTC) resistance gene (*nat1*) were artificially synthesized and cloned into the pUC57 vector. A plasmid containing ClP4, a promoter for a protein of unknown function from a virus infecting the marine diatom *Chaetoceros lorenzianus*, and a plasmid containing the ClP4 3′ region (ClP4-3′) (193 bp) (Kadono et al. 2022), were used. The promoter and terminator sequences of the putative *fcp* gene of *N. palea* were amplified from genomic DNA. DNA fragments were amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara Bio) and assembled using a GeneArt Seamless Cloning and Assembly Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

Nourseothricin sensitivity assay

Approximately 2.0×10^7 cells of the *Nitzschia* sp. strain NIES-4635 grown under standard conditions in f/2 medium to the logarithmic growth phase were plated on f/2 agar plates containing different concentrations of NTC (Jena Biosciences GmbH, Jena, Germany) and cultured for 14 days at 20°C under light illumination (20 µmol photons/m^2/s). After 14 days, cell growth was confirmed using a CKX53 microscope (Olympus, Tokyo, Japan).

Genetic transformation of diatoms by electroporation

Cells from the *Nitzschia* sp. strain NIES-4635 grown to the logarithmic growth phase were harvested by centrifugation (2000 × *g*, 20°C, 4 min). Cell pellets were washed twice in 0.77 M mannitol solution containing 1.5% (v/v) IMK medium. Finally, 2.0 × 10^7 cells were suspended in 150 µL of the same solution. Constructs containing 5 µg of DNA linearized by PCR were added to cell suspensions and thoroughly mixed. DNA constructs were PCR amplified using TaKaRa Ex *Taq* (Takara Bio) and purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega) in accordance with the manufacturer's instructions. The DNA constructs introduced into NIES-4635 are listed in Fig. 1. The DNA construct and cell mixture were transferred to a cuvette with a 2 mm gap, and were subjected to multi-pulse electroporation using a NEPA21 electroporator (NEPAGENE, Chiba, Japan) under the conditions listed in Table S2. After electroporation, cells were collected, transferred to 4 mL of non-selective IMK medium, and incubated at 20°C for 20–24 h under low light illumination (7–8 µmol photons/m^2/s). After illumination, cells were collected by centrifugation (2000 × *g*, 20°C, 4 min) and resuspended in 200 µL of IMK medium. The cell suspension was applied to IMK agar plates containing 500 µg/mL NTC for approximately 14 days at 20°C with light illumination (20 µmol photons/m^2/s) to select transformants. The transformation of *P. tricornutum* (UTEX-642) was performed in the same manner as for NIES-4635, except that pulse conditions and 200 µg/mL zeocin were used for transformant selection. The pulse conditions used for the transformation of *P. tricornutum* (UTEX-642) were as described by Miyahara et al. (Miyahara et al. 2013).

Analysis of introduced DNA

For the PCR analysis, genomic DNA extracted from the wild-type and transformants was used with the primers listed in Table S1 and TaKaRa Ex *Taq* (Takara Bio). *gfp* and *nat1* were amplified from the wild-type and transformants by PCR, with 18S rDNA used as a positive control.

For Southern blotting analysis, genomic DNA was digested with *Hin*d III and separated by agarose gel electrophoresis on a 0.9% agarose gel. The digested genomic DNA was then transferred from the agarose gel to a Hybond N^+ nylon transfer membrane (Amersham International, Slough, UK) by capillary transfer. Using a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions, the PCR-amplified *gfp* probe was then labeled with digoxigenin. The *gfp* probe was incubated with the membrane and hybridized probes were detected using Image Quant LAS500 (GE Company, Marlborough, MA, USA) and Tropix® CDP-Star® Ready-to-Use (Thermo Fisher Scientific, Waltham, MA, USA).

