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Transformation of Nonselectable Reporter Genes in Marine Diatoms

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Abstract: We report the genetic transformation of two marine diatoms by microparticle bombardment. The pennate diatom *Phaeodactylum tricornutum* was transformed with the bacterial gene *Sh ble* from *Streptoalloteichus hindustanus,* which confers resistance to the antibiotics phleomycin and zeocin. Transformants contained between 1 and 10 copies of the exogenous DNA integrated into the genome by illegitimate recombination at apparently random locations. Transformation efficiencies were around 10^{-6}, and individual cell lines could be maintained at −80°C following cryopreservation. Also, *P. tricornutum* could be transformed simultaneously with two different plasmids, one containing the *Sh ble* gene and another containing the firefly *luciferase* gene (*LUC*) under control of a promoter derived from a *fucoxanthin, chlorophyll a/c-binding protein* gene (*FCP*). In these cotransformants, LUC activity was light inducible. The transient transformation of the centric diatom *Thalassiosira weissflogii* with the bacterial β-*glucuronidase* (*GUS*) gene has also been achieved using similar transformation technology. The availability of gene transfer protocols for marine diatoms, together with a range of functional reporter genes and regulated expression systems, will permit molecular dissection of their biology and allow an assessment of the biotechnological potential of these organisms.

Key words: cryopreservation; diatoms; fucoxanthin, chlorophyll a/c-binding protein; genetic transformation; luciferase; microparticle bombardment.

INTRODUCTION

Diatoms are important components of marine phytoplankton, being particularly important for biogeochemical cycling of minerals such as silica, and for global carbon fixation (Werner, 1977; Tréguer et al., 1995). Their success is not well understood although it is known that diatoms are remarkably flexible in adjusting their photosynthetic reactions to allow maximal growth rates over a wide range of light intensities. In spite of their enormous ecologic importance, only recently have they attracted the attention of molecular biologists. Consequently, knowledge of genome structure is extremely limited, and only a few genes have been isolated, among them those encoding fucoxanthin, chlorophyll a/c-binding proteins (*FCP*) from *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* (Bhaya and Grossman, 1993; C. Leblanc, A. Falciatore, and C. Bowler, manuscript in preparation), ATP synthase from *Odontella sinensis* (Pancic and Strotmann, 1993), and acetyl–coenzyme carboxylase (*ACCI*) from *Cyclotella cryptica* (Roessler and Ohlrogge, 1993). Owing to the unusual na-

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ture of diatom cell walls, composed of rigid silicaceous material, some attention has also been given to genes encoding major cell wall proteins such as frustulins in *Navicula pelliculosa* (Kröger et al., 1996) and in *Cylindrotheca fusiformis* (Kröger et al., 1994), and in identifying silicon-responsive genes such as those encoding silicon transporter proteins (Hildebrand et al., 1993, 1997).

The absence of adapted methodologies that permit molecular and cell biological studies in diatoms has also been a serious limitation. In particular, the lack of a transformation system has been a major bottleneck for studying basic biological processes such as the regulation of gene expression and subcellular protein targeting. Problems with introducing foreign DNA into diatom cells have been largely due to difficulties in controlling and manipulating diatom life cycles and to their rigid cell walls. Furthermore, no one has yet succeeded in obtaining naked protoplasts able to regenerate their cell walls.

The availability of a transformation protocol would also permit evaluation of marine diatoms for biotechnological applications. Diatoms are a potential source of biodiesel (Dunahay et al., 1996) and specialty chemicals such as ω-3 fatty acids (Barclay et al., 1994), an essential component of formula milk for neonates (Gerwick et al., 1994). Engineered diatoms can potentially also be used as vectors to introduce vaccines and improve nutritional value of feedstuffs for aquacultured fish and crustaceans (Gladue and Maxey, 1994). Two groups have succeeded in transforming diatoms. Dunahay et al. (1995) utilized helium-accelerated particle bombardment to introduce the *nptII* gene for kanamycin resistance in *C. cryptica* and *Navicula saprophila,* whereas Apt et al. (1996) utilized similar protocols to introduce the *Sh ble* gene for phleomycin resistance in *P. tricornutum.* In both cases transformation efficiencies were low, around 10^{-6}/μg DNA, and the foreign DNA was shown to be stably integrated into the genome in one to five copies.

We have independently established a system for *P. tricornutum* transformation that is highly similar to that reported by Apt et al. (1996). In addition, we have performed a thorough molecular analysis of the transformants in order to better characterize the integration patterns of the foreign DNA in the diatom genome. Furthermore, we describe cotransformation with two different plasmids, one containing a selectable marker gene and another containing the firefly *luciferase* gene (*LUC*). We also present results from transient transformation experiments in the centric diatom *T. weissflogii* using the β-*glucuronidase* (*GUS*) gene as a screenable marker. Foreign gene expression in *T. weissflogii* is particularly important because this species represents a group of high ecological relevance.

Materials and Methods

Strains and Media

Phaeodactylum tricornutum Bohlin clone CCMP 632, *T. weissflogii* (Grunow) Fryxell et Hasle clone CCMP 1336 (ex clone ACTIN), and *C. fusiformis* clone CCMP 343 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, Me.). Cells were grown axenically in filtered seawater (SW) enriched with nutrients as in f/2 medium (Guillard, 1975) at 20°C and with continuous illumination (150 μmol m^{-2} s^{-1}). For growth on solid media, cultures were grown on 100% or 50% SW containing f/2 nutrients, 1% bactoagar (Difco). Cultures were periodically tested for bacterial contamination by culturing in the dark in medium (100% SW plus f/2) supplemented with 1 g/L peptone (Oxoid S.p.A.).

Plasmid Construction

All plasmids were maintained in *Escherichia coli* strain SURE (Greener, 1990). Plasmids were isolated using the Maxiprep Plasmid Isolation Kit (Qiagen, Inc.). Construction of the plasmids pFCPBp-Sh ble and pFCPFp-Sh ble used for transformation of *P. tricornutum* is shown in Figure 1. The 420-bp *Sh ble* gene from pUT58 (Drocourt et al., 1990) was isolated as a *Cla*I-*Not*I fragment and cloned in pBluescript KS^+ (Stratagene) to form pKS-Sh ble. *FCP* promoter and terminator sequences for *P. tricornutum* were subsequently inserted. A 230-bp transcription terminator sequence from the *FCPA* gene (*FCPA3′*) (Bhaya and Grossman, 1993) was obtained by polymerase chain reaction (PCR) performed on cells as described below, using the primers 5′-TGGCGGCGGCCGCAACAACTACC-3′ and 5′-GGCATCCGCGGTGAAGACGAGC-3′, which created *Not*I and *Sac*II sites at the 5′ and 3′ ends, respectively, of the *FCPA3′* sequence. This amplification product was digested with *Not*I and *Sac*II, purified by Geneclean (Bio 101, Inc.), and cloned into *Not*I and *Sac*II-digested pKS–Sh ble. An *FCPB* promoter fragment of 490 bp (*FCPBp*) was PCR amplified using the primers 5′-CACCATGGGTACCAATCTCGCC-3′ and 5′-GGGGGTCGAGTCGACTTCTGGCAACCG-3′, and a 660-bp promoter fragment (*FCPFp*) was amplified with 5′-GGGGGTACCTAACAG-

