UNIVERSITY OF CALIFORNIA, SAN DIEGO

Microscopic Studies in Nascent Frustule Formation from Protoplasts of the Marine Pennate Apochlorotic Diatom *Nitzschia alba* and Production of Novel Transformation Vectors for Studies in *N. alba* and the Marine Centric Photoautotrophic Diatom *Thalassiosira pseudonana*

A Thesis submitted in partial satisfaction of the requirements for the degree of

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by

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The Thesis of Jeffrey Carlson is approved and it is acceptable in quality and form for publication on microfilm:

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Dedicated to my God and Savior Jesus Christ and to my Good Friends Thanh Ngo, Ha Huynh, Ed Koo, and My Whole Family for Helping Me to Make it Through this Project

In Loving Memory of Chester Carlson

For now we see through a glass, darkly; but then face to face: now I know in part; but then shall I know even as also I am known.

1 Corinthians 13:12

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LIST OF ABBREVIATIONS

ACCase……..……..acetyl-Coa carboxylase/biotin carboxylase

clonNAT…….....nourseothricin

DIC…….....differential interference contrast (microscopy)

eGFP…….....GFP w/ codon bias for *Phaeodactylum tricornutum*

fcp…….fucoxanthin, chlorophyll a/c-binding protein

gDNA……..….genomic DNA

NAT……....……..nourseothricin acetyl-transferase

PDMPO……..silicic acid tracer

PDMS……..……polydimethylsiloxane

R123……...……rhodamine 123 dye

SDV…… silica deposition vesicle

SEM…….scanning electron microscopy

Sh ble…….……...*ble* gene of *Streptoalloteichus hindustanus*

SV……...SV40 early viral promoter or poly-A

TEM……...transmission electron microscopy

TMOS……..……..tetramethylsiloxane

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ABSTRACT OF THE THESIS

Microscopic Studies in Nascent Frustule Formation from Protoplasts of the Marine Pennate Apochlorotic Diatom *Nitzschia alba* and Production of Novel Transformation Vectors for Studies in *N. alba* and the Marine Centric Photoautotrophic Diatom *Thalassiosira pseudonana*

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Biosilicification is an area of growing interest in marine biology, nanotechnology, and nanoscience. The process is ubiquitous in nature, with diverse species displaying siliceous structures. This comes as small wonder

when one considers the immensity of silicon available to biogeochemical processes. No where is the process of silicification so highly specialized as in the production of cell walls by diatoms. Ongoing research in electron microscopy to follow the processes of silicification and current efforts to sequence genomes of marine diatoms and produce genetic transformation systems in some model species are unlocking the secrets of biosilicification. Already, biomimetic chemistry has enabled the mimicry of the silicification processes of diatoms *in vitro*. Surely, further understanding of the proteins, metabolic processes, and cellular structures tied to biosilicification in diatoms will lead to new frontiers in science and technology. In order to communicate pertinent information regarding aspects of diatom cell wall morphogenesis and biosilicification, a general introduction to diatom structure and cell wall formation will be supplied (Chapter I); including an introduction to the two species used in this study. In this work, efforts are made to study the process of biosilicification in naked protoplasts of the marine apochlorotic chemoheterotrophic species *Nitzschia alba* as they regenerate valves using microscopy methods for observation (Chapter II). Simultaneously, new vectors are generated for future studies in transformation of both *N. alba*, and the marine centric photoautotrophic diatom *Thalassiosira pseudonana* (Chapter III).

CHAPTER I

AN INTRODUCTION TO DIATOM BIOSILICIFICATION

1.1 The Nature of Diatoms

Diatoms are ubiquitous unicellular marine microalgae that are found all over the world in various biomes. Benthic and pelagic marine species, as well as freshwater, and even volcanic (Brown and Wolfe, 2006) species have been discovered. Both photosynthetic and apochlorotic (non-photosynthetic) species have been described. Altogether, these diverse microbes comprise a class of organisms known as Bacillariophyceae and are famous for their intricate nano-structured bio-silica cell walls termed 'frustules' (Round et. al., 1990). Like snow-flakes, these cell walls contain highly-ordered species-unique surface and subsurface geometry that is both intriguing and mysterious. The subject of cell wall formation in Bacillariophyceae has been an area of intensifying study as the biosynthesis of these complex nano-structures may be of use in diverse biotechnological applications in the future (see review: Parkinson and Gordon, 1999). Concurrently, studies of the metabolic pathways involved in cell wall synthesis may elicit useful information in areas of proteomics (Hildebrand et al., 1997), biosilicification (Hazelaar et al., 2003), and other biochemical fields.

1.2 Cell wall Silicification

Diatoms take up silicic acid ($Si[OH]_4$) from the environment for use in cell wall formation (Azam et al., 1973; Lewin, 1955; Richter, 1906), and possibly DNA synthesis (Darley and Volcani, 1969). The rate of cell wall silicification of diatoms has been tied to aerobic respiration of the cells (Martin Jézéquel et al., 2000), and limits the growth rate of diatoms in silicon-limiting conditions (Falkowski et al., 2004; Roessler, 1988). Although heterologous species differ in the extent of silicification of cell walls (Li and Volcani, 1984), silica is a key component in diatom cell walls. Silicic acid that is taken up by the cell is sequestered into a silica-deposition vesicle (SDV) (Stoermer et al., 1965) which is bounded by a lipid bilayer membrane termed the 'silicalemma' (Reimann et al., 1966). Following cellular mitosis, the SDV is observed to expand intracellularly near the cleavage furrow. Silica deposition within the SDV following mitosis is responsible for production of new cell wall material within each daughter cell (Pickett-Heaps et al., 1990).

To date, most studies dealing with cell wall synthesis have focused on the production of new cell wall material in daughter cells immediately following cytokinesis. Without question, a genetic understanding of the expression patterns, and processes of cell wall silicification in diatoms will yield a wealth of information concerning the actual course of cell wall synthesis (Li and Volcani, 1984). Additionally, microscopic studies of the cell wall throughout various stages of formation can provide a more comprehensive picture of cell

wall formation (Pickett-Heaps et al., 1990; Chiappino and Volcani, 1977; Lauritis et al., 1968). Already, many studies have explored aspects of silicification (Hildebrand et al., 2007; Hazelaar et al., 2005).

Although no comprehensive model has yet been established, microscopic images offer a wealth of information concerning the possible steps involved in cell wall synthesis. In one model, for example, the cell produces a sheet of carbohydrates and lipids within the SDV, which is layed out as a template in advance of silicification, followed by gradual filling-in by silicate particles under the control of specialized proteins called silaffins, long chain polyamines, and probable involvement of the cytoskeleton linked to mobile proteins, followed by final filling-in of the surface characterized by deposition of more silica upon the established framework (reviewed in: Zurzolo and Bowler, 2001). This model, termed 'spatial cementing,' is said to have both horizontal (x/y-phase), and vertical (z-phase) stages of growth (Hildebrand et al., 2006).

1.3 Cell Wall and General Characteristics

The diatom cell wall, or frustule, is an intricately structured shell composed of silica, lipids, proteins, and other common components of the eukaryotic cell wall (Scala and Bowler, 2001). The shell is composed of two-halves in all species described thus far, with one half fitted over the other like the two parts of a Petri dish. This shell contains the protoplast of the cell

which is bounded by a lipid bilayer membrane called the plasmalemma. The larger half is termed the epitheca, because it fits over the other half, while the smaller half is similarly termed the hypotheca. Each theca may have a variety of distinct patterned holes called areolea which are generally set in well-ordered rows called striae, or in concentric patterns (Anonymous, 1975). These areolea function as passages for food, water, and waste passing to and from the protoplast of the cell which is housed within the frustule (Round et al., 1990).

The two theca are further separated by additional rings that run around the circumference, along the rim of each theca. These rings may be stacked one upon the other, one lapping over the joint of the next, as a carpenter might design a clever joint. These additional rings are called copulae, or 'girdle-bands' (Round et al., 1990). Thus, the cell can be seen, simply, of being composed of a protoplast having a soft protoplasmic membrane (plasmalemma)—which is perhaps similar in many ways to other plant protoplasts that have been previously described—housed within the protective armor of the frustule which protects the protoplast from mechanical stress (Hamm et al., 2003), while providing a clever means to filter outside materials coming into the cell (Pickett-Heaps et al., 1990), as well as providing a measure of protection from predators or the elements (Pondaven et al., 2006; Smetacek, 1999).

Given that the frustule is most often composed to a large degree of silica, it is transparent to light; allowing photosynthetic species to flourish in low-light conditions with chloroplasts which are functional analogues of chloroplasts found in other eukaryotic species. Practically, this means that the cells appear transparent in light microscopy when viewed in the brightfield, but having a dark color when chloroplasts are present. In apochlorotic species,

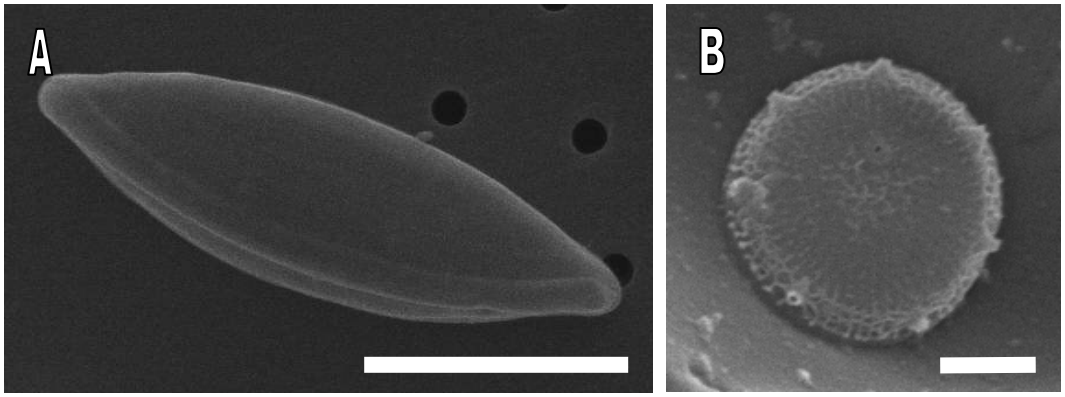
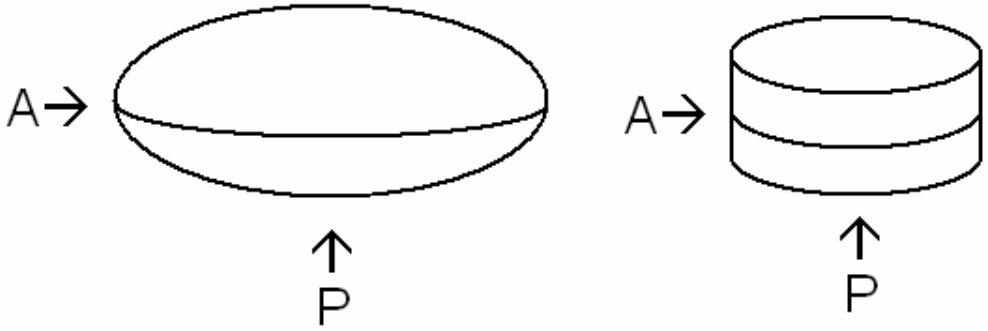


Figure 1.1: SEM images of diatoms. A. Pennate diatom *Nitzschia alba* (scale bar=5μm). B. Centric diatom *Thalassiosira pseudonana* (scale bar=1 μm)



Scheme 1.1: Schematic view of pennate and centric forms illustrating apical "A" and pervalvar "P" axes. Terminology by Anonymous (1975)

lacking the chlorophyll pigments found in chloroplasts, the cells appear transparent throughout. Additionally, cells seen in bright field can be distinguished to some degree on the basis of morphology, with some cells

having a round, or ‘centric’ morphology, while other species possess an oblong, or ‘pennate’ morphology (Van Den Hoek et al., 1997).