Fluorescence microscopic analysis of transformants

Transformed diatom cells were observed using a BZ-X710 fluorescence microscope (KEYENCE, Osaka, Japan). GFP and chlorophyll fluorescence were determined by applying filters specific to GFP (excitation filter 470/40 nm; emission filter 525/50 nm) and tetramethylrhodamine (TRITC) (excitation filter 545/25 nm; emission filter 605/70 nm). The GFP fluorescence-positive rate was calculated as the percentage of colonies containing cells that clearly emitted GFP fluorescence in 10 randomly selected antibiotic-resistant colonies. The proportion of GFP-fluorescent cells in the culture of the obtained clones was calculated from fluorescence microscopy images. At least 50 cells were observed for each clone and the numbers of cells with and without GFP fluorescence were counted.

Measurement of GFP fluorescence intensity

Fluorescence micrograph images were separated into three color channels (red (R), green (G), and blue (B)) by ImageJ; the green image channel was used for the subsequent analyses. The area of one diatom cell was selected and the integrated density (IntDen,

total fluorescence intensity of the region) was measured from the image. The fluorescence of the background without cells was then measured and the corrected total cell fluorescence (CTCF) was calculated using the following formula: CTCF = IntDen − (area of selected cell × background brightness). In each condition, 10 cells were used for CTCF measurements. Tukey's multiple test was used for statistical analysis.

Genetic transformation of diatoms by microparticle bombardment

The transformation of diatoms using microparticle bombardment was performed according to a previously reported method (Poulsen et al. 2006). Briefly, pure tungsten (3 mg, diameters of 0.6 µm, and 1.0 µm, Kojundo Kagaku Kenkyujo, Sakado, Japan) was coated with 5 µg plasmid DNA using the $CaCl_2$-spermidine method. Diatoms grown to the logarithmic growth phase were collected by centrifugation and 1.0×10^8 cells were plated on f/2 agar medium. Plated diatom cells were placed 6 cm from the macrocarrier and bombarded with 600 µg of tungsten particles coated with plasmid DNA using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a 1550 psi rupture disk. Bombardment was performed in the chamber under a negative pressure of 28 in of Hg. Particle-bombarded cells were collected and incubated in non-selective liquid f/2 medium for 24 h under low light illumination (7–8 µmol photons/m^2/s). The cells were centrifuged again and plated on selective f/2 agar medium containing 100 µg/mL zeocin for *P. tricornutum* (UTEX-646), 200 µg/mL zeocin for *C. fusiformis* (NIES-2351), 500 µg/mL zeocin for *Nitzschia* sp. (NIES-3877), and 100 µg/mL NTC for *N. palea* (NIES-487) to select transformants.

Phylogenetic analysis

Multiple sequence alignment was performed with MAFFT 7.455 (Katoh and Standley 2013). Alignments were trimmed using TrimAl (ver. 1.4.1) (Capella-Gutiérrez et al. 2009). RAxML (version 8.2.12) was used to construct a phylogenetic tree using

maximum likelihood (ML) methods (Stamatakis 2014). The best model of the phylogenetic analysis was determined using the GTRGAMMAI option in RaxML. Bootstrap analysis was performed with 1,000 replications. The constructed phylogenetic tree was plotted using iTOL (ver. 5.0) [https://itol.embl.de/]. The sequences used for phylogenetic analysis were obtained from NCBI. The accession numbers of the genes used in the phylogenetic analysis are listed in Table S3.

Results

Construction of plasmids containing the putative *fcp* promoter/terminator from *N. palea*

This study was designed to establish a transformation system for *N. palea*, a widely used diatom of the genus *Nitzschia*. First, an endogenous promoter/terminator of the *fcp* gene was searched for in *N. palea*. To do this, six *fcp* gene sequences from different diatoms were aligned and degenerate primers were designed to amplify the *fcp* gene from the *N. palea* genome (Table S1). The putative *fcp* gene of *N. palea* was amplified by PCR using the designed degenerate primers. The obtained sequence showed good homology with the *fcp* gene of the diatom *S. costatum* (E-value: 4e-37). These *fcp* genes encode a LHC protein belonging to the *Lhcf* subfamily in diatoms (Kumazawa et al. 2022). Using genome walking, the *fcp* promoter region was defined as the 500 bp sequence upstream of the start codon and the *fcp* terminator region was defined as the 300 bp sequence downstream of the stop codon (Fig. S1). A transformation vector, pBle/Npfcp-gfp, was constructed to co-express the *Sh ble* and GFP genes under the control of the *N. palea fcp* promoter/terminator (Fig. 1a). Additionally, pNat/Npfcp-gfp was constructed by replacing *Sh ble* in pBle/Npfcp-gfp with *nat1* (Fig. 1b).