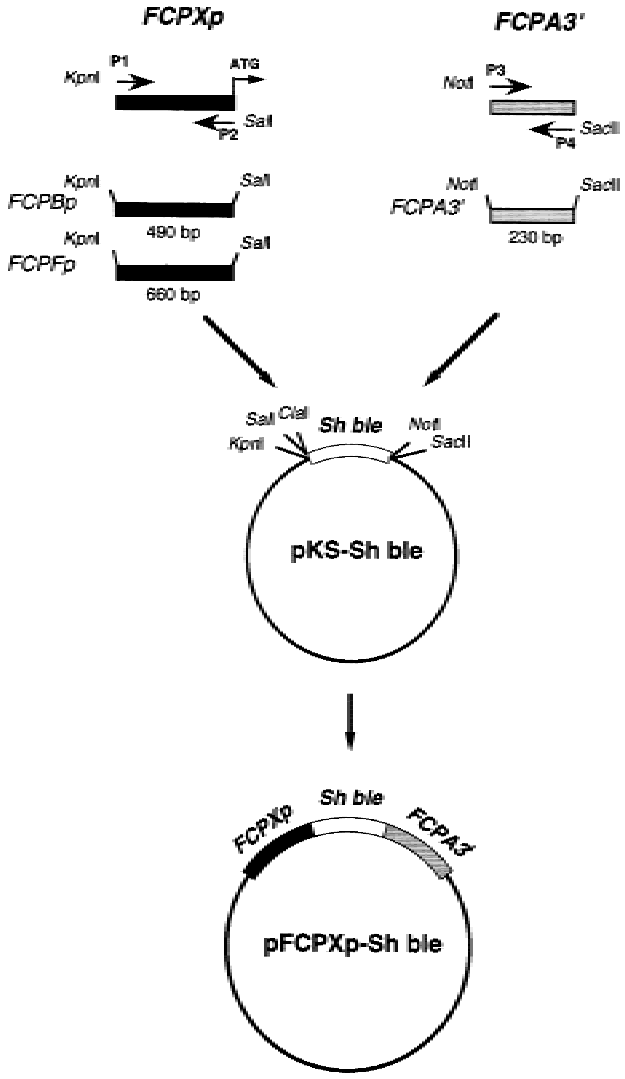

Figure 1. Construction of plasmids pFCPBp–Sh ble and pFCPFp–Sh ble used to transform *P. tricornutum*. The 420-bp *Sh ble* gene from pUT58 was isolated as a *Cla*I-*Not*I fragment and cloned in pBluescript KS$^+$ (Stratagene) to generate pKS–Sh ble. *FCP* promoter and terminator sequences obtained by PCR from *P. tricornutum* were subsequently inserted (see Materials and Methods for more information).

GATTAGTGCAATTCGAG-3′ and 5′-GGGGGTCGACTTGGTTAATTTTTCGATTTC-3′ (Bhaya and Grossman, 1993). Each of these fragments contained engineered *Kpn*I and *Sal*I sites at their 5′ and 3′ ends, respectively. These *Kpn*I-*Sal*I promoter fragments were then cloned into pKS–Sh ble–FCPA3′ and digested with *Kpn*I-*Sal*I to obtain the final plasmids (respectively denoted pFCPBp–Sh ble and pFCPFp–Sh ble) used to transform *P. tricornutum* cells.

The pFCPBp-LUC and pFCPFp-LUC plasmids used to cotransform *P. tricornutum* were obtained, respectively, by replacing the *Sal*I-*Not*I *Sh ble* fragment of pFCPBp–Sh ble and pFCPFp–Sh ble with a *Sal*I-*Not*I fragment containing the firefly luciferase coding region from plasmid pJD261 (Luehrsen et al., 1992). The *FCPA3′* transcription terminator sequences were replaced in both cases with a *Not*I-*Sac*II fragment from plasmid pACCNPT10 containing the acetyl–coenzyme A carboxylase 3′ end (*acc13′*) (Dunahay et al., 1995).

The pFCPBp-GUS plasmid used to transform *T. weissflogii* was obtained by replacing the *Sal*I-*Not*I *Sh ble* fragment of pFCPBp-Sh ble with a *Sal*I-*Not*I fragment containing the complete *GUS* gene, originally denoted *uidA* (Jefferson et al., 1987).

Transformation of *P. tricornutum* and Selection of Resistant Clones

Sensitivity to phleomycin and zeocin (Cayla SARL) was determined empirically for *P. tricornutum*. Different numbers of cells (10^5 and 10^6) were spread on agar plates containing different salt concentrations (100% and 50% SW) and different amounts of phleomycin and zeocin (10, 50, 100 μg/ml). The sensitivity range was determined after 2 weeks of growth.

Cells were bombarded using the Biolistic PDS-1000/He Particle Delivery System (BioRad). Approximately 5×10^7 cells were spread on agar plates containing 50% SW and allowed to dry under a sterile hood (approximately 1 hour). Plasmid DNA (1 μg) was coated onto M5, M10, or M17 tungsten particles (0.4 μm, 0.7 μm, and 1.1 μm diameter, respectively, BioRad), as described in the PDS/1000 He instruction manual. Agar plates containing the diatom cells were positioned at level 2 in the chamber (6 cm from the stopping screen), and burst pressures of 650, 1100, and 1550 psi were used. After bombardment the cells were illuminated for 48 hours at 150 μmol m^{-2} s^{-1} at 20°C (the cells divided once during this period) and then resuspended in 1 ml of SW. Approximately 10^7 cells (300 μl) were spread on plates containing 50% SW and 100 μg/ml phleomycin. Resistant colonies were obtained after 10 to 14 days of incubation at 20°C in constant white light (150 μmol m^{-2} s^{-1}). Putative transformants were restreaked on plates containing 100 μg/ml phleomycin.