In this study, both photosynthetic and apochlorotic species were used. Additionally, studies were conducted in both centric and pennate forms. The two species researched were the well-known centric photosynthetic model species *Thalassiosira pseudonana* which is the subject of much current research in our laboratory since both the genome of this species, and an established method of genetic transformation have been described (Poulsen et al., 2006); and the marine apochlorotic pennate *Nitzchia alba* which has also been extensively studied (Chiappino et al., 1977; Lauritis et al., 1968) and is unique for having a previously described method for producing protoplasts from cultured cells (Hemmingsen; dissertation, 1971).

1.4 The Species: *Thalassiosira pseudonana*

T. pseudonana is currently an excellent model for studies of biosilification because the genome sequence has been published (Armbrust et al., 2004), and because tools for genetic transformation have already been developed. Although the actual protocols vary, the common method of transformation involves the ‘biolistic’ approach. In this method, gold or tungsten microparticles are coated with the desired DNA to be used in transformation, and these ‘bullets’ are fired into a lawn of cells spread on an agar plate. The microparticles pierce the cells, wounding them and allowing

the DNA to pass into the cell to be taken up by chromosomes within the nucleus of the cell where they are integrated into the genome to await expression, either as constitutively expressed genes, or as inducible genes, depending on the nature of the promoter used (summarized in: Kroth, 2007).

Studies have shown that inducible gene expression is possible using the nitrate reductase gene promoter which is inactive in medium until nitrates are added. More commonly employed, the fucoxanthin chlorophyll a/c binding protein promoter, LHCF9 from *T. pseudonana* has been used as a promoter to drive constitutive expression in cells incubated under constant light. The promoter is ideal for expression in the photoautotrophic species *T. pseudonana* since the cells are commonly cultured under continual light (Poulsen et al., 2006). However, this promoter may not drive constitutive expression in heterologous species. The effort to produce a 'universal promoter' for heterologous gene expression in 'all' species of diatoms has been the motivation behind the development of heterologous constructs in this study using the constitutive promoter acetyl-CoA carboxylase in attempts to transformation both *T. pseudonana* and *Nitzschia alba*, and the development of a construct utilizing the SV40 early promoter used to transform *T. pseudonana*.

Detection of transformed cells often depends upon selection of putative transformants in selective medium containing antibiotics. Previous studies have proven the usefulness of a number of antibiotics in inhibiting the growth

of *T. pseudonana* and other species. Of particular interest to this work is the antibiotic clonNAT (nourseothricin) produced by Werner BioAgents (Jena, Germany) which is primarily a mixture of streptothricins D and F produced by and harvested from the bacteria *Streptomyces noursei* (Bocker and Bergter, 1986). The NAT gene, also known as nat1, derived from *S. noursei* confers resistance to clonNAT. The neurseothricin *N*-acetyltransferase (NAT) gene product catalytically converts the toxic streptothricins into harmless byproducts (Krügel et al., 1993). Poulsen et al. (2006) have shown this drug and resistance gene combination to be applicable to studies of *T. pseudonana* and so the combination was selected for the current work.

The development of additional selectable markers for use with *T. pseudonana* could aid in studies involving transformations with multiple vectors, allowing selection of clones containing both genes introduced into the cell by either concurrent or step-wise selection of putative transformants on medium containing antibiotics appropriate to each resistance gene. Several transformation systems have already been developed in diatoms such as *Phaeodactylum tricornutum*. The *P. tricornutum* system uses the *sh ble* gene of *Streptoalloteichus hindustanus* as a selectable marker for resistance to the antibiotics zeocin and phleomycin. Both of these antibiotics were shown to destroy wild-type *P. tricornutum* with high efficiency. The system utilizes the native fucoxanthin, chlorophyll a/c-binding protein (*fcp*A) promoter and terminator elements to drive transcription (Zaslavskaia et al., 2000).

1.5 The Species: *Nitzchia alba*

N. alba is an apochlorotic (non-photosynthetic) marine pelagic pennate species. This species is chemoheterotrophic, thriving on a diet of minerals and degraded proteins. In nature, these diatoms are found in relatively cold and dark waters, and rest upon any available substrate (Lauritis et al., 1968). The cells exhibit rapid gliding motility which appears to be aided in some way by the production of a thick, predominantly carbohydrate substance termed 'mucilage' which often appears as flocculation in liquid culture (Lewin and Lewin, 1967). The mucilage is excreted from a long, thin slit which runs the length of the apical axis of the valve; dividing it into two halves. The raphe fissure comprises the overlapping joint which is seen on the valve exterior, and the internal structures associated with the raphe; visible in microscopic images of the cellular interior (reviewed in: Hildebrand and Wetherbee, 2003). A sessile clone of this species, designated strain cc3 in this work, has been cultured and is useful in transformation, making possible selective screening in agar plates.

Hemmingsen (1971) has previously described a method for producing protoplasts from *N. alba* by incubating cultured cells in special hypotonic medium. This unique method makes this species useful for studies of cell wall regeneration from protoplasts using a variety of microscopic techniques and novel silica-tracing fluorescent dyes which can aid in localizing newly incorporated siliceous material within the regenerating cell wall. Research has

also been carried out to attempt to confine protoplasts as cell walls regenerate in order to conform the nascent valve to a microscopic mold. Protoplast confinement, if achieved, would present an attractive means for inexpensively and rapidly producing micro-scale silica structures from diatom protoplasts shaped into various geometries.

In order to generate vectors for future genetic studies of cell wall silicification in *N. alba*, several promoters have been considered as useful candidates for transformation studies. A vector having the acetyl-CoA carboxylase (biotin carboxylase) promoter and terminator elements has been used in attempts to transform both *T. pseudonana* and *N. alba* in order to explore potential application as a candidate universal transformation vector for diatom research. Preliminary transformation attempts using the biolistic transformation method provide evidence for the effectiveness of this vector in producing clonNAT resistant putative transformants.

1.6 Purpose of the Current Research

Far from exploring further models of silicification in valve formation, the aim of this current work is to provide more data toward understanding the morphological stages of silicification involved in the formation of new cell walls in protoplasts of *N. alba* within the context of established models of nascent valve formation outlined above (Chapter II), while simultaneously developing further tools for genetic studies of diatoms which may ultimately aid in

elucidating biochemical pathways of biosilicification in diatoms at the genetic level (Chapter III). Thus, the goals of this project are two-fold: 1) to produce useful protoplast samples of *Nitzschia alba* for microscopic analysis of cell wall silicification, and 2) to conduct preliminary studies to generate genetic tools for the transformation of both *Thalassiosira pseudonana* and *Nitzschia alba* to aid in future research.

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CHAPTER II

CELL WALL REGENERATION STUDIES OF *N. alba* PROTOPLASTS

2.1 ABSTRACT

Although previously described by Hemmingsen, protoplast induction of *N. alba* as described in this thesis, is truly a novel procedure since protoplasts had to be purified as whole cells, rather than as vesicularized cell particles, which were acceptable products in the ATP studies carried out by Hemmingsen. Axenic cultures of *N. alba* were grown to density before transfer to hypotonic medium for incubation and induction of protoplast extrusion. Protoplasts harvested using either a percoll density gradient, or a novel three-step density gradient were washed before being returned to growth medium for incubation and observation of the reforming cell walls. Staining by PDMPO and rhodamine 123 dye showed silica deposition patterns in nascent protoplast valves suggesting that the new cell wall is constructed in a step-wise manner beginning with the encirclement of the protoplast by the raphe. SEM further suggests that raphe formation occurs concurrent to generation of a valve-like form which encircles the protoplast, which is subsequently followed by construction of a spherical shell of apparently heterogeneous construction.

2.2 INTRODUCTION

Diatom reproduction usually proceeds via asexual mitotic division of cells. During mitosis, one parent cell produces two daughter cells through mitotic division of the protoplast, followed immediately by formation of two valves at the cleavage furrow; one for each daughter cell within the parent cell. Following successful production of the daughter valves, each valve is exocytosed by the daughter protoplasts. The parent cell becomes two daughter cells each consisting of one valve of the parent cell, which serves as the epitheca of the daughter cell, and the nascent valve which becomes the hypotheca in the nascent cell (Pickett-Heaps et al., 1990). This process has been studied extensively in diverse species, including microscopic observations using TEM to study valve formation in *Nitzschia alba* (Chiappino et al., 1977). This species is particularly useful for studies of valve formation owing to the lack of cellular pigmentation common to photosynthetic species, but absent from apochlorotic *N. alba* cells (Lauritis et al., 1968).

Valve formation really begins with uptake of silicic acid into the cell by transmembrane silicon transporters (SITs). *Xenopus laevis* oocytes transformed with genes encoding SITs from *C. fusiformis* took up germanic acid, which competitively inhibits silicon uptake (Hildebrand et al., 1997). These novel membrane spanning proteins have been described in heterologous species (Thamatrakoln and Hildebrand, 2007), although more characterization is needed to fully understand their role in silicification. The

formation of a silicon deposition vesicle near the cleavage furrow signifies the beginning of nascent valve formation (Chiappino and Volcani, 1977). The SDV has been described as: "an eccentrically located flat round bag with a siliceous ring inside it, surrounded by a few small vesicles (16nm in diameter)" (Li and Volcani, 1984). In the early stages of valve formation, as the SDV is expanding, the vesicle is very thin, being no thicker than the particles of silica diffusing throughout the interior (Gordon and Drum, 1994; Coombs et al., 1968). Interest in the composition of the silicalemma during this time has spurred efforts to isolate the SDV or silicalemma for examination. However, after over 30 years of research attempting to isolate this organelle in diatoms, the structure remains elusive. Without doubt, an understanding of the composition of the silicalemma would greatly improve understanding of the process of biosilicification in diatoms.

The entire process of nascent valve formation happens within the confines of a daughter protoplast which is, moreover, housed within the protective confines of a parent cell. Thus, the protoplast is not exposed to extracellular conditions during valve formation. However, observations of live cultures of *Nitzschia alba* have confirmed that this species sometimes produces protoplasts during normal culturing in growth medium. Under hypoosomotic conditions, the protoplasts can be made to burst their frustules (Hemmingsen, 1971). With the protoplasts freed from the confines of the frustule, a unique opportunity arises to study the process of protoplast valve

regeneration—the process whereby cells regenerate their entire cell wall from within the naked protoplast. Using novel fluorescent silica tracers, the process of silica deposition within the SDV can be observed. Also, scanning electron microscopy (SEM) of nascent valves can yield clues about the process of protoplast valve regeneration. Altogether, these data supply the beginnings of a morphological model for nascent valve formation in protoplasts of *N. alba*.