The two constructs, pBle/Npfcp-gfp and pNat/Npfcp-gfp, were first used to transform *N. palea* by microparticle bombardment and electroporation methods. However, zeocin- or NTC-resistant transformants of *N. palea* were not obtained using either transformation method (Table S4). Conversely, zeocin-resistant transformants with clear GFP fluorescence were obtained

when pBle/Npfcp-gfp was introduced into the diatoms *C. fusiformis* (NIES-2351) and *P. tricornutum* (UTEX-646) using microparticle bombardment (Fig. S2, Table S4). Transformants with clear GFP fluorescence were also obtained by the introduction of pBle/Npfcp-gfp into *P. tricornutum* (UTEX-642) using electroporation (Fig. S2, Table S4). In addition, cells with transient GFP fluorescence were observed when pBle/Npfcp-gfp was introduced into *Nitzschia* sp. (NIES-3877) (Fig. S2, Table S4). GFP fluorescence was found to be distributed throughout the cytoplasm in all transformants of *C. fusiformis* (NIES-2351), *P. tricornutum* (UTEX-646), *P. tricornutum* (UTEX-642), and *Nitzschia* sp. (NIES-3877) (Fig. S2). The proportion of GFP-fluorescent cells in the culture of the obtained clones was 3 out of 67 observed cultured cells, 88 out of 98 observed cultured cells, and 74 out of 76 observed cultured cells for the *C. fusiformis* (NIES-2351), *P. tricornutum* (UTEX-646), and *P. tricornutum* (UTEX-642) transformants, respectively. These results confirmed that the putative *fcp* promoter and terminator sequences obtained from *N. palea* displayed functional in different diatom species.

Isolation of the marine pennate diatom *Nitzschia* sp. from the environment

To investigate whether the plasmids constructed in this study could be used more widely, a new species of *Nitzschia* was isolated from the coastal area of the Seto Inland Sea. Optical and scanning electron microscopy analyses revealed that the diatom strain NIES-4635 isolated from New Okayama Port contains the structure known as keel, which is present in diatoms of the genera *Entomoneis*, *Surirella*, and *Nitzschia* (Fig. S3). PCR amplification and DNA sequencing determined approximately 1.8 kbp of 18S rDNA for strain NIES-4635 (accession No. LC776760) and this sequence had the highest homology (99.61%) with the sequence from the diatom *Nitzschia supralitorea* (accession No. MN696703.1). Consequently, the NIES-4635 strain was classified as a *Nitzschia* species and used for the subsequent experiments.

Transformation of *Nitzschia* sp. strain NIES-4635 by electroporation

To develop a transformation system for *Nitzschia* sp. strain NIES-4635, the growth curve of strain NIES-4635 was first established at 23°C for 9 days under light illumination. The specific growth rate of strain NIES-4635 was 0.680/day, and the doubling time was 1.02 days (Fig. 2).

Next, the NTC sensitivity of strain NIES-4635 was investigated by culturing the strain on agar medium containing 50–1000 μg/mL of NTC for 14 days at 20°C under light illumination. The growth of strain NIES-4635 was completely inhibited by 250 μg/mL NTC (Table 1). Therefore, transformants of strain NIES-4635 were selected using medium containing 500 μg/mL NTC.

The pNat/Npfcp-gfp plasmid was linearized by PCR and used to transform strain NIES-4635 by electroporation. Then, NTC-resistant colonies were isolated. The genomic DNA was extracted from the NTC-resistant clones, and the presence of *nat1* and *gfp* was confirmed by PCR amplification (Fig. S4). However, GFP fluorescence was not detected when the transformants were observed using a fluorescence microscope (Fig. S5). The transformation of strain NIES-4635 with pBle/Npfcp-gfp was unsuccessful.