Cotransformation of *P. tricornutum*

Cells were bombarded using conditions described above with large particles (1.1 μm) and the highest pressures (1550

psi). Particles for bombardment were coated with 0.5 μg pFCPBp–Sh ble plus 0.5 μg pFCPFp-LUC or with 0.5 μg pFCPFp–Sh ble plus 0.5 μg pFCPFp-LUC. Putative cotransformants were obtained after 10 to 14 days of incubation at 20°C on plates containing 100 μg/ml phleomycin. Colonies resistant to phleomycin were grown in f/2 medium containing 100 μg/ml phleomycin. To verify the presence of LUC activity, 10^4 cells (50 μl) in the exponential phase of growth were tested in vivo. Cells were placed in a luminometer (LB 9507, Berthold), and 50 μl 200 mM D-luciferin (sodium salt) was added (Sigma). Photon emission was measured in cotransformants as relative light units (RLU).

Luciferase Extraction and Analysis

In vitro LUC assays were performed on cotransformants grown in darkness for 2.5 days and then exposed to 150 μmol m^{-2} s^{-1} continuous white light for 24 hours. Samples were taken from light, at time zero (dark) and after 5, 8, 12, and 24 hours of light. Total protein was extracted from 5 ml of cultures using a 2:1 volume ratio of ice-cold 1 × Cell Lysis Reagent, supplied as part of a luciferase assay kit (Promega). The extracts were cleared by 5 minutes of centrifugation at 10,000 *g*, and supernatants were tested for luciferase activity. Cell extracts (5 μl) were mixed with 50 μl of Luciferase Assay Reagent (Promega), and luciferase activity was measured in a luminometer (LB 9507, Berthold) for 20 seconds. Protein content of the cell extracts was determined using the BioRad protein assay kit according to the manufacturer's instructions. Luminescence was normalized using a standard curve prepared with serial dilutions of purified recombinant firefly luciferase (Promega).

Cryopreservation

Transformed and nontransformed clones of *P. tricornutum* could be stored indefinitely at −80°C. Prior to cryopreservation, cells were grown in liquid culture to exponential phase, and approximately 10^7 cells were concentrated by centrifugation in medium (1.5 ml) containing 10% dimethyl sulfoxide (plus antibiotic if appropriate). The cells were frozen to −80°C gradually with intermediate steps of 1 hour each at 4°C and −20°C. Following storage at −80°C, cells were replated on agar plates with and without phleomycin to verify percentage survival and stability of the transformed phenotype after cryopreservation. Cryopreservation of *C. fusiformis* and *T. weissflogii* was performed in the same way.

Transformation of *T. weissflogii* and Histochemical *GUS* Assay

The microparticle bombardment system was also used to introduce foreign DNA into *T. weissflogii* cells. Cells were bombarded with a plasmid containing the *GUS* gene under control of the *FCPB* promoter described previously (pFCPBp-GUS). Plasmid DNA (1 μg) was coated onto gold (Aldrich) or tungsten (BioRad) particles and shot onto plates containing a lawn of cells (approximately 10^6 cells). Plates were subsequently incubated for 1 day at low light intensity at 20°C and then stained for GUS activity following standard procedures (Jefferson et al., 1987). Briefly, a piece of agar was placed in a 5.5-cm Petri dish containing 200 μl X-Gluc solution (100 mM Na-phosphate, pH 7.0, 10 mM Na_2 ethylenediaminetetracetic acid (EDTA), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.1% Triton X-100, 0.3% X-Gluc (Clontech)), and incubated at 37°C overnight. Blue cells were detected by microscopy.

PCR Amplification from Diatom Cells

PCR was performed on cell lysates, obtained by centrifugation of diatom cells from 1.5-ml culture volumes followed by resuspension of the pellet in 50 μl of lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 5 mM KCl). Lysis was performed on ice (2 minutes), after which the cells were centrifuged for 5 minutes. The pellet was resuspended in distilled water (20 μl), and 10 μl was used in a 20-μl PCR reaction. The PCR mixture contained PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), dNTPs (2 mM), 120 ng of each primer, and 1 unit of Ampli *Taq* DNA Polymerase (Perkin Elmer). The thermal profile was as follows: 94°C for 5 minutes (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute) × 35 cycles, 72°C for 10 minutes. The *Sh ble* gene from putative transformant clones was PCR amplified using primers 5′-TCGAGTTCTGGACCGACCGGCT-3′ and 5′-ACGAAGTGCACGCAGTTGCCGG-3′. Amplification products were analyzed on agarose gels.

Preparation of Nucleic Acids and Southern Blot Analysis of Transformants

To prepare total DNA, pelleted cells from 100-ml cultures were resuspended in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodiumdodecyl sulfate (SDS), 10 mM dithiothreitol, 10 μg/ml proteinase K, and incubated at 37°C for 15 minutes to induce cell lysis. The lysate was extracted with

1 volume phenol:chloroform (1:1) and 1 volume of chloroform. The aqueous phase was then precipitated with 2.5 volumes of ethanol, 1/10 volume 3 M sodium acetate (pH 5.6), washed twice with 70% ethanol, dried, and resuspended in distilled water. Complete digestion of genomic DNA with restriction enzymes was achieved by overnight incubation at 37°C. Usually 10 U of enzyme was utilized per microgram of DNA. The DNA fragments were separated on 0.8% agarose gels and transferred to nylon membranes (Duralon-UV, Stratagene), which were cross-linked with UV light using a Stratalinker (Stratagene). Hybridizations were performed overnight at 50°C in a rotary oven in 7% SDS, 0.5 M Na_2HPO_4 (pH 7.2), 1 mM EDTA. The filters were washed three times for 10 minutes in a washing solution containing 40 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1 mM EDTA, and exposed to x-ray film (Kodak).

Western Blotting

A rapid assay of Sh ble protein in putative transformed clones was performed by Western blotting. Protein samples were prepared according to Dunahay et al. (1995) and subjected to electrophoresis in SDS–polyacrylamide gels containing 15% acrylamide. Separated proteins were transferred to a nitrocellulose membrane (Amersham) by electroblotting and incubated with a rabbit polyclonal antibody against the Sh ble protein (Cayla SARL). The ECL Western blotting system (Amersham) was used to detect immunoreactive proteins using an antirabbit secondary antibody.

Results

Microparticle Bombardment of *P. tricornutum*

Apt et al. (1996) previously reported the stable transformation of *P. tricornutum* using microparticle bombardment to introduce chimeric genes containing the *Sh ble* gene from *Streptoalloteichus hindustanus,* which confers resistance to the antibiotics phleomycin and zeocin. In independent experiments, we confirmed the sensitivity of *P. tricornutum* to these antibiotics (Table 1). In addition, we found that antibiotic sensitivity was dependent on salinity of the media and cell plating density (Table 1). For example, although *P. tricornutum* grew equally well in both 100% and 50% SW, in the latter zeocin (e.g., 100 μg/ml) and phleomycin (e.g., 10 μg/ml) were more effective at inhibiting growth. Also, although no colonies formed when 10^5 cells were plated on 50% SW containing 50 μg/ml zeocin or 10 μg/ml phleomycin, these antibiotic concentrations were not sufficient to inhibit algal growth when 10^6 cells were plated (Table 1). Because phleomycin was more effective at low concentrations, it was used in all subsequent experiments.