2.3 MATERIALS AND METHODS

2.3.1 *N. alba* Cells and Culturing

Nitzchia alba, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, CCMP2426, was originally isolated from La Jolla Shores near the Scripps Pier by Mrs. Linda Tennent (Hemmingsen, 1971). Strain dc2 is a highly motile strain which was used in initial protoplasting experiments for this study and is relatively long and slender in morphology. The sessile strain cc3, used in most transformations because of the advantages of a non-motile strain in selective plating procedures, was isolated from a surface-plated culture of dc2 *N. alba* as a compact colony, and is relatively short and stout in morphology. Both strains dc2 and cc3 were stored in stock solutions of 50 mL at 18°C in the dark as axenic cultures in ASW medium (Darley and Volcani, 1969) supplemented with 100 µg/mL vitamin B12, 100 µg/mL biotin, and 1 mg/mL tryptone (the total medium hereafter referred to as ‘ASWT’) with 1x

Cellgro® Pen/Strep Mix (e.g. 100 IU/mL penicillin; 100 µg/mL streptomycin) to prevent bacterial growth. New stock cultures were inoculated approximately once every three months. Cells were cultured in 1.0 L to 1.5 L batches of either ASWT or "N. alba medium," (Hemmingsen, 1971) at either 18°C or 31°C, with stirring and minimal lighting, with or without aeration. Typically, cultures reached a density of 0.5-1.0 x 10^6 cells/mL in 7-10 days.

2.3.2 *N. alba* Protoplasts

Two distinct methods were employed to induce and purify protoplasts. The second method was developed in order to reduce the steps and time involved in the procedure, and improve the purity of the protoplast sample in anticipation of future applications.

Method 1: Cells of *N. alba* strain dc2 were cultured to high density in "N. alba medium" at 18°C with stirring in low light, sometimes with rhodamine 123 (R123) dye (Sigma, St. Louis, MO) 1:1000 part (e.g. 1 µg/mL) in solution to aid in visualizing cells with fluorescent microscopy. Cultured cells were pelleted in 40 mL centrifuge tubes at 10,000 x g for 10 min at 4°C. Following centrifugation, medium was aspirated off, and the pellet resuspended in "L+3% Medium," a hypotonic, protoplast-inducing medium designed by Hemmingsen (1971) which contains salt, tryptone, and yeast extract. 40 mL centrifuge tubes containing cells in L+3% Medium were covered in aluminum foil to minimize exposure to light, and were incubated at 18°C for 24 hrs on a

horizontal agitator, followed by incubation at c.a. 22°C for 24-48 hrs on a vertical rotor running at c.a. 15 rpm. Protoplasts were purified by Percoll (Sigma) density gradient centrifugation. Briefly, 70% (v/v) Percoll, 3% (w/v) NaCl solution was ultracentrifuged for 30 min at 30,000 x g to establish the gradient. Then, the cell sample containing the protoplasts was loaded onto the gradient and centrifuged for 10 min at 1,000 x g. Fractions were drawn off of the gradient and analyzed for protoplast content. Typically, a starting sample containing 20-50% protoplasts, with 100% damaged cells (e.g. partially or fully opened) was run through the gradient yielding a relatively pure protoplast fraction determined by brightfield and fluorescent microscopy.

***Method 2*:** This method is similar in concept to the first, but employs a novel 3-step gradient made from the solutions described in table 2.1.

Table 2.1: Composition of the 3-Step Gradient

	Solution B (Wash)	Solution C	Solution D
Monomeric sugar:	500 mM Mannitol	--	100 mM Mannitol
Dimeric sugar:	--	500 mM Sucrose	400 mM Sucrose
Osmotic stabilizer:	300 mM NaCl	300 mM NaCl	300 mM NaCl
pH Buffer:	10 mM Tris.Cl pH 8.0	10 mM Tris.Cl pH 8.0	10 mM Tris.Cl pH 8.0

Cells of *N. alba* strain cc3 were cultured to high density in 1.5 L of ASWT medium at 31°C with aeration and stirring in moderate lighting. Cultured cells were harvested by collection in 250 mL centrifuge buckets and pelleted by centrifugation at 3,000 x g for 15 min. The six pellets were collected and consolidated into 500 mL of L+3% medium prepared in sterile square

polystyrene media bottles with screw caps. The diatoms were then incubated, lid slightly open, at c.a. 22°C with vigorous stirring (forming a vortex) for 24-72 hrs, depending on rate of protoplast formation. The cells were then harvested by collection in 250 mL buckets and centrifugation at 3,000 x g for 10 min, aliquoted into 15 mL Falcon Tubes (BD Biosciences, San Jose, CA) and centrifuged for an additional 5 min at 3,000 x g. Supernatant was decanted, and cell pellet resuspended in 1 mL "solution C." "Solution C" was then overlayed with 125 µL each "Solution D" and "Solution B," in sequence. The gradient was centrifuged at c.a. 300 x g for 10 min. The top 600-800 µL was transferred by pipet to a 1.7 mL microcentrifuge tube and one volume of "Solution B" was added to wash out additional waste. The microcentrifuge tube containing the gradient fraction diluted in "Solution B" was centrifuged in a horizontal rotor at 300 x g for 5 min, after which the supernatant was removed, leaving a pellet containing the protoplasts. Protoplasts could be resuspended in desired medium for regeneration of the cell wall. From this procedure, typically 2-5 x 10^5 pure protoplasts could be recovered from 1.5 L of cultured *N. alba* cells.

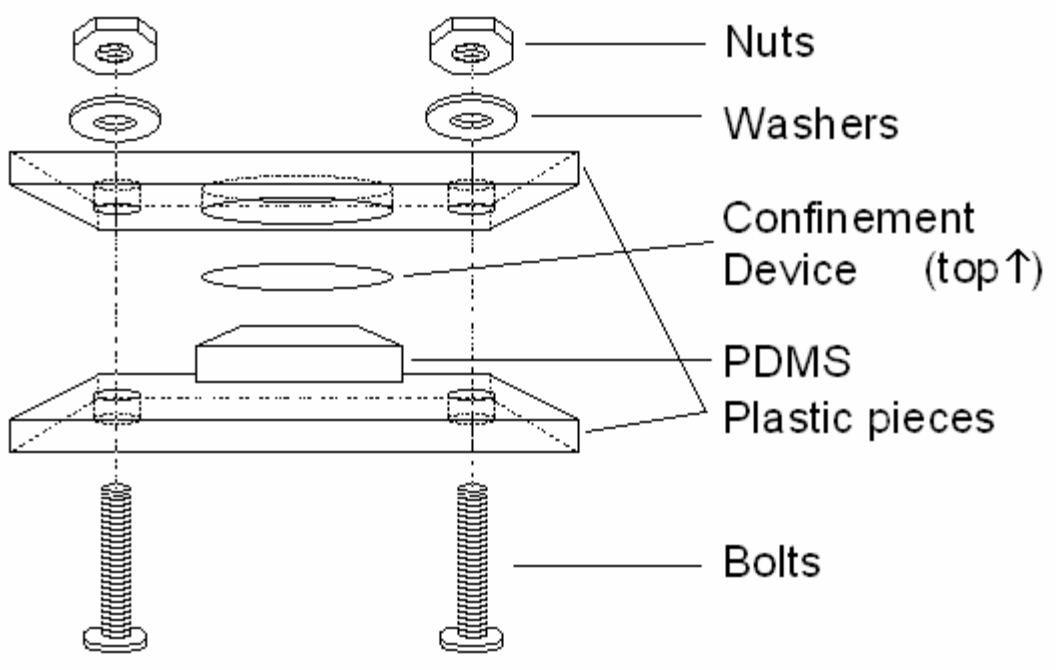
2.3.3 Cell Confinement

In some cases, protoplasts were confined to geometric containers while regenerating valves. Protoplasts prepared using "Method 1" (above) were resuspended in ASWT medium. The 'confinement device' graciously provided

to our laboratory by a collaborator, Dr. Derek Hansford of Ohio State University, comprised a small round filter etched with parallel rows of hexagonal chambers of micro-scale proportions arrayed in three unique regions according to size: 15, 20, and 25 μm which served as the bottom-half of a mold for the nascent valves. Each chamber had nano-scale holes at the bottom to allow medium (but not cells) to pass through. The confinement device was placed chamber-side up on a vacuum filter placed in an Erlenmeyer flask with an arm connected to a hand-pump vacuum. The filter was clamped between the vacuum filter and cylindrical glass housing tube through which the sample of protoplasts was applied by pipet as evenly as possible across the surface of the confinement filter. The protoplasts were forced into the chambers of the confinement device in a laminar flow hood under constant vacuum of 15-20 psi with a variable time of 15-60 mins required to pass the sample through the device. To prevent drying of the sample, and cell death, fresh ASWT media was periodically applied to the top of the device during filtration to any dry regions on the surface. Once all medium had apparently passed through, the confinement device was either directly prepared for SEM, in the case of one experiment; or was otherwise transferred to the “clamping jig” (scheme 2.1).

The device was placed with the open (top) side of the microchambers facing a polydimethylsiloxane (PDMS) stop which served as the top-half of each micro-mold by sealing the cells into the chambers. These two halves of

the mold were sandwiched between two plastic pieces joined by screws and bolts which were tightened to ensure that the confinement device remained securely pressed to the PDMS stopper throughout subsequent incubation in ASWT. The plastic part which abutted the filter was fashioned with a circular



Scheme 2.1: Clamping jig to hold confinement device

hole only slightly lesser in diameter to the confinement device, which fitted around the circumference of the device, holding it in place, while simultaneously allowing passage of liquid media through the microchamber nanopores so that the cells would have a fresh supply of ASWT throughout regeneration. The entire confinement device had to be sterilized with 95% (v/v) ethanol prior to use. After assembly of the clamping jig in a laminar flow

hood, the entire assembly, containing protoplasts, was submerged in a sterile glass baking pan containing liquid ASWT medium with either 2-4-pyridyl-5-{[4-(2-dimethylaminoethylamino-carbomoyl)methoxyl-phenyl]}oxazole (PDMPO) (Molecular Probes, Carlsbad, CA) 1:10,000 part (e.g. 100 nM) in medium, or R123 dye, 1:1000 part in medium; covered with sterile aluminum foil; and left to incubate for 12-24 hrs. Following incubation, the confinement device was removed from the assembly using sterile tweezers and cells were either removed from the chambers by repeated rinses of ASWT using a pipet to mechanically wash the cells out into a 15 mL Falcon tube for collection and fluorescent microscopy, or left within the filter in preparation for SEM.

2.3.4 Microscopy

2.3.4.1 DIC Microscopy to Measure Protoplast Size

Protoplasts purified by “Method 1” (section 2.3.2) were viewed under 40x magnification using an Axioskop epifluorescence microscope (Zeiss, Oberkochen, Germany) operating in DIC mode. A stage micrometer was used to calibrate dimensions, and 48 separate cells were measured to determine an average size for the protoplasts. This served as a control value in future observations to ensure that objects viewed were, in fact, protoplasts.

2.3.4.2 Flourescent Microscopy for Viewing of PDMPO and R123 dye

Incorporation of silica into nascent valves was observed for protoplasts after 24 hrs regeneration in ASWT medium containing 1:10,000 part PDMPO. Live cells were mounted on slides and viewed with an Axioskop epifluorescence microscope with 40x non-immersion objective operating in UV mode, photographed with a color camera attachment, and captured in tagged image file format (i.e. .tiff) for future observation. For viewing of regeneration in confined cells, the cells were grown for 24 hrs in the confinement device and clamping jig described in section 2.3.3 immersed in liquid ASWT medium having either 1:10,000 volume PDMPO, or 1:1,000 part R123 dye. Cells were rinsed from the confinement device as described, and mounted for viewing.