Investigation of the optimal promoter for GFP expression in strain NIES-4635

The optimal promoter for GFP expression was determined to obtain a transformant of strain NIES-4635 with GFP fluorescence. In this study, we examined the *fcpA* promoter from *P. tricornutum* (pPtfcp), which has been used in many studies (Buhmann et al. 2014; Kira et al. 2016; Kadono et al. 2022), and the recently reported highly active virus-derived promoters, ClP4 and ClP4 3′ regions (Kadono et al. 2022). Hereafter, the NTC resistance gene and the *gfp* expression sequences are referred to as the *nat* cassette and *gfp* cassette, respectively. A *nat1* cassette under the putative *fcp* promoter/terminator from *N. palea* (pNat) and a *gfp* cassette under ClP4, ClP4-3′, or pPtfcp were co-transformed into strain NIES-4635 using electroporation (Fig. 1c–e). GFP-fluorescent cells were observed in the pNat+ClP4-3′-gfp and pNat+Ptfcp-gfp transformed conditions (Fig. 3b, c, Table 2). GFP fluorescence was

distributed throughout the cytoplasm in the transformants obtained by the introduction of either construct (Fig. 3b, c). From the image analysis, cells transformed with pNat+ClP4-3′-gfp had higher GFP fluorescence than cells transformed with pNat+Ptfcp-gfp (Fig. S6). The transformation frequency of cells transformed with pNat+ClP4-3′-gfp and pNat+Ptfcp-gfp was up to 150 and 365 per 10^8 cells, respectively (Table 2). The GFP fluorescence-positive rates following transformation with pNat+ClP4-3′-gfp and pNat+Ptfcp-gfp were 3.7% and 4.5%, respectively (Table 2). The proportion of GFP-fluorescent cells in the culture of the clones transformed with pNat+Ptfcp-gfp was 41 out of the 145 cultured cells observed. In clones transformed with pNat+ClP4-3′-gfp, GFP fluorescence was observed in all cultured cells.

Construction of plasmids for transformation of strain NIES-4635

Cells transformed with pNat+ClP4-3′-gfp and pNat+Ptfcp-gfp displayed GFP fluorescence (Fig. 3b, c); however, both constructs showed low GFP fluorescence-positive rates (Table 2). As the *nat* and *gfp* cassettes were introduced into the cells as different DNA fragments, the probability of both cassettes being transformed into the cells simultaneously was likely quite low. Consequently, two new plasmids, pNat/ClP4-3′-gfp and pNat/Ptfcp-gfp, were constructed with the aim of introducing the *nat* and *gfp* cassettes into strain NIES-4635 as a single fragment (Fig. 1f, g). By replacing the *gfp* cassette promoter of pNat/Npfcp-gfp with the ClP4-3′ and *P. tricornutum fcpA* promoters, respectively, pNat/ClP4-3′-gfp and pNat/Ptfcp-gfp were formed (Fig. 1f, g). The newly constructed plasmids were linearized (Fig. 1f, g) and then transformed into the strain NIES-4635 by electroporation. The transformation frequency and GFP fluorescence-positive rate of cells transformed with pNat/ClP4-3′-gfp reached 93 per 10^8 cells and 44%, respectively (Table 2). The GFP fluorescence-positive rate of cells transformed with pNat/ClP4-3′-gfp was approximately 10-fold higher than that of cells co-transformed with pNat and ClP4-3′-gfp as separate fragments (Table 2). In contrast, no GFP fluorescence was observed in cells transformed with pNat/Ptfcp-gfp (Table 2, Fig. 3e). The highest GFP fluorescence of all transformants obtained

in this study was observed in cells transformed with pNat/ClP4-3′-gfp (Fig. S6). In clones transformed with pNat/ClP4-3′-gfp, 21 out of 229 cultured cells showed GFP fluorescence.

The presence of *nat1* and *gfp* in the genomes of cells transformed with pNat/ClP4-3′-gfp was confirmed by PCR amplification (Fig. 4a). Southern blotting analysis of *Hind* III-digested genomic DNA from pNat/ClP4-3′-gfp-transformed cells with a *gfp* probe revealed transgene integration (Fig. 4b).