Table 1. Effect of Salt Concentration and Plating Density on Zeocin and Phleomycin Sensitivity in *Phaeodactylum tricornutum**

	Plating density	
Antibiotic concentration	10^5 cells	10^6 cells
100% Seawater	++	+++
+ 10 μg/ml zeocin	++	++
+ 50 μg/ml zeocin	−	+
+100 μg/ml zeocin	−	+
+ 10 μg/ml phleomycin	−	++
+ 50 μg/ml phleomycin	−	−
+100 μg/ml phleomycin	−	−
50% Seawater	++	+++
+ 10 μg/ml zeocin	++	++
+ 50 μg/ml zeocin	−	+/−
+100 μg/ml zeocin	−	−
+ 10 μg/ml phleomycin	−	+/−
+ 50 μg/ml phleomycin	−	−
+100 μg/ml phleomycin	−	−

*Antibiotic sensitivity was determined on plates containing different salt concentrations (100% and 50% SW) and different amounts of zeocin and phleomycin. The signs + and − are indicative of growth or nongrowth, respectively.

For construction of chimeric genes for diatom expression containing the *Sh ble* resistance gene, we used PCR to amplify *FCP* promoters and 3′ regulatory sequences from *P. tricornutum* cells (Bahya and Grossman, 1993). Specifically, the *FCPB* and *FCPF* promoters were fused upstream of *Sh ble,* and the *FCPA3′* transcription terminator sequences were fused downstream to generate pFCPBp–Sh ble and pFCPFp–Sh ble (Figure 1).

Using a protocol similar to that reported previously (Apt et al., 1996), we used microparticle bombardment to transform *P. tricornutum* with the plasmids pFCPBp–Sh ble and pFCPFp–Sh ble. Different experimental conditions tested are summarized in Table 2. We modified particle size and particle bombardment velocity (i.e., pressure). The most transformants were obtained using the maximal pressure of 1550 psi. It therefore appeared that forces with very high impact were required to introduce foreign DNA into *P. tricornutum* cells. This was supported by the observation

Table 2. Recovery of Resistant Colonies After Bombardment Under Different Conditions*

Construct	Pressure (psi)	Particle size	Colonies per bombardment (minimum, maximum)	*n*
FCPBp–Sh ble	650	M5 (0.4 μm)	5 (0, 10)	2
FCPFp–Sh ble			1 (1, 1)	2
FCPBp–Sh ble		M10 (0.7 μm)	10 (0, 20)	2
FCPFp–Sh ble			3 (0, 6)	2
FCPBp–Sh ble		M17 (1.1 μm)	9 (3, 15)	2
FCPFp–Sh ble			11.5 (1, 22)	2
FCPBp–Sh ble	1100	M5 (0.4 μm)	1 (0, 3)	3
FCPFp–Sh ble			2 (0, 6)	3
FCPBp–Sh ble		M10 (0.7 μm)	12.3 (2, 40)	4
FCPFp–Sh ble			4.8 (1, 16)	4
FCPBp–Sh ble		M17 (1.1 μm)	19.3 (0, 58)	3
FCPFp–Sh ble			10 (2, 25)	3
FCPBp–Sh ble	1550	M5 (0.4 μm)	14 (13, 15)	2
FCPFp–Sh ble			17.5 (16, 19)	2
FCPBp–Sh ble		M10 (0.7 μm)	17.1 (2, 61)	3
FCPFp–Sh ble			13.1 (4, 27)	3
FCPBp–Sh ble		M17 (1.1 μm)	23.8 (5, 49)	3
FCPFp–Sh ble			12.4 (2, 30)	3

*Representative examples of transformation efficiencies obtained with different FCP promoters, particle sizes, and bombardment pressures are shown. Final column shows average number of colonies per transformation, the minimum and maximum numbers from individual transformation experiments (in parentheses), and number (*n*) of transformations from which data are derived. Bombardments were performed on plates containing 1×10^7 cells. After 2 days of growth, cells were replated on phleomycin (100 μg/ml) to select for transformant colonies.

that transformed colonies were obtained only when the cells were placed closest to the stopping screen of the biolistic device (data not shown). Particle size became an important factor at low pressures (650 and 1100 psi): under these conditions large particles (M10–M17) were more effective than small particles. Maximal transformation efficiencies were between 2 and 20 transformants per 10^7 cells/μg DNA, in agreement with previous reports of diatom transformation (Dunahay et al., 1995; Apt et al., 1996). However, a lot of variation was observed from one experiment to another, even when using identical bombardment conditions (Table 2). This could be due to many factors, but the level of humidity within the petri dishes was probably a key determinant.

Both the *FCPB* and *FCPF* promoters were capable of driving expression of the *Sh ble* gene at levels sufficient to confer resistance to phleomycin, although the *FCPB* promoter was the most efficient under the optimal conditions found to transform *P. tricornutum* (Table 2). Apt et al. (1996) also reported the effectiveness of *FCPBp–Sh ble* fusions for transformation, but did not test activity of the *FCPF* promoter.

Molecular Analysis of Transformants

PCR analysis was performed directly on putative transformed cells using primers to amplify the *Sh ble* gene (Figure 2A). In most of the antibiotic-resistant clones, a 420-bp fragment was amplified, whereas in wild-type cells, no amplification product was obtained.

To confirm that the resistant phenotype of the transformants was due to expression of a functional Sh ble protein, cell extracts were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using an anti–Sh ble antibody. Most resistant clones contained a protein of approximately 14 kDa that was not present in untransformed cells (Figure 2B). The size of this protein corresponds with the reported size of the Sh ble protein (Drocourt et al., 1990).