2.3.4.3 SEM of Confined Protoplasts

Confined protoplasts (Section 2.3.3) and whole-cell 'controls' were prepared for SEM. Some whole cells of *N. alba* strain cc3 were prepared for SEM by acid cleaning. Briefly, samples were pelleted and washed once in water, and the cell pellets were frozen at –80°C until further processing. Organic material was removed by first disbursing the cell pellets in 50 µL water, then adding 400 µL concentrated sulfuric acid, and placing the microcentrifuge tubes into a boiling water bath for 10 min. The tubes were removed, allowed to cool, then a small amount (c.a. 20 mg) of solid KNO_3 was added and gently mixed, and the tubes placed back in the boiling water bath

for 10 min. After boiling, the tubes were removed, allowed to cool, two volumes of water added, and tubes were then centrifuged at 10,000 x g for 10 min. The pellets were then washed twice with water, and resuspended in 1 ml of 95% (v/v) ethanol and were stored at –20°C until use.

For confined cells, the confinement filter containing the newly filtered, or regenerating cells, was critical point dried and sputter coated (Au/Pd). In separate experiments, aliquots containing approximately 2 x 10^6 whole cells of *N. alba* of heterogeneous morphology, and the same number of cells of of *N. alba* strain cc3, were filtered onto separate Whatman Cyclopore 1 µm PC10 membranes which were also critical point dried and sputter coated as described. All samples were subsequently observed in an FEI Quanta 600 SEM at the Scripps Institution of Oceanography Unified Laboratory Facility.

2.4 RESULTS

2.4.1 SEM of *N. alba* Reveals Morphologies and Fine Structure of the Frustule

Nitzchia alba of heterogeneous morphology were grown in liquid ASWT medium and prepared for SEM in order to observe the fine structure of the cell wall. For these images, the cells were prepared by fixation in formaldehyde and stored at 4°C until use. Fixed cells were prepared for microscopy by the standard method of sputter coating, and viewed with an FEI Quanta 600 SEM. Detailed views of the cell interior are pictured in figure 2.1. Strikingly, the

interior views reveal a series of amorphous structures lining the raphe fissure.

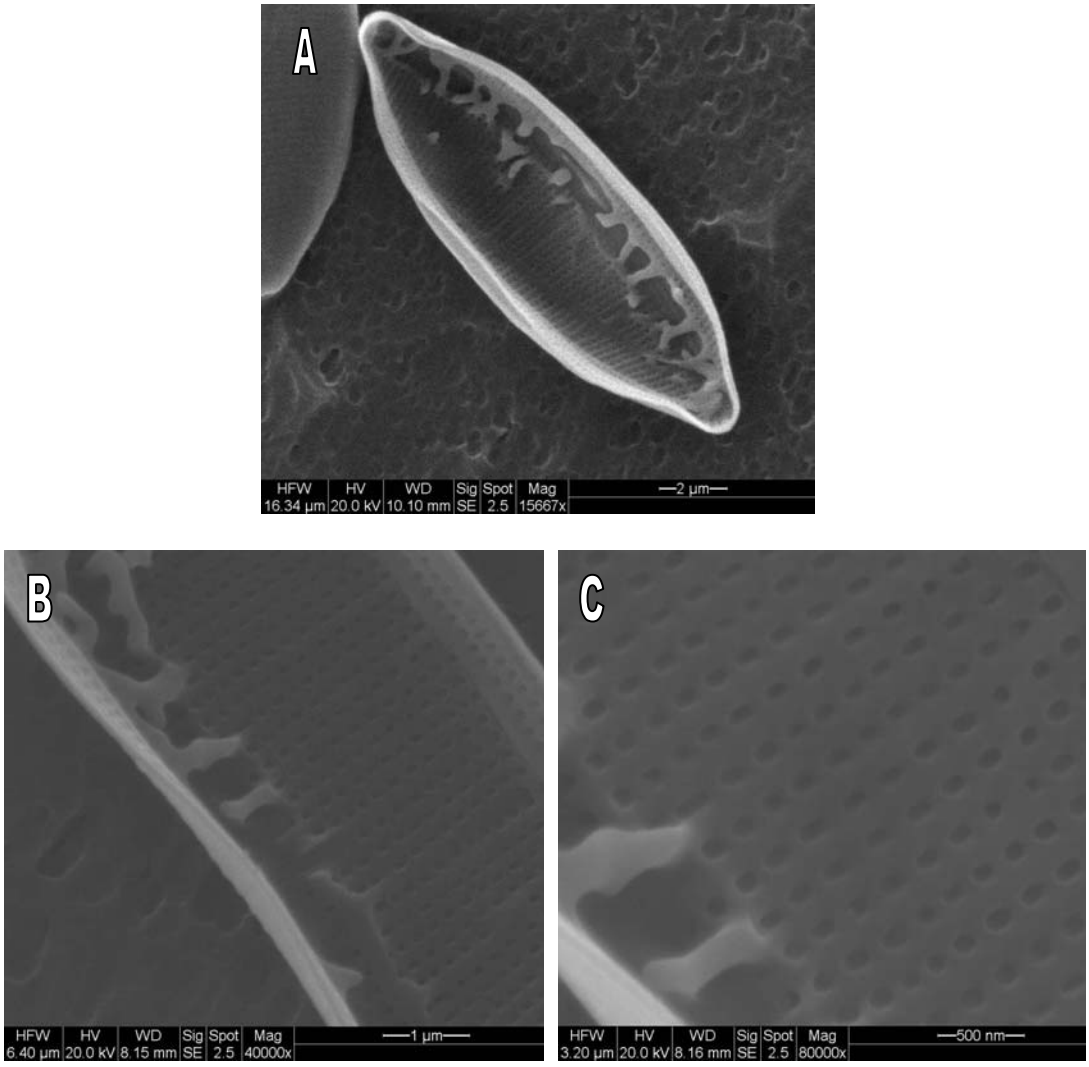


Figure 2.1: SEM showing fine structure of cultured *N. alba*. A. Pervalvar axial view of the interior of the frustule. The amorphous structures along the length of the valve line the interior of the raphe fissure. B. Detail view of the amorphous structures lining the raphe fissure. Areolea are seen in rows to the right of the fissure. C. Detail view showing the precise ordering of areolea in parallel striae which follow the pervalvar axis around the circumference of the cell

The raphe fissure is involved in cellular gliding motility in pennate species through secretion of mucilage to the cellular exterior (Wetherbee et al., 1998;

Hoagland et al., 1993; reviewed in: Edgar and Pickett-Heaps, 1984). Mucilage is an extrapolymeric substance (EPS) excreted by diatoms, which has roles in both binding and motility.

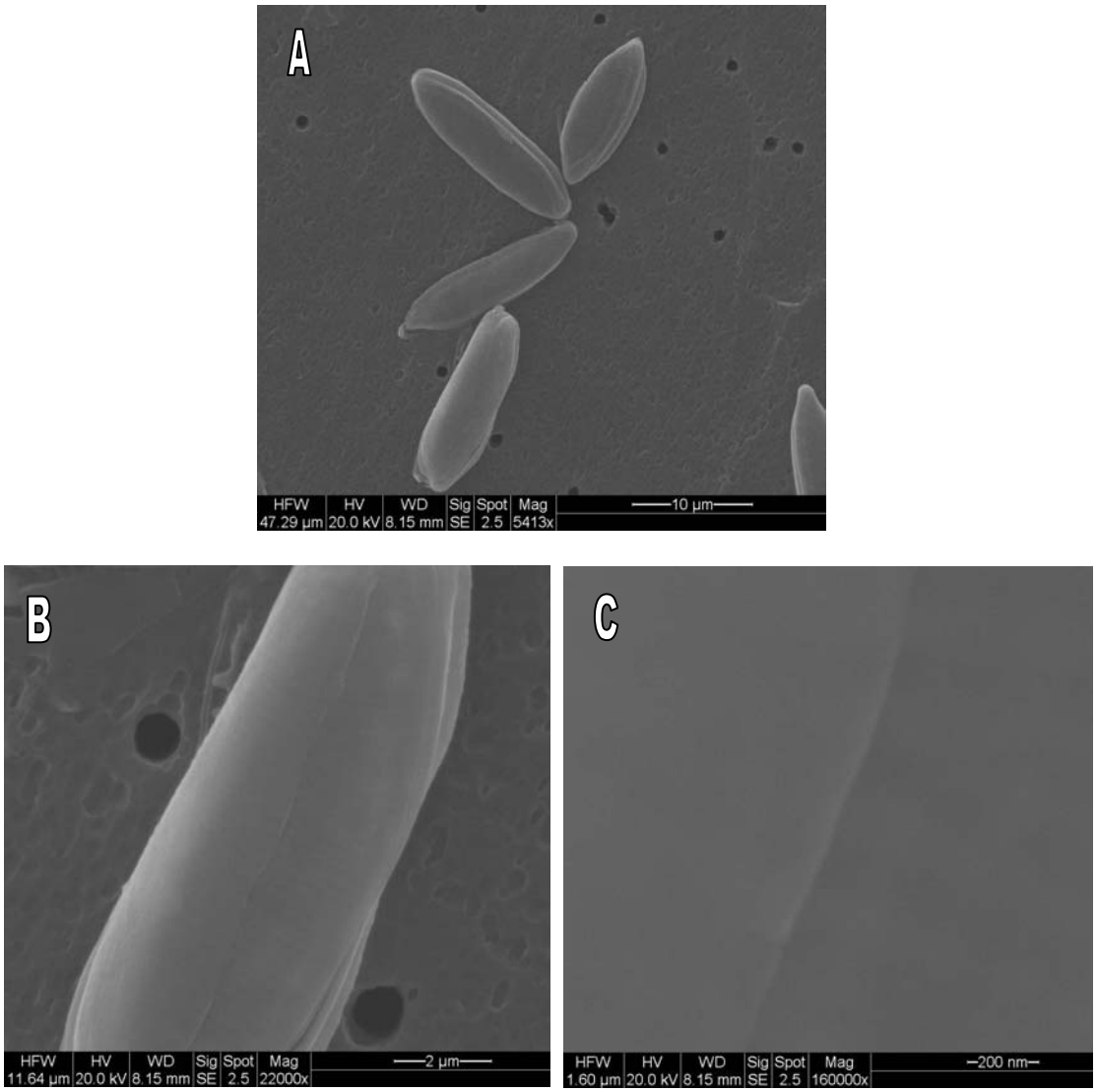


Figure 2.2: SEM of smooth-frustuled *N. alba* from heterogeneous culture. A. Contextual view showing a grouping of smooth-walled cells. Size and morphological variance are apparent among the cells pictured. B. Pervalvar axial view showing exterior of a smooth-walled frustule. Striae can be seen through the translucent silica wall running in the direction of the pervalvar axis; following the circumference of the cell. C. Detail view of the smooth-walled frustule showing a seam in the silica covering.

Also striking to see is the regularity and clarity of the areolea which line the cell wall in rows, called striae, spaced at precise intervals. The rows parallel the pervalvar (short) axis of the cell; following the circumference of the frustule. The openings are clearly defined on the interior face of the cell. These structures act as channels through which nutrients, water, and waste are exchanged with the environment surrounding the cell. Pickett-Heaps et al. (1990) have described these channels as possessing a flap in the interior which helps to regulate the passage of materials into and out of the cell. Interestingly, the openings seems somewhat uniform between interior, and exterior views (figure 2.2) seen through the smooth silica covering of the cell wall. These types of areolae, passing through the cell as a single unit are known as poroid aerolae. Figure 2.3 shows a cell with a layer of organic material surrounding the frustule. This coating is not related to the thin silica layer seen to cover the cells in figure 2.2.