Discussion

In this study, a transformation system for the marine pennate diatom *Nitzschia* sp. strain NIES-4635 was established using multi-pulse electroporation. To establish a transformation system for *Nitzschia*, new plasmids containing the *N. palea fcp* promoter/terminator were constructed (Fig. 1). The *fcp* gene obtained by degenerate PCR encodes a putative Lhcf-subfamily protein; many of these accumulates abundantly in diatoms (Kumazawa et al. 2022).

Initially, experiments were conducted to establish a transformation system for *N. palea*. The transformation experiments were investigated using microparticle bombardment and multi-pulse electroporation. However, transformants of *N. palea* were not obtained, even once (Table S4). While attempting to transform *N. palea*, variations in Si concentration in the culture medium, particle size and pressure for microparticle bombardment, and different buffers for suspending algal cells for multi-pulse electroporation were investigated, but successful transformation was not achieved. *N. palea* was the only freshwater diatom used in this study. Previous studies have reported that the cell walls of freshwater diatoms are thicker than those of marine diatoms (Conley et al. 1989). This may have made the introduction of DNA into the cells more difficult. Another contributing factor may be the extracellular polymeric substances formed by *N. palea* (Laviale et al. 2019). In any case, it is possible that the *N. palea* transformation experiment did not introduce DNA constructs into the cells.

Although the plasmids in this study could not successfully transform *N. palea*, the transformation of *C. fusiformis* and *P. tricornutum* was achieved (Fig. S2, Table S4). The *N. palea*-derived *fcp* promoter/terminator is functional in *C. fusiformis* and *P. tricornutum*, possibly because of the phylogenetic relationship between these three species (Fig. S7) (Nemoto et al. 2020; Theriot et al. 2010). These results suggest that the putative *fcp* promoter/terminator sequences from *N. palea* could be used to transform other related diatom species. Several studies have reported that promoters from certain diatoms are also functional in other diatoms (Buhmann et al. 2014; Miyagawa et al. 2009; Muto et al. 2013; Johansson et al. 2019). In this study, the transformation of *T. pseudonana* (CCMP-1335) using a plasmid containing the *N. palea fcp* promoter/terminator was unsuccessful (data not shown). This may be partly because of the phylogenetic distance between the pennate diatom *N. palea* and the centric diatom *T. pseudonana*. However, a promoter derived from a pennate diatom was reported to be functional in centric diatoms (Johansson et al. 2019). However, there are cases in which promoters from centric diatoms do not function in other centric diatoms (Ifuku et al. 2015; Miyagawa-Yamaguchi et al. 2011). This suggests that variation in the specificity of the promoters: certain diatom promoters are versatile and can function in different species, while others only function in specific species.

Subsequently, pNat/Npfcp-gfp containing the *N. palea*-derived fcp promoter/terminator was introduced into the *Nitzschia* sp. strain NIES-4635 isolated from the Seto Inland Sea using multi-pulse electroporation, and antibiotic-resistant clones without GFP fluorescence were obtained. Genomic PCR analysis of the transformants revealed the presence of the introduced genes (*nat1* and *gfp*) (Fig. S4). The phylogenetic analysis revealed that *N. palea* and strain NIES-4635 were less closely related than *N. palea* and *C. fusiformis* or *N. palea* and *P. tricornutum* (Fig. S7). Therefore, it possible that the activity of the *N. palea*-derived promoter is low in the phylogenetically distant strain NIES-4635, causing *gfp* not to be expressed. Transgene silencing is another possible explanation; it has been reported to occur in diatoms when antisense or inverted repeat sequences of the target gene are introduced (De Riso et al. 2009). It has also been reported that introduced DNA constructs are truncated or inserted into the genome in a

different orientation during the transfection process in diatoms (Angstenberger et al. 2019). It is possible that the same situation occurred in this study, and transgene silencing occurred due to unintended DNA insertions into the genome. In the red macroalga *Pyropia yezoensis*, when antibiotic resistance and reporter genes are expressed under the control of the same promoter, the reporter gene is not expressed because of gene silencing (Shin et al. 2016). Because the vector pNat/Npfcp-gfp expressed *gfp* and *nat1* under the control of the same promoter, it is possible that transgene silencing occurred in strain NIES-4635, similar to what occurred in *P. yezoensis.*