Southern blot analyses were then used to verify the

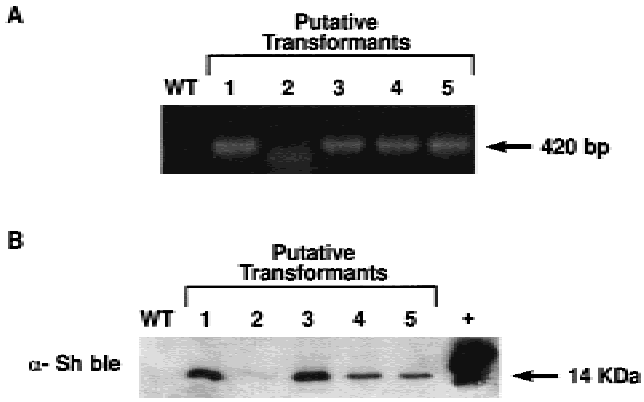

Figure 2. Molecular analysis of phleomycin-resistant transformants. **A:** PCR analysis performed directly on putative transformed cells (lanes 1–5) to amplify the *Sh ble* coding sequence. The arrow marks the 420-bp amplification product expected in transformed clones. **B:** Western blot analysis of transformants. Cell extracts from transformed and wild-type (WT) cells were analyzed by western blotting using a rabbit polyclonal antibody against the Sh ble protein. The size of the protein (14 kDa) is shown. Lane + shows a positive control of the purified Sh ble protein. In both assays, four of the five putative transformants are true transformants; that in lane 2, however, may be an escape.

presence of the *Sh ble* gene and to analyze integration patterns in DNA isolated from two antibiotic-resistant clones transformed with pFCPFp–Sh ble (clones 1 and 3 in Figure 2). As probes in the hybridization experiments, we used the *Sh ble* gene, the *FCPF* promoter, and the *FCPA3′* sequences. The presence of the *Sh ble* gene in high molecular weight undigested DNA from the two transformants confirmed the presence of the exogenous gene in the diatom genomes and excluded the possibility that it was being maintained on a plasmid (Figure 3B, lane u). In digested DNA from both transformants, the presence of multiple *Sh ble*–hybridizing fragments indicated the presence of several copies (between 7 and 10). The hybridization patterns observed with the promoter and terminator probes confirmed this hypothesis. In addition, these probes did not reveal any evidence for recombination of the transgene into homologous sites within the genome. For example, in the case of homologous recombination within *FCPFp,* we would expect a mobility shift of the 1.4-kb *Bst*XI-hybridizing fragment in transformed cells. Conversely, recombination within *FCPA3′* would result in a similar shift of the 1.3-kb *Bst*XI fragment. This did not occur in either case (Figure 3B, lanes Bs). The same conclusion can be drawn from the *Bgl*II-hybridizing fragments (Figure 3B, lanes Bg).

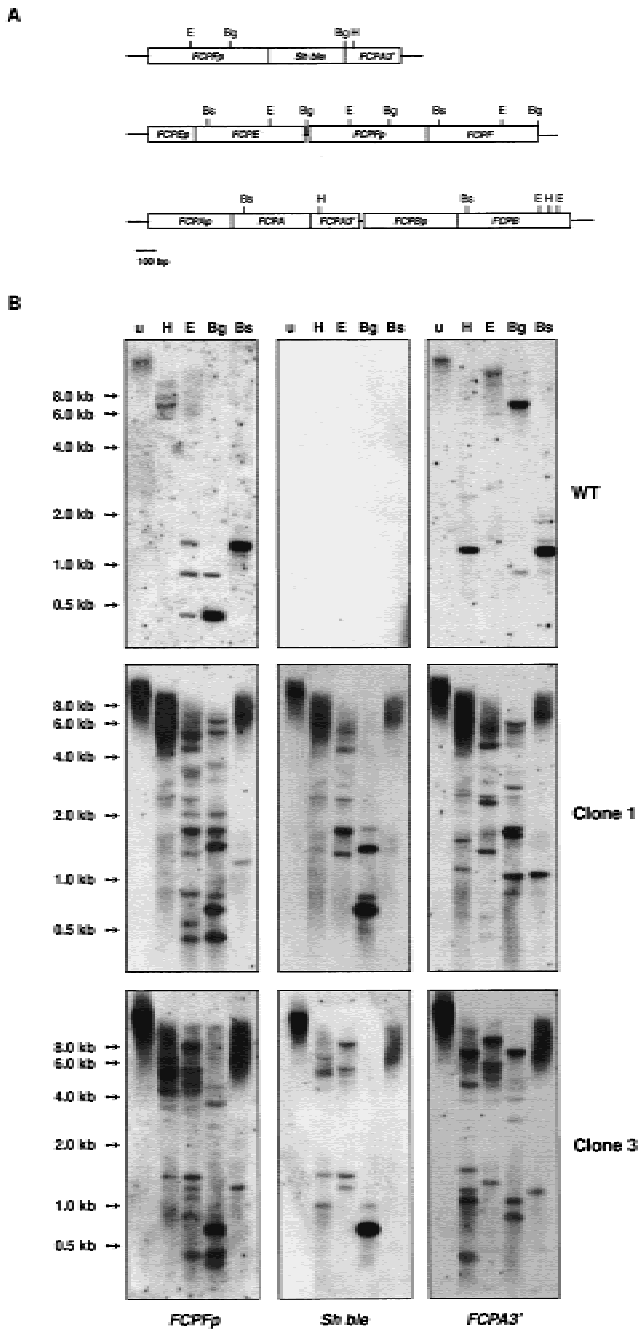

Figure 3. Southern blot analysis of *P. tricornutum* transformants. **A:** Schematic maps of chimeric Sh ble construct used for transformation (*top*), *FCPFp* region (*middle*), and *FCPA3′* region (*bottom*) within the *P. tricornutum* genome. Sites of restriction enzymes used in Southern blot analyses are indicated, and the size bar is given below. **B:** Southern blot analysis of the wild-type (WT) (*top panel*), transformant clone 1 (*center panel*), and transformant clone 3 (*lower panel*) using *FCPFp* (*left*), *Sh ble* (*middle*), and *FCPA3′* (*right*) sequences as probes. Molecular weights are indicated. Abbreviations: u, uncut; H, *Hin*dIII; E, *Eco*RI; Bg, *Bgl*II; Bs, *Bst*XI.

We also found no clear evidence for the presence of multiple inserts at single insertion sites. If this had occurred, we would predict, for example, that the *Sh ble* probe would hybridize strongly to the same-sized *Hind*III and *Eco*RI fragments, as both enzymes recognize only one site within the foreign DNA at opposite sides of the resistance gene. Such a fragment would be expected to be the same size as the introduced plasmid (4.3 kb). Figure 3B (lanes H and E) demonstrates that this did not occur.

Southern blot analyses of other phleomycin-resistant clones confirmed these general observations and indicated copy numbers between 1 and 10 within the host genome (data not shown). We therefore propose that integration of foreign DNA into the *P. tricornutum* genome occurs by illegitimate recombination at several independent sites.

Stability of the Transformed Phenotype

Transformed *P. tricornutum* clones were maintained on agar plates containing 100 μg/ml phleomycin. The presence of the *Sh ble* gene was confirmed in cells grown on antibiotic selection for more than 1 year (data not shown). To test the stability of the phleomycin-resistant phenotype under nonselective conditions, clones were grown in liquid medium without the antibiotic, and the cells were tested periodically for phleomycin resistance by plating them on selective media. In contrast to *C. cryptica* (Dunahay et al., 1995), approximately 80% of transformed clones were found to have lost their antibiotic resistance after growth under nonselective conditions for longer than 3 months. This loss of resistance could be correlated to loss, rearrangement, or modification of the foreign DNA sequences in the genome. Selective pressure may therefore be an important factor for the maintenance of foreign DNA in the *P. tricornutum* genome.