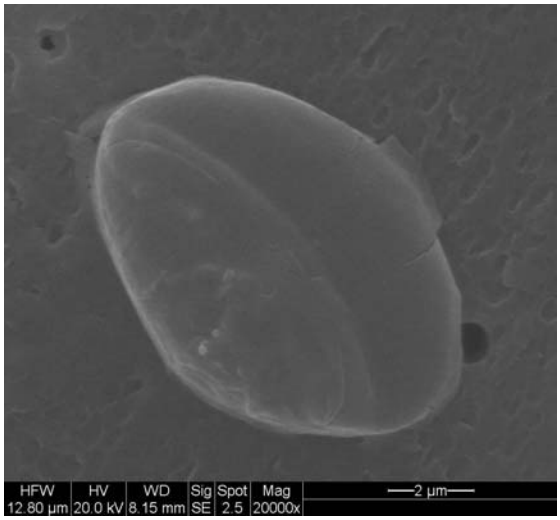


Figure 2.3: SEM of *N. alba* displaying organic material surrounding the frustule.

Taken together, these images present an interesting baseline from which to conduct SEM studies in *N. alba*. The cells pictured here were acid treated prior to microscopy so that organic material was degraded, which is why no intracellular material is visible in the samples. Thus, the heterogeneous culture, when viewed with the SEM, was 'pure' in frustules; cellular debris and vesicular parts including plastids, vacuoles, and partial cells, were absent.

2.4.2 SEM of *N. alba* strain cc3 Reveals Unique Morphology

Cells of *Nitzschia alba* strain cc3 were cultured in liquid ASWT medium and prepared for SEM by acid cleaning of the frustules. Cleaned frustules were primed for SEM using the standard sputter-coating method. Compared to the cells pictured throughout section 2.4.1, cells pictured in figure 2.4 lack a thin silica layer over the frustule. The silica layer is formed last in silicification, and can be absent in silicon-limiting conditions. In these images, the limiting by silicon is evidenced by the lack of complete bridging structures. The method of acid cleaning used here was gentler than those that have been used to *dissolve* the frustule in order to identify associated lipids and proteins. In those studies, stronger hydrofluoric acid or ammonium fluoride were used (Kröger et al., 2002; 2000; 1999). The poroid areolea are clearly visible in cleaned frustules, having a clean bore clear through the frustule; no secondary structures are observed.

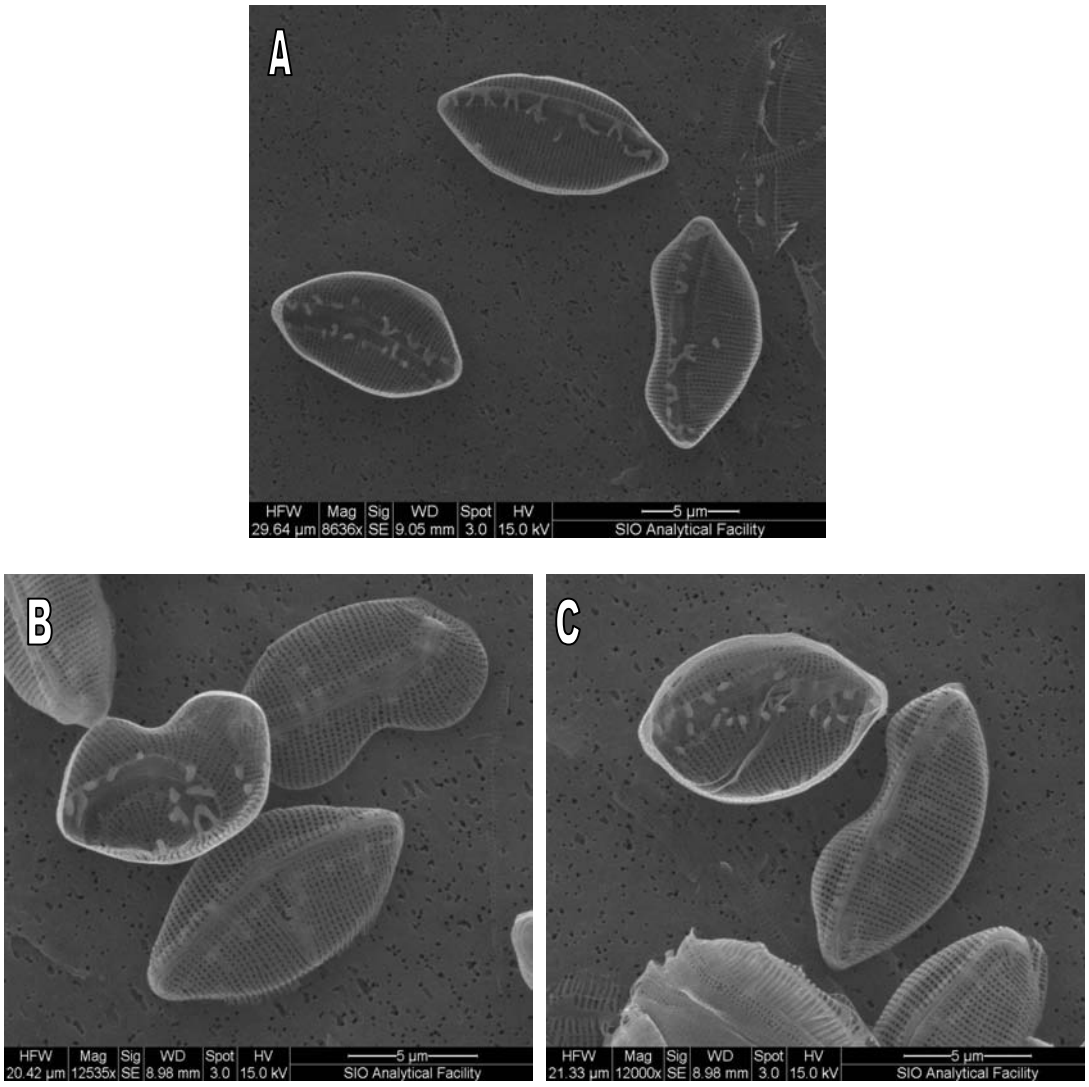


Figure 2.4: SEM of *N. alba* strain cc3. Acid cleaned cells shown in contextual view. A. Pervalvar view of the interior of three cc3 frustules. Note again the presence of the amorphous structures along the raphe fissure. Bridging is evident in the uppermost valve. Striae are clearly visible as parallel rows. B. Malformed raphe fissure which veers from the midline of the cell, turning downward ninety degrees to run parallel to the pervalvar axis of the cell. Note the variance in the striae which do not follow the pervalvar axis throughout, but instead seem to radiate centrifugally from the curve of the raphe fissure. C. Interior view of a cell showing abnormal morphology; note the absence of the characteristic indentation in the valve which is typical of the 'peanut' morphology of this strain. Also, a pair of ribbon-like structures line the interior of the frustule, but do not affect the arrangement of aerolea as the raphe fissure does in (B).

Strikingly, strain cc3 not only exhibits decreased cell size, but shape is altered in this strain, which has a much more pronounced mid-pervalvar

indentation when compared to the heterologous culture. This 'peanut' morphology is characteristic of this strain. Strain cc3 was originally isolated as a 'compact colony' on an ASWT agar plate, and was chosen because of the desirable trait of forming close-packed; rather than diffuse colonies. This trait is ideal for transformation applications since selection is hindered in motile strains, which fail to form clonal groups in 0.8% (w/v) agar medium. The cell pictured in figure 2.4 (B) has a malformed raphe. Other cells pictured have bent raphe fissures (B; lowermost frustule exterior), roundness (A), or aberrations in interior structure (C).

2.4.3 Culturing Conditions Affect Protoplast Formation

2.4.3.1 Temperature Effects on Protoplast Formation

Initially, cultures of *N. alba* were grown under standard conditions of 18°C with stirring and aeration. However, later observations suggested that growth at c.a. 22°C without aeration was conducive to protoplast formation in cultures of *N. alba* growing in ASWT (data not shown). Experiments were carried out to determine whether temperature of the growth medium could affect protoplast formation in *N. alba* strain dc2 cells (table 2.2). Cultures were grown at 18°C and c.a. 22°C concurrently; whole cells and protoplasts were counted with a heamocytometer under 40x magnification. Protoplasts were distinguishable in the brightfield as translucent spheres with visible organelles;

whole cells were either pennate, or opaque spheres when viewed end-on (i.e. down the apical axis).

Table 2.2: Comparison of Growth of *N. alba* dc2 Under Different Temperature Conditions†

	18°C			22°C		
Days	Number of Cells	Number of Protoplasts	% proto-plasts	Number of cells	Number of Protoplasts	% proto-plasts
3	$1.63 \times 10^5 \pm 0.36 \times 10^5$ ‡	$5.00 \times 10^3 \pm 0$	3.08	$2.20 \times 10^5 \pm 0.58 \times 10^5$	0	0
4	$5.50 \times 10^5 \pm 0.57 \times 10^5$	$1.50 \times 10^4 \pm 0.09 \times 10^4$	2.73	$2.86 \times 10^5 \pm 0.32 \times 10^5$	$2.63 \times 10^4 \pm 0.17 \times 10^4$	9.17
5	$5.95 \times 10^5 \pm 0.72 \times 10^5$	$3.50 \times 10^4 \pm 3.0 \times 10^4$	5.88	$6.45 \times 10^5 \pm 1.0 \times 10^5$	$2.50 \times 10^4 \pm 1.3 \times 10^4$	3.88

† Data from a single experiment; values represent averages of a minimum of four counts
‡ Standard deviation of each cell count

The data suggest that there is no great difference in either overall growth of each culture, nor in number of protoplasts present on successive days. Although, the culture grown at c.a. 22°C does perhaps show more overall growth by the fifth day.

2.4.3.2 Growth Medium Effects on Protoplast Formation

L+3% medium, as described by Dr. Barbara Hemmingsen (1971), was successful early on in generating protoplasts from *N. alba*. However, various growth media were experimented with as alternatives for protoplast induction, with varying degrees of success. The first protoplasts were observed in experiments using “N. alba Medium” lacking B12/biotin supplements. However, the conditions of growth in that experiment were atypical, and subsequent comparison with protoplasting medium showed that the N. alba

medium without biotin was relatively weak in protoplast induction compared to L+3% Medium (table 2.3).

Table 2.3: Comparison of Protoplast Yield from *N. alba* in Special Growth Media vs. Protoplasting Media

Medium	*N. alba* dc2	*N. alba* dc1a	*N. alba* cc3
N. alba (-)*	Good growth (no protoplasts)	Good growth	Good growth
L+3%	Approx. 10% protoplasts	Fractured cells only	None observed

*medium lacking biotin and B12 supplements

In later experiments, the possibility of preserving more protoplasts by adding citrate buffer to balance the pH of the L+3% Medium was tested, but showed that little was gained by the buffering since both buffered, and non-buffered control cultures contained “low yield of protoplasts,” a “high number of fractioned cells,” “bits of protoplasts,” and “good overall yield of cells.” Hemmingsen (1971) also noted that pH seemed to have little effect on protoplast yield outside of a narrow optimum range.