Different promoters were examined to obtain NIES-4635 transformants with obvious GFP fluorescence. Consequently, transformants with obvious GFP fluorescence were obtained with a *gfp* cassette containing the diatom-infecting virus-derived promoter, the ClP4 3′ region (ClP4-3′), and the *P. tricornutum*-derived *fcpA* promoter (pPtfcpA) (Fig. 3b, c). No transformants with GFP fluorescence were obtained with the *gfp* cassette containing ClP4. ClP4 is less active than ClP4-3′ in *P. tricornutum* (Kadono et al. 2022). It is possible that ClP4 has a lower promoter activity than ClP4-3′ in strain NIES-4635, resulting in the failure to detect transformants with GFP fluorescence. Next, a new plasmid was constructed by replacing the promoter for *gfp* in pNat/Npfcp-gfp with ClP4-3′ or pPtfcpA (Fig. 1f, g) and transformed into strain NIES-4635. Consequently, the GFP fluorescence-positive rate of the transformants increased significantly when pNat/ClP4-3′-gfp with ClP4-3′ as a promoter for *gfp* was used for transformation (Table 2). This may be because introducing the transgene as a single DNA fragment results in a higher probability of the insertion of both *nat1* and *gfp* into the genome. In contrast, transformation of pNat/Ptfcp-gfp with pPtfcpA as the promoter for *gfp* did not result in transformants with GFP fluorescence (Fig. 3e, Table 2). The introduction of a *gfp* cassette containing pPtfcpA and the *P. tricornutum*-derived *fcpA* terminator (tPtfcpA) resulted in transformants with GFP fluorescence, indicating that the *N. palea*-derived *fcp* terminator (tNpfcp) may affect the expression level of pNat/Ptfcp-gfp. In yeast studies, changes of up to 11-fold in expression levels were reported when the terminator was changed (Curran et al. 2013). ClP4-3′ was reported to be 10 times more active than

pPtfcpA in *P. tricornutum* (Kadono et al. 2022). Accordingly, it is possible that GFP expression under control of the less active pPtfcpA was affected by tNpfcp, whereas GFP expression under the more active control of ClP4-3′ was not affected by tNpfcp.

Transformation of the genus *Nitzschia* was recently reported (Sprecher et al. 2023), with plasmids transformed into *Nitzschia captiva* using microparticle bombardment. The maximum transformation frequency of *N. captiva* was 88 colonies/10^8 cells, which was comparable to that of strain NIES-4635. Although the transformation efficiency was comparable to that of Sprecher et al. (Sprecher et al. 2023), the multi-pulse electroporation method used in this study is simpler and less expensive than microparticle bombardment.

The present study has established an electroporation transformation system for *Nitzschia* sp. strain NIES-4635 that was isolated from the coastal Seto Inland Sea. The genus *Nitzschia* contains evolutionarily and ecologically unique species; in recent years, some species have been reported to be useful in the field of biotechnology. The transformation system established in this study may be useful for analyzing the function of genes or improving the functionality of these *Nitzschia* species. Furthermore, the plasmids produced in this study can be used to transform *C. fusiformis* and *P. tricornutum*, which are closely related to strain NIES-4635. This plasmid has the potential to be used for other closely related species and may be a useful versatile plasmid.

Statements and declarations

Ethics approval and consent to participate:

Not applicable.

Availability of data and material:

The 18S rDNA sequence of strain NIES-4635 is available under DDBJ accession number LC776760. The sequences of *N. palea fcp* promoter and terminator are available under DDBJ accession number LC776935, and LC776936.

Competing interests:

The authors declare that they have no conflict of interest.