Cotransformation of Plasmids in *P. tricornutum*

To introduce other genes of interest into diatom cells, we developed a system to cotransform *P. tricornutum* with two different plasmids, one containing a selectable gene conferring resistance to phleomycin (pFCPBp–Sh ble or pFCPFp–Sh ble) and another containing a nonselectable *LUC* gene (pFCPFp-LUC or pFCPBp-LUC). Equal amounts of the two different plasmids (i.e., 0.5 μg of pFCPBp–Sh ble plus 0.5 μg of pFCPFp-LUC, or 0.5 μg of pFCPFp–Sh ble plus 0.5 μg of pFCPBp-LUC) were coated onto M17 particles and shot into diatom cells at high pressure (1550 psi). Phleomycin-resistant cells were tested for the presence of *Sh ble* by PCR, and the presence of the second chimeric gene was analyzed by performing in vivo LUC assays directly on the putative cotransformants grown under selective pressure.

Efficiencies of cotransformation were surprisingly high. Between 60% and 70% of transformed clones contained both markers, and no differences were observed between the *FCPB* and *FCPF* promoters (data not shown). PCR analysis using primers to amplify the *LUC* coding region confirmed this result (data not shown).

Molecular Analysis of Cotransformants

Southern blot analysis was performed on the cotransformed clones that initially tested positive in the LUC assay, to verify the presence of the second foreign gene and to study integration patterns. Total DNAs from wild-type and from 14 putative cotransformants (1–14) were digested with *Eco*RV, which recognizes only one site in the *LUC* gene, and hybridized with a *LUC* gene fragment representing sequences downstream of the *Eco*RV restriction site. The results of this analysis (Figure 4A) indicated significant variations in the number of copies of the chimeric *LUC* gene in the different cotransformants, as previously shown for the selectable marker *Sh ble* (Figure 3). Some clones contained only a few copies (e.g., one copy in clones 5 and 12, and two copies in clones 2 and 10), while others contained multiple hybridizing fragments, probably owing to the integration of the foreign DNA at multiple sites. No bands were detected in DNA extracted from untransformed clones (Figure 4A, lane wt).

In vitro LUC assays were performed on the same cotransformant clones shown in Figure 4A to compare luciferase activity with the number of copies of the chimeric *LUC* gene (Figure 4B). Although the lowest LUC activity was detected in clones 10 and 12, containing, respectively, two copies and one copy of *LUC,* in similar clones (e.g., clones 2, two copies; and 5, one copy) LUC activity was comparable to that found in cotransformants containing multiple copies. It therefore appeared that the expression level of the chimeric gene was only loosely correlated with copy number and may therefore be more dependent on the integration site of the transgene into particular sites in the diatom genome. Luciferase activity was undetectable in wild-type cells (wt in Figure 4B) and in clones transformed with pFCPBp–Sh ble or pFCPFp–Sh ble alone (data not shown).

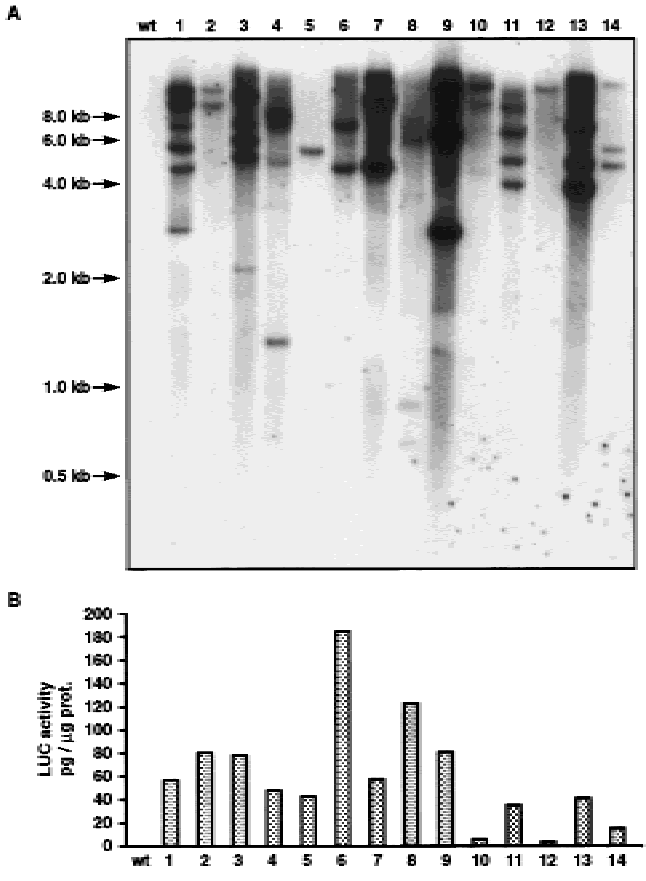

Figure 4. Molecular analysis of *P. tricornutum* cotransformants. **A:** Southern blot analysis of genomic DNA extracted from wild-type (lane wt) and from 14 independent LUC-positive cotransformants (lanes 1–14), using a *LUC* gene fragment as probe. Genomic DNA was digested with *Eco*RV, which recognizes one site in the *LUC* coding sequence. Molecular weights are shown in kilobase pairs. **B:** Luciferase activity in extracts of the cotransformant clones 1–14. LUC activity is given as picograms of LUC per microgram of total protein.

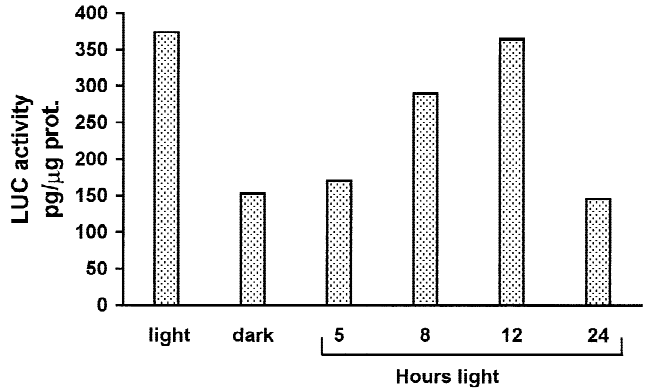

Figure 5. Analysis of light-regulated LUC activity in cotransformant 10, which contains two copies of the *FCPFp-LUC* chimeric gene. Protein extracts were prepared from light- and dark-adapted cells and from dark-adapted cells subsequently exposed to light for 5, 8, 12, and 24 hours.