2.4.4 Density Gradients for Purification of Protoplasts

2.4.4.1 Sucrose Gradient Centrifugation

Prior to the development of the Percoll gradient for purification of protoplasts, a 5-step sucrose gradient was used to separate protoplasts from cellular debris and whole cells. *N. alba* were grown with R123 dye to aid in distinguishing protoplasts from silica frustules and resuspended in L+3% Medium for protoplasting. The protoplast-containing fraction was pelleted and

loaded into the top of the sucrose gradient, made isotonic with 3% (w/v) NaCl, and centrifuged for 1,000 x g for 10 min. Fractions were drawn off in 500 µL increments and observed in brightfield and fluorescent microscopy, and the protoplasts were separated into the 5% (v/v) fraction.

2.4.4.2 Isotonic 70% Percoll Gradient

In order to achieve greater purity in protoplast separation, cell samples grown with R123 dye were prepared and loaded into 70% (v/v) percoll gradients with 3% (w/v) NaCl. Fractions drawn off of the gradients in 250 µL increments were observed with brightfield and fluorescent microscopy. Protoplasts were observed in the second and third fractions drawn from the top of the column.

2.4.4.3 Better Purity and Yield with a Novel 3-Step Gradient

Greater yield was achieved using a 3-step flotation gradient designed to separate cells between fractions of monomeric and dimeric sugars supplemented with buffer and salts. Using this gradient, separation of ½ of protoplasts generated from a 1.5 L starting culture of *N. alba* strain cc3 (condensed to 500 mL for protoplasting, and condensed to 1 mL prior to density gradient centrifugation) was possible on a single column in a 15 mL Falcon tube; the percoll gradient fouled when such dense samples were applied to the top of the gradient. However, the 3-step gradient also failed to

achieve good separation when an entire 1.5 L starting batch was applied to a single column (data not shown).

2.4.5 Average Size of Protoplasts Determined from DIC Images

Measurement of average protoplast size was achieved by imaging of a culture of *N. alba* strain dc2 using a Zeiss Axioskop epifluorescence microscope in DIC mode at 40x magnification (figure 2.5). 16 separate

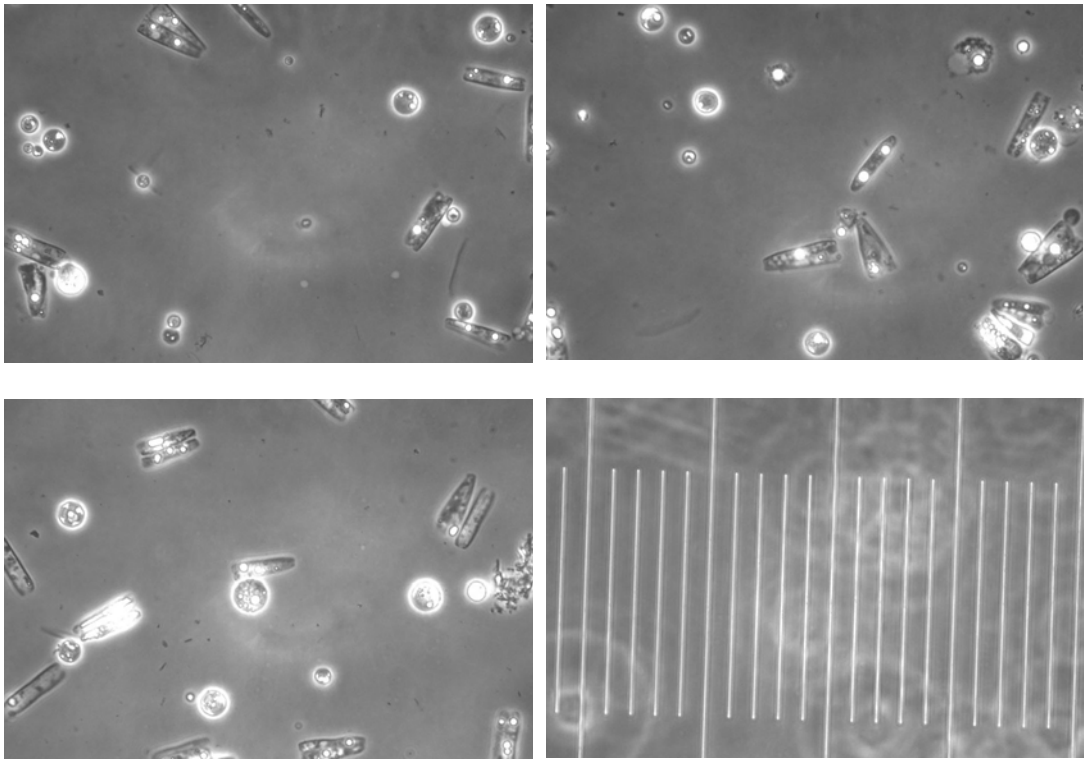


Figure 2.5: DIC microscopy; 40x mag. of live protoplasts purified in 70% percoll gradient. The contextual images provide an accurate representation of the purity routinely achieved using this method, with whole cells nevertheless present in the enriched protoplast fraction. Protoplasts are clearly seen as bright spheres. All images taken were from the same preparation; the lower right image shows the stage micrometer

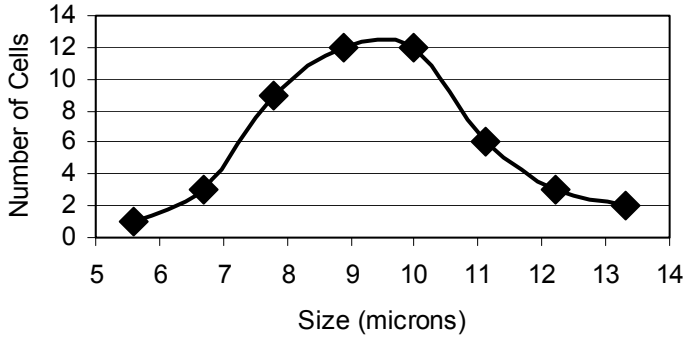
images, having an average of 5 protoplasts per field, were captured and stored in tagged image file format (.tiff), along with one image taken of a stage

micrometer with 10 µm intervals which was used to measure cells from other images. From this data, the average protoplast diameter was calculated to be c.a. 9.3 µm (table 2.4).

Table 2.4: Size Measurents of *N. alba* Protoplasts

Size (µm)	No. of cells
5.6	1
6.7	3
7.8	9
8.9	12
10	12
11.1	6
12.2	3
13.3	2

Graph 2.1: Size Distribution of *N. alba* Protoplasts



This measured diameter is in good agreement with the size of the intact cells which were also observed in the captured images. From brief examination of the images, intact cells appear to measure 20 µm in length (i.e. along the apical axis), and 5 µm in width (i.e. along the pervalvar axis). By way of crude estimation, the cell is assumed to be a cylinder of volume $\pi r^2 \cdot h$ where r=5/2 µm, and h=20 µm. This gives an estimated volume of 392.7 μm^3. Assuming a perfect sphere for the protoplasts, volume is given by $4/3\pi r^3$, where r=5 µm. Calculating yields an average volume of 523.6 μm^3 per protoplast. Thus, the calculated volume for the protoplasts is greater than the overestimated volume of the intact cell. This data suggests that the protoplasts measured here represent the entire protoplast of the cell; not just a

part. The vesicles observed in culture are therefore likely remnants of deceased protoplasts; not portions of viable ones. Hemmingsen (1971) observed similar results in microscopic observations, but concluded that the variability in protoplast diameter was due to splitting of many of the protoplasts, and that some of these cells did not represent viable protoplasts.

2.4.6 SEM shows Protoplasts in Microchambers of the Confinement Device

Nitzschia alba protoplasts were prepared and filtered into microchambers on the surface of a custom designed filter. Each chamber is of defined dimensions and hexagonal shape. Figure 2.6 depicts a typical microchamber. The hexagonal shape has been slightly distorted in the

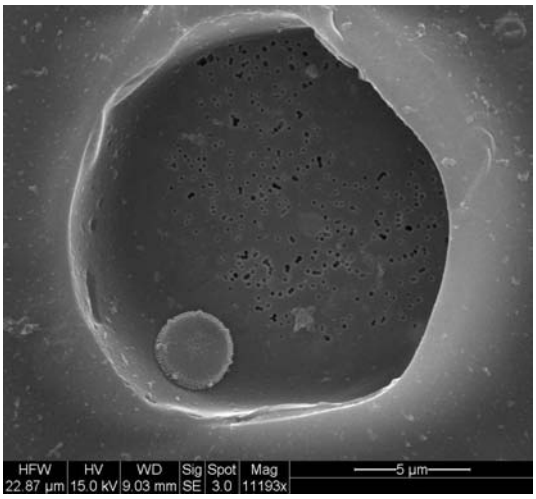


Figure 2.6: SEM depicting a single microchamber. The small cell seen in the bottom of the image is a single cell of *Thalassiosira pseudonana*. Notice the nanopores in the bottom of the microchamber which allow passage of media, but not cells, through the chamber under vacuum

preparation of the filter for SEM. The filter containing protoplasts was immediately prepared for SEM imaging in order to determine whether

protoplasts had been successfully drawn into the microchambers under vacuum. Figure 2.7 pictures a number of protoplasts that were successfully drawn into the microchambers under vacuum. The cells have clearly been ablated, and only the remnants are visible as a ‘pancake’ within the mould of the microchamber. Notable is the absence of any of the small holes which are seen in the bottom of the microchamber in figure 2.6; proving that the bottom of the microchambers are filled with the protoplasts.

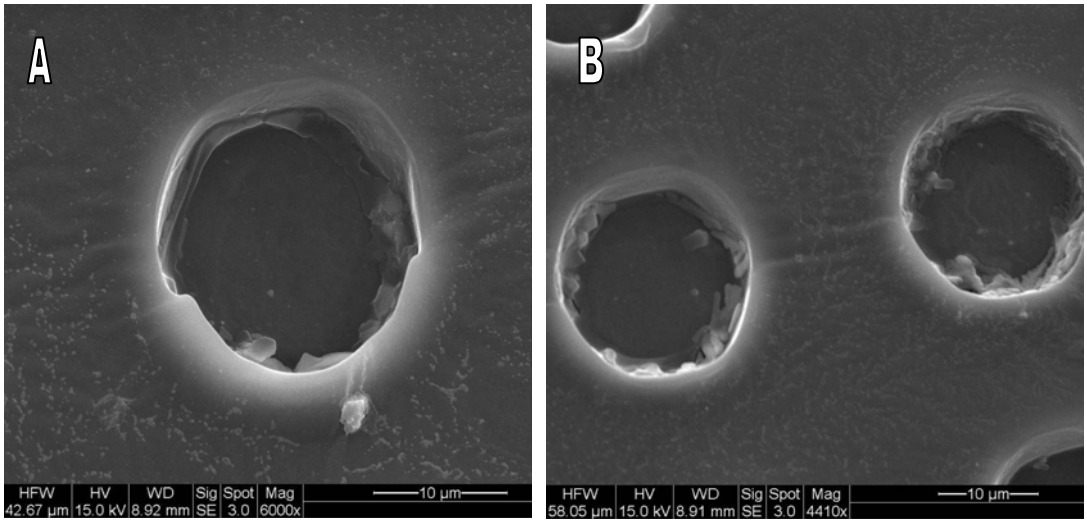


Figure 2.7: SEM of confined protoplasts of *N. alba* strain dc2.