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Author contributions:

Michiko Nemoto designed the research; Koki Okada, Yu Morimoto, and Yukine Shiraishi performed the experiments; Koki Okada, Yu Morimoto, Yukine Shiraishi, Takashi Tamura, Shigeki Mayama, Takashi Kadono, Masao Adachi, Kentaro Ifuku and Michiko Nemoto analyzed the data; Koki Okada, Takashi Kadono, Masao Adachi, Kentaro Ifuku, and Michiko Nemoto wrote and revised the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Fig. 1 DNA constructs transformed into the *Nitzschia* sp. strain NIES-4635. a) pBle/Npfcp-gfp, b) pNat/Npfcp-gfp, c) pNat +ClP4-gfp, d) pNat+ClP4-3′-gfp, e) pNat+Ptfcp-gfp, f) pNat/ClP4-3′-gfp, and g) pNat/Ptfcp-gfp were introduced into strain NIES-4635. a), b), f), g) were transformed as a single DNA fragment. For c) and d), *nat* and *gfp* were co-transformed as different DNA fragments. pNpfcp, *N. palea fcp* promoter; *Sh ble,* the zeocin resistance gene; tNpfcp, *N. palea fcp* terminator; *nat1*, the nourseothricin resistance gene; ClP4, a promoter for a protein of unknown function from a diatom-infecting virus that infects the marine diatom *C. lorenzianus*; *gfp*, GFP gene; tCffcpA, *C. fusiformis fcpA* terminator; ClP4-3′, the 3′ region of ClP4; pPtfcpA, *P. tricornutum fcpA* promoter; tPtfcpA, *P. tricornutum fcpA* terminator.

Fig. 2 Growth curve of the *Nitzschia* sp. strain NIES-4635 cultured in IMK medium (*n*=3)

Fig. 3 Bright field and fluorescence images of the *Nitzschia* sp. strain NIES-4635 cells transformed with different DNA constructs. GFP, GFP fluorescence images; Chlorophyll, chlorophyll autofluorescence; GFP+Chlorophyll, merged images of the GFP fluorescence and chlorophyll.

Fig. 4 Analysis of the genome of *Nitzschia* sp. strain NIES-4635 transformed with pNat/ClP4-3′-gfp, which expresses *gfp* under the control of the ClP4 3′ region. a) The 18S rDNA (1,791 bp), *nat1* (555 bp), and *gfp* (546 bp) were amplified by PCR. Genomic DNAs from the wild-type (WT) and transformants (TF) were used as the template DNA. M1: 1,000 bp marker. M2: 100 bp marker. b) Southern blotting analysis of genomic DNAs from the WT and transformants (TF). Total genomic DNA (10 μg) digested with *Hind* III was electrophoresed and blotted onto a nylon transfer membrane. Plasmid pNat/ClP4-3′-gfp (P) (1 ng) was used as a positive control.

Tables

Table 1 Effect of nourseothricin (NTC) concentrations on the growth of the *Nitzschia* sp. strain NIES-4635 plated on agar

NTC concentration (µg/mL)	0	50	100	250	500	1,000
Cell growth	+	+	+	−	−	−

Cells (2.0 × 10^7) of *Nitzschia* sp. strain NIES-4635 were plated on f/2 agar plates containing various NTC concentrations and incubated at 20°C under light illumination (20 µmol photons/m^2/s) for 14 days.

(+) and (-) indicate detectable and undetectable growth on the agar plate, respectively.

Table 2 Transformation frequency and GFP fluorescence-positive rate of the *Nitzschia* sp. strain NIES-4635 transformed by multi-pulse electroporation

Introduced DNA construct	Transformation efficiency (Colonies per 10^8 cells) Average (minimum, maximum)	GFP fluorescence-positive rate (Number of colonies that contained cells with GFP fluorescence/Number of colonies observed) × 100 (%)	Number of trials
pBle/Npfcp-gfp	0, (0, 0)	-	3
pNat/Npfcp-gfp	9.58, (0, 240)	0.0%	26
pNat+ClP4-gfp	35.0, (10, 60)	0.0%	2
pNat+ClP4-3′-gfp	50.0, (0, 150)	3.7%	3
pNat+Ptfcp-gfp	125, (0, 365)	4.5%	7
pNat/ClP4-3′-gfp	23.8, (0, 93)	44%	4
pNat/Ptfcp-gfp	64.3, (0, 133)	0.0%	3