Analysis of FCP-LUC Expression in Response to Light

Diatom FCP proteins are functional equivalents of chlorophyll a/b-binding proteins (CAB) of higher plants and green algae. CAB protein synthesis has been shown in numerous instances to be controlled by light quality and quantity, and this regulation acts primarily at the level of gene expression (Terzaghi and Cashmore, 1995). For this reason, we used cotransformed cells containing the *LUC* reporter gene fused to an *FCP* promoter to examine the light-mediated induction of *FCP* gene expression. Cotransformant 10 (which contains two copies of an *FCPFp-LUC* chimeric gene) was grown in a light-dark cycle of 12 hours light and 12 hours dark for 1 week, adapted for 2.5 days in darkness, and subsequently exposed to continuous white light. In vitro LUC assays were performed on cell extracts taken from light- and dark-adapted cells and from cells reexposed to light for up to 24 hours (Figure 5). LUC activity decreased in cells maintained in the dark for 2.5 days, but after subsequent light exposure activity increased steadily up to a maximum at around 12 hours. At this time point LUC activity was comparable to that observed prior to dark treatment. Interestingly, after 24 hours in the light, LUC activity decreased (Figure 5), perhaps suggesting circadian regulation of *FCP* gene expression in diatoms, similar to that observed for higher plant *CAB* genes (Millar and Kay, 1991).

Cryopreservation

The maintenance of large numbers of transformed clones could be greatly facilitated by the possibility of freezing cells for indefinite periods of time. We therefore established a protocol for cryopreservation of *P. tricornutum* cells. Cells were grown in liquid medium with antibiotic selection until exponential phase and then concentrated in 1.5 ml of growth medium to which 10% dimethyl sulfoxide had been added. The temperature was lowered gradually to −80°C using intermediate steps of 1 hour each at 4°C and −20°C. Following storage at −80°C, the cells were replated on agar plates with and without phleomycin to verify survival percentage and the stability of the transformed phenotype after

Table 3. Antibiotic Sensitivity in *Thalassiosira weissflogii**

Antibiotic	Concentration	Growth
—	—	++
Chloramphenicol	25–50–100 μg/ml	+
Phleomycin	10–50–100 μg/ml	+
Zeocin	10–50–100 μg/ml	+
Streptomycin	50–75 μg/ml	++
Spectinomycin	50–75 μg/ml	++
Ampicillin	100 μg/ml	++
G418	50–100–250 μg/ml	+++
Hygromycin	20–100 μg/ml	++

*Assays were performed using 10^6 cells plated on SW. The + signs are indicative of growth.

cryopreservation. Although the majority of cells died, about 10% survived. PCR analysis was performed to verify maintenance of the foreign DNA within the *P. tricornutum* genome. Thus far, we have not found cryopreservation to affect the stability of the transformed phenotype for at least 1 year, and all transformed clones tested have maintained their phleomycin-resistant phenotype after the freezing process (data not shown).

We have also tested the cryopreservation procedure on other species of diatoms, such as the centric diatom *T. weissflogii* and the pennate diatom *C. fusiformis.* Although the protocol was successful for *C. fusiformis,* we have been unable to cryopreserve *T. weissflogii* cells (data not shown).

Transformation of *T. weissflogii*

Although *P. tricornutum* has become a useful experimental system for laboratory studies of diatoms, it cannot be considered to be representative of this group of algae because of its very restricted ecological distribution and its unusual cell wall, which is not highly silicified (in contrast to the majority of diatoms, it does not even have a strict dependence on silica for growth (Lewin et al., 1958)). In order to understand the ecological success of diatoms, it will therefore be necessary to develop other experimental systems. To this end, we attempted to transform the centric diatom *T. weissflogii,* which is widely distributed in the world's oceans. Surprisingly, *T. weissflogii* was extraordinarily insensitive to a wide range of antibiotics, even at high concentrations (Table 3), making it impossible to attempt to select for transformant clones by selection on antibiotics. Furthermore, this diatom did not grow well on diluted SW medium (data not shown).

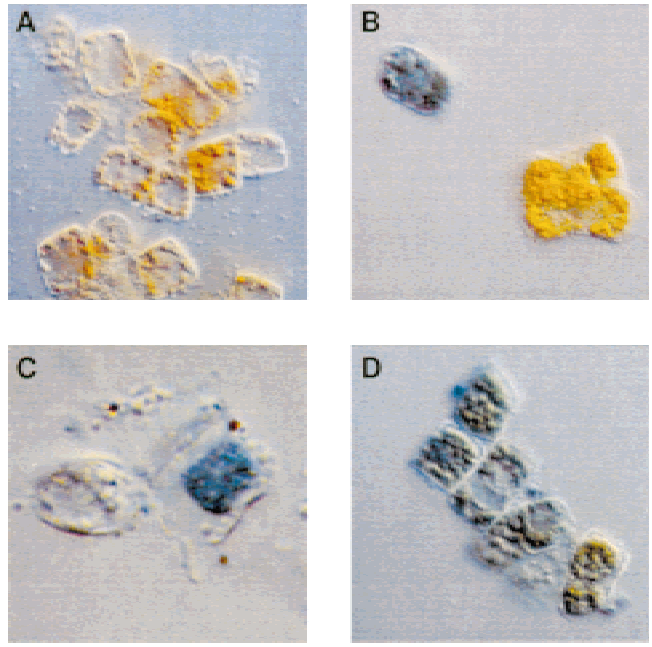

Figure 6. Transient transformation of *T. weissflogii*. Cells were transfected with pFCPBp-GUS and stained for GUS activity as described in Materials and Methods. **A:** Nontransformed cells. **B:** A GUS-positive cell (*top left*) with a group of GUS-negative cells. **C:** A GUS-positive cell with gold particle still attached (*at top right of cell*). **D:** A group of cells showing transmission of the GUS-positive phenotype through three generations.

To determine whether foreign DNA could be introduced into *T. weissflogii* by particle bombardment, we used the screenable *GUS* reporter gene, widely used in plant molecular biology (Jefferson et al., 1987). A plasmid containing the *GUS* gene fused between the *FCPBp* and *FCPA3′* sequences of *P. tricornutum* was coated on gold or tungsten particles and shot onto plates containing a lawn of *T. weissflogii* cells (approximately 5×10^6 cells). Following bombardment the cells were incubated for 1 day at low light intensities at 20°C, after which a piece of agar containing putative transformed diatoms was placed in a solution containing X-Gluc substrate and incubated at 37°C overnight. The *T. weissflogii* cells expressing the *GUS* gene were stained blue and could be detected in the microscope (Figure 6). Efficiencies were lower than for transformation of *P. tricornutum* (by almost an order of magnitude), and analysis of cells 1 week after bombardment failed to reveal any positive cells (data not shown), indicating that *GUS* expression was only transient. Nonetheless, GUS expression was in some cases transmitted through three cell divisions (Figure 6D).