The images clearly show that protoplasts were successfully drawn into microchambers in the confinement device, and fit well within the mould. One may well ask if the ‘pancakes’ seen here are the result of the vacuum treatment that the cells received. Ideally, during vacuum filtration, mechanical stress on the cells would be avoided by the continual addition of liquid medium to the filter membrane so that only liquid medium would be drawn through the

filter, preventing adhesion and suction of the cells to the microchamber bottom. Protoplasts are spherical in shape, and quite stable under mechanical stress; withstanding centrifugation of 10,000 x g, and vigorous vortexing. So, the flattening shown here is unlikely to have resulted from the vacuuming process, and is more likely an effect of the critical point drying procedure used immediately prior to SEM imaging.

2.4.7 Flourescent Imaging Reveals Structure of Nascent Raphe

Protoplasts were allowed to regenerate valves for 24 hrs in the presence of PDMPO. Staining of the cells revealed morphology of the nascent raphe. In dividing cells of *N. alba*, the formation of the raphe preceeds the silicification of the rest of the new valve. Therefore, the shape of the raphe during valve formation can be viewed as a good indicator of the final morphology of the valve. Figure 2.8 depicts fluorescent staining of newly incorporated silica by PDMPO in a non-confined protoplast. The bright, thin

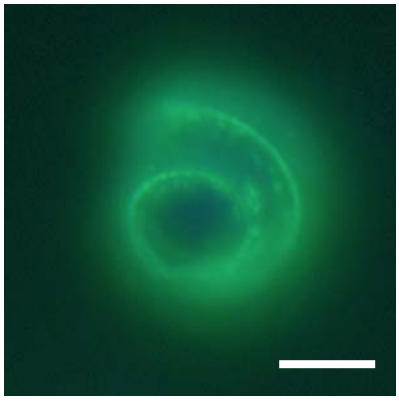


Figure 2.8: Fluorescent microscopy of silica incorporation into the regenerating cell wall of a protoplast of *N. alba*, visualized with PDMPO; 40x mag, scale bar=5 µm

line which traces the shape of a backwards ‘6’ is the raphe of the newly developing cell wall. Small points of fluorescense which seem to follow the inner arc of the raphe probably correspond to the amorphous silica structures observed in SEM of the interior of whole *N. alba* cells.

2.4.8 Microscopic Morphology of Nascent Valve in Confined Protoplasts

2.4.8.1 Fluorescent Images of Show Minute Structural Effects

In contrast to the round shape of the raphe seen in the non-confined cell in figure 2.8, several confined cells observed exhibited clearly defined

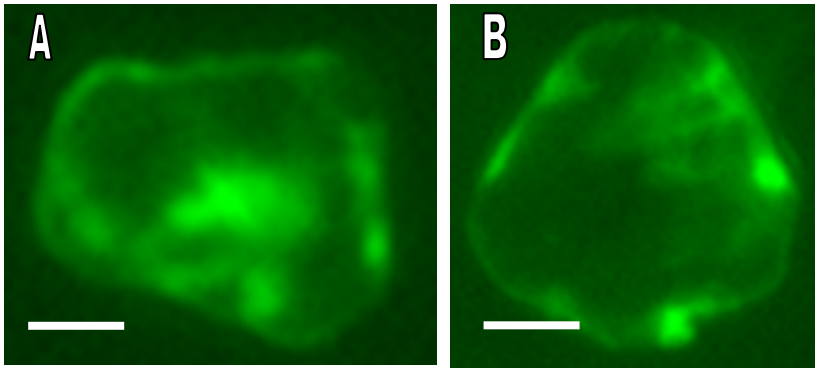


Figure 2.9: Fluorescent microscopic images of confined cells stained with R123 dye; 40x mag.; shown in transapical view. A, B. New cell walls of confined protoplasts showing distinctive angular morphologies. Scale bar=2 μm

boundaries corresponding to the geometry of the microchambers. Figure 2.9 depicts two such cells viewed with fluorescent microscopy, where newly incorporated silica has been stained with R123 dye.

2.4.8.2 SEM Shows Angular Features in Valves of Confined Protoplasts

In order to further investigate the effectiveness of the confinement

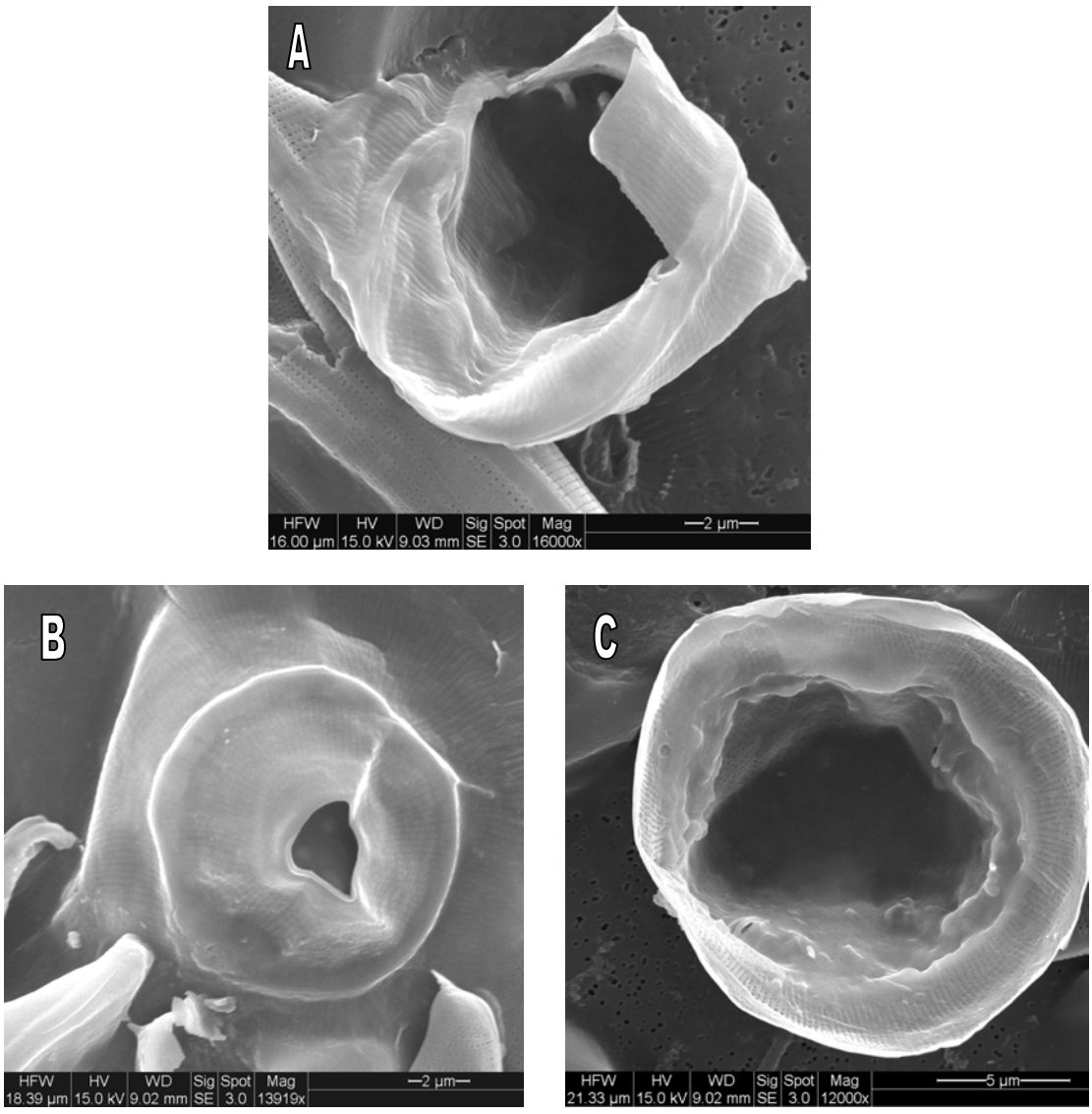


Figure 2.10: SEM of confined protoplasts after 24 hrs of cell wall regeneration in the confinement device suggest a mechanism of cell wall regeneration. A. partially regenerated valve; the striae are visible in the portion of the valve in the upper right quadrant. B. Flattened valve displays the pervalvar view of a nascent cell wall with a clearly defined ring delineating the circumference of the cell. Notice the hole seen in the center of the valve where the wall has not yet silicified. C. Pervalvar view of the interior of a regenerated cell wall. Striae are distinguishable around the periphery of the valve. The bottom of the valve appears to be composed of a more amorphous type of silica, when viewed from the interior, lacking the typical areolar patterning. Notice also the nanopores of the microchamber bottom plate visible in the background of A,C.

procedure in producing novel structures in nascent valves of protoplasts, protoplasts were confined to microchambers and allowed to regenerate their valves for 24 hrs in liquid ASWT medium. Following the incubation period, the confinement device was retrieved, mounted on a stub, and sputter-coated in preparation for SEM. The goal of the project was to obtain a more complete picture of the surface of the confinement device during regeneration, and to view the morphological results of confinement.

Figure 2.10 depicts a number of valve morphological forms resulting from confinement, following 24 hrs of regrowth after purification. The cell pictured in figure 2.10 (B), moreover, has a white circular line which traces the circumference of the new valve, allowing measurement of cell size. The diameter of this ring is approximately 10 μm, which correlates well with the average diameter of the protoplasts viewed with DIC microscopy (section 2.4.5). The valves pictured in figure 2.10 suggest a mechanism whereby the cell wall regenerates around the sphere of the protoplast. In figure 2.10 (A), the striae, typically parallel to the pervalvar axis in *N. alba*, now define the polarity of the new valve. Figure 2.10 (B) depicts the same sort of structure as in (A), however, the new valve has been flattened so that only the upper part remains intact to the viewer. A couple of facts are suggested by the image: 1) the new valve is still somewhat ‘soft’ since the flattened portion appears to have bent to conform to gravitational or other mechanical stress, and 2) the valve, after encircling the protoplast as a ring, appears to grow in a pervalvar

orientation (i.e. upward; toward the viewer in figure 2.10, B) in order to close the ‘ends’ of the valve around the protoplast. This latter conclusion is supported by the small (relative to the rest of the cell) hole pictured in the center of figure 2.10 (B) which appears to denote a point of closure of the nascent valve. Similarly, in figure 2.10 (C) the interior of the cell wall shows complete encirclement of the protoplast by the nascent valve structure, including the formation of an amorphous cell wall to the rear of the valve pictured. This cell wall probably represents the point where the edges of the circularized valve have met and enclosed the protoplast. Presumably, if left to grow longer, the cell pictured in figure 2.10 (C) would similarly enclose the proximally viewed end of the valve as well.