Discussion

In this report we have demonstrated that the diatoms *P. tricornutum* and *T. weissflogii* can be transformed using microparticle bombardment, a technique used to introduce DNA into a wide variety of organisms recalcitrant to transformation by other methods. Our results with *P. tricornutum* transformation are an independent confirmation of experiments reported by Apt et al. (1996) using the *Sh ble* bacterial resistance gene as a selectable marker for transformation. Moreover, we have established that *P. tricornutum* antibiotic sensitivity is dependent on different factors such as cell plating density and salt concentration (Table 1), as previously observed for kanamycin sensitivity in the diatom *C. cryptica* (Dunahay et al., 1995).

Molecular analysis of transformed *P. tricornutum* clones by Southern blot hybridization revealed multiple fragments (up to 10 copies of foreign DNA) in digested DNA extracted from different clones. These multiple hybridizing fragments are probably due to integration of the foreign DNA at multiple sites. Detailed analysis performed with different restriction enzymes and with different probes on two transformants excluded the occurrence of homologous recombination and indicated that the exogenous DNA had integrated randomly by illegitimate integration at several sites (Figure 3). However, analysis of the data should not be considered to be conclusive because assessing integration patterns is difficult when the ends of the foreign DNA are not known. Furthermore, it is possible that the inclusion of longer sequences of homologous DNA would result in homologous recombination events. In mouse embryonic stem cells and in the moss *Physcomitrella,* several thousand base pairs are required for efficient gene targeting (Deng and Capecchi, 1992; Schaefer and Zrÿd, 1997). The controlled manipulation of the diatom genome by homologous recombination would be extremely important for understanding diatom biology, and should be pursued actively.

Putative transformants were also examined by PCR performed directly on cells. This methodology is very sensitive, requires only small amounts of starting material (1.5 ml culture), and is extremely rapid. We have found this to be the best method to screen for the presence of exogenous DNA in diatoms, and it is now used routinely in our laboratory.

The cotransformation of *P. tricornutum* simultaneously with two different plasmids, one containing a selectable marker and the other not, was found to be very efficient (up to 70%), and all the clones analyzed retained the nonselectable reporter gene under conditions selective only for the antibiotic resistance gene. Because it was important to maintain selection pressure to retain *Sh ble* activity (see Results), the stable expression of the nonselectable gene may therefore indicate that the two plasmids were integrated together at the same site in the genome, as most likely occurs in the moss *Physcomitrella patens* (Kammerer and Cove, 1996).

We have used the cotransformation protocol to introduce firefly luciferse-based chimeric genes into diatoms. Luciferase activity could be readily detected in vitro (Figure 4B) and in vivo (data not shown), although there was little correlation between LUC activity and *LUC* gene copy number (Figure 4), suggesting that position effects are an important factor constraining foreign gene expression in *P. tricornutum*.

Using cells cotransformed with *FCPFp-LUC,* we have shown for the first time the regulated expression of a chimeric gene in diatoms. In particular, luciferase activity in *FCPFp-LUC* cotransformants was reduced in 2.5-day-dark-adapted cells and subsequently increased following a shift to white light (Figure 5). Although LUC activity steadily increased during the first 12 hours of light irradiation, after 24 hours it was again at basal levels. Such observations reveal the versatility of the LUC reporter for gene expression studies in diatom cells, in that it can be used to monitor both induction and repression events. This versatility has been previously shown in plant and animal cells, and is due to the short half-life (approximately 3 hours) of the luciferase protein in vivo (Millar et al., 1992). Furthermore, these results indicate that the *FCPF* promoter is sensitive both to the absence and to the presence of light. In addition, the initial increase followed by subsequent decrease in *FCPF* promoter activity in light-exposed cells may be indicative of circadian control of this promoter. Such phenomena have previously been reported for *CAB* genes in higher plants (Millar and Kay, 1991), and *Arabidopsis* mutants are now available that are defective in these mechanisms (Millar et al., 1995). It will be of great interest to verify the existence of a circadian oscillator controlling *FCP* gene expression in marine diatoms. Light-inducible promoter fragments fused to the *LUC* reporter gene should greatly facilitate such studies and will also be of utility to determine the active wavelengths of light utilized for photoperception in the marine environment.

In this work we have also described a cryopreservation system to store the pennate diatoms *P. tricornutum* and *C. fusiformis* for indefinite periods. The possibility of storing transgenic diatoms greatly facilitates the maintenance of large numbers of transformed lines and reduces costs re-

sulting from the use of expensive antibiotics to maintain selective pressure on transformants. Cells can be maintained for indefinite periods of time at −80°C, without any apparent effect on the stability of the transformed phenotype.

This is also the first report describing the transient transformation of the centric marine diatom *T. weissflogii,* using the screenable *GUS* reporter gene. Although *GUS* expression was only transient and efficiencies of transformation in *T. weissflogii* were lower than for *P. tricornutum,* our results reveal that there is no fundamental impediment to introduction of foreign DNA in this organism. However, it is possible that current biolistic technology is not sufficient to generate the high-impact velocities required to obtain higher numbers of transformants because *P. tricornutum* transformation requires maximal settings of all parameters. Large particles of 1.1 μm were more efficient than small particles, even though *P. tricornutum* has a diameter of only 2 to 3 μm. The larger size of *T. weissflogii* may therefore indicate that larger particles will be required for successful transformation. Although GUS is a very sensitive reporter enzyme, it is not ideal because material must be fixed and any bacterial contamination can result in erroneous GUS staining (Jefferson et al., 1987). Moreover, with increasing numbers of cell divisions, the blue coloration is lost. This may be due to dilution of the GUS enzyme through cell division or could be indicative of the loss of plasmid DNA in the transiently transformed clones. Nonetheless, the observed antibiotic resistance in *T. weissflogii* will be a major stumbling block for the establishment of a reliable protocol for stable transformation.

The ability to generate transgenic diatoms makes them amenable for a wide range of molecular studies aimed at dissecting their basic biology and ecology. For example, microparticle bombardment can be used to generate transformants containing multiple foreign genes such as *LUC* and the *green fluorescent protein* (*GFP*) gene to study diatom gene expression and protein trafficking. Moreover, the availability of transgenic technologies opens up a variety of potential biotecnological applications dependent on the expression of genes of specific interest. Practical approaches could be of value for the aquaculture industry or for large-scale production of valuable specialty chemicals.

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