Further evidence for this model of cell wall formation was present in other cells, where the interior of the point of closure of edges of the valve form were observed to meet. The cell depicted in figure 2.11 (A) has clearly visible features on the distal valve interior. The patterning of the striae along the interior of the bottom of the new cell wall reveal the blueprint of cell wall formation in this part of the valve. Apparently, the edges of the valve form have been pushed together at the apex of the cell. An apparently random pattern of twistings and intertwinings can be seen creating projections of the valve into the protoplasmic space. Some of the projections of the valve seem to be crowned with amorphous features which are torn bits of silica created by

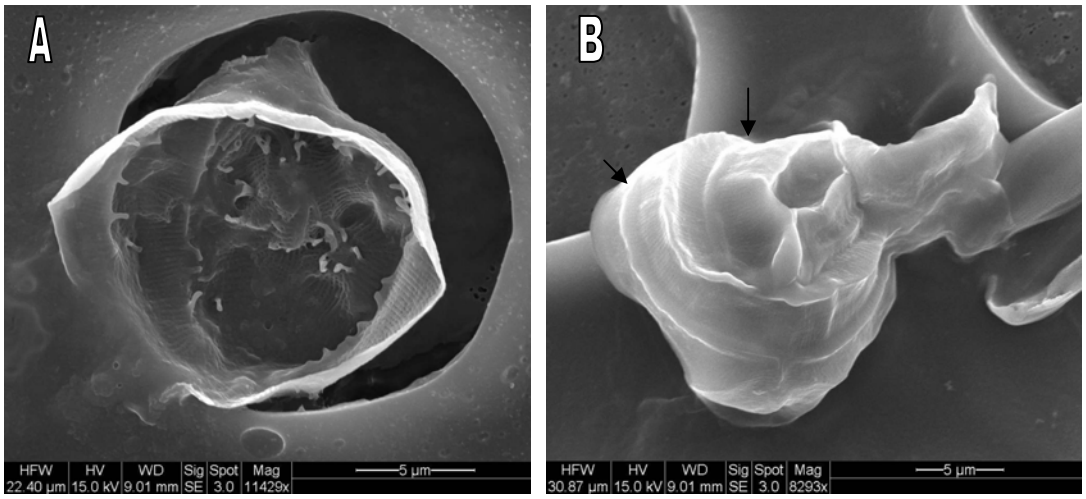


Figure 2.11: SEM of confined protoplasts showing angular morhpolgy. A. Putative closure mechanism for valve formation is depicted. Notice the presence of aerolar rows on all visible surfaces of the nascent valve. B. Completed valve with three corners. Note the presence of two separate raphes (arrows) around the circumference.

mechanical stress. The cell pictured in figure 2.11 (B) shows a fully formed valve with apex enclosed. Two raphes are visible on the valve exterior surface, flanked by perpendicularly oriented striae which run to the boundary of the valve form which seems to comprise the majority of the new cell wall. The two valve forms appear to be joined at an interface creating a projection outward from the cell wall. Also, each valve form seems to be joined to some regions of amorphous silica which comprise the top and bottom of the cell. The cell seems to be constructed much like a 'coil pot' in ceramics; by winding of the linear valve form into a cylindrical shape which is completed by pressing the edges together at either end to form a complete enclosure around the protoplast. The cells pictured in figure 2.11 also provide contrast between the clearly defined aerolae seen in the interior of the cell in (A), and the relatively smooth exterior surface of the cell pictured in (B), serving as a reminder of the

presence of an organic layer surrounding partially completed valves observed here.

2.4.8.3 SEM Reveals Spherical Conformation of Nascent Valve

In one cell observed in the confinement procedure, the developing valve took on a spherical shape, rather than the angular morphology observed in part of the population. The cell wall, nevertheless, seemed to develop by a method analogous to the one outlined in section 2.4.8.2. In this cell, the raphe seemed to twist around the protoplast, rather than forming a coil or loop, in order to completely enclose the cell. In the cell, pictured in figure 2.12, the raphe was seen to curl around the cell in order to enclose the protoplast.

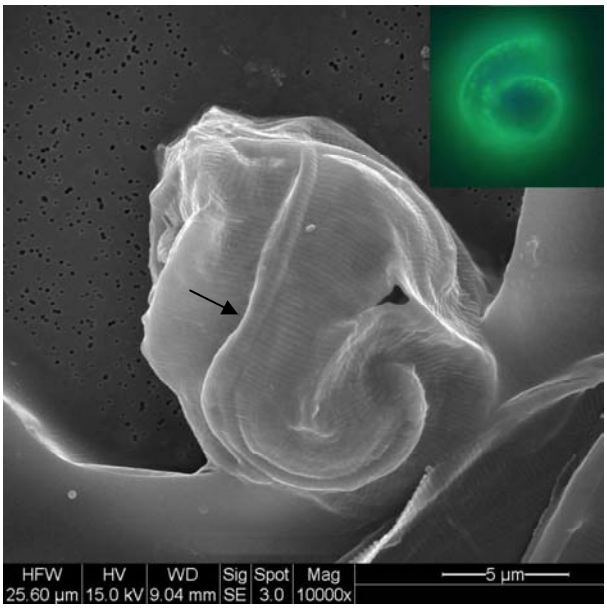


Figure 2.12: Comparison of SEM image and fluorescent image obtained with PDMPO staining (figure 8; reversed for comparison). SEM of regenerating cell wall showing the wrapping of the valve form into a spherical conformation; the raphe is visible in the shape of a hook. Inset: Fluorescence image of a developing cell wall; the raphe is visible as a ‘6’ (arrow)

The valve form here could be compared to an orange peel when removed from the orange, yet still holding the shape of the fruit inside. The raphe is particularly distinguishable on this form, and is reminiscent of the fluorescent image acquired by PDMPO staining pictured in figure 2.12 (inset). It is likely that the cell observed with PDMPO would have a similar structure if viewed with SEM.

Most remarkable, however, was the discovery of a completely regenerated valve clearly viewed from the exterior showing the incorporation of the valve form with a well-formed raphe encircling the circumference of the cell in an equatorial manner, and joining seamlessly with the amorphous portions at the apical end of the valve (figure 2.13).

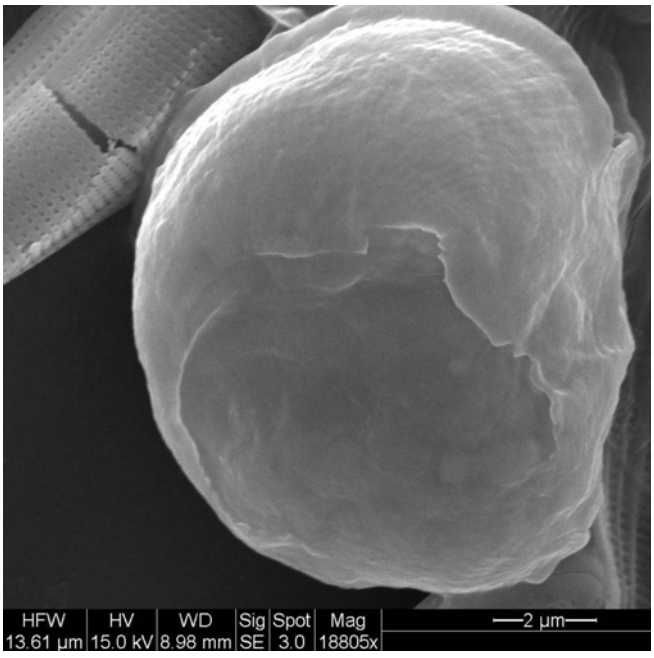


Figure 2.13: SEM of regenerated spherical cell wall showing incorporation of valve-form and amorphous portion completing encapsulation of the protoplast.

2.4.9 PDMPO Staining Reveals Apparent Time-Course of Regeneration

In order to better understand the process of cell wall regeneration in *N. alba*, studies were conducted to observe the restoration of the silica valve by staining with PDMPO, followed by observation with an epifluorescent microscope. Cells of *N. alba* strain dc2 were protoplasted, then allowed to regrow for 24 hrs in liquid ASWT medium with 1:10,000 part PDMPO stain in order to visualize incorporation of silica into the growing valve. Cells were purified in 70% (v/v) percoll gradient by density gradient centrifugation and visualized using a Zeiss Axioskop epifluorescence microscope. A series of images, taken from this time point appear to constitute stages in cell wall development. The pictures captured were merged into a single image, presented as figure 2.14.

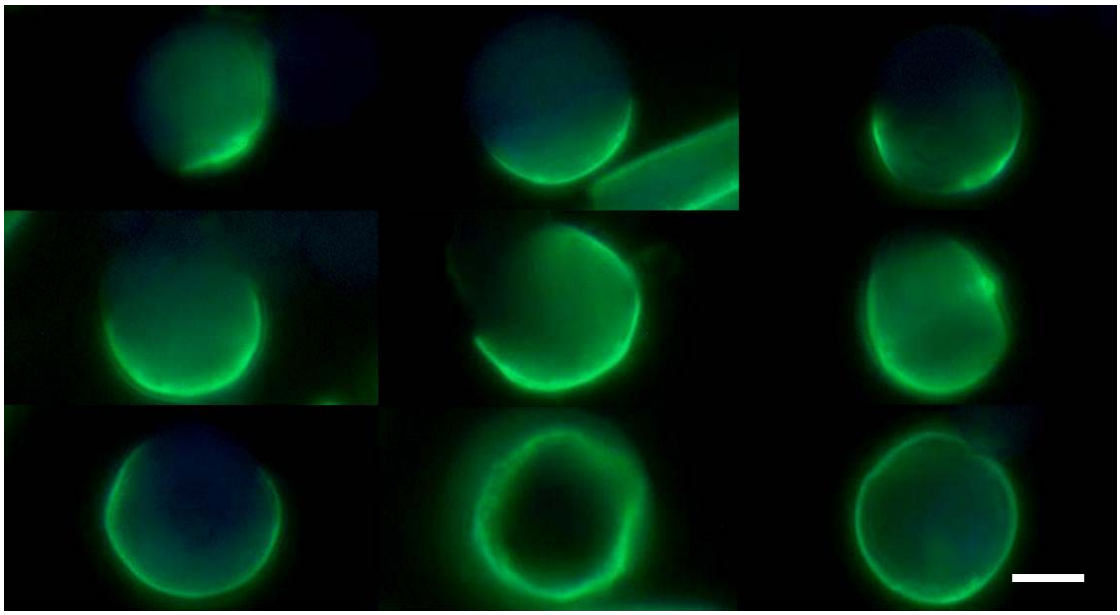


Figure 2.14: Stages in cell wall morphogenesis in the marine apochlorotic diatom *Nitzchia alba*. Scale bar=5 µm; images are sequential, beginning from the upper left-hand corner through the lower right

The images depict the expansion of the fluorescent silica within the SDV as it grows to envelope the whole cell. Beginning with the first appearance of the concentrated silica just below the plasmalemma, the silica expands to engulf the entire protoplast. At various stages in the development of the cell wall, intricate structures can be discerned associated with the silica. SEM studies in this work suggest that these small structures may comprise the amorphous silica structures associated with the interior of the raphe fissure. The blue color of PDMPO in some images is due to circumneutral conditions.

Certainly, these images appear to illustrate expansion of the raphe encompassing the cell. What is unclear from this study is whether the encirclement visualized in figure 2.14 constitutes three dimensional spherical encapsulation of the protoplast, or planar encirclement by the valve-form described in SEM work. The visibility of fluorescence from the plasmalemma would seem to suggest that this is encirclement, rather than encapsulation.

2.5 DISCUSSION

2.5.1 A Novel Method for Purification of Viable Protoplasts from *Nitszchia alba*

The method of protoplast induction starting from liquid suspension cultures of *Nitzschia alba* is based upon work conducted by Hemmingsen (1971). Her Doctoral Dissertation was heavily relied upon in the early stages

of the work presented here. Of particular note in her research was the creation of a method for protoplasting without the use of digestive enzymes, greatly simplifying the ability to maintain protoplast viability throughout the protocol. However, numerous modifications were made to her methods to obtain the protoplasts used in these studies. First, in order to produce viable protoplasts for use in cell wall regeneration studies, changes were made in the makeup of liquid culture medium used for growth. Next, a new method of purification of the protoplasts from broken frustules and other cellular debris was devised in order to produce a more pure culture for microscopic studies.

2.5.1.1 Optimization of Growth Conditions to Increase Protoplast Yield

Hemmingsen described a liquid culture medium with characteristics similar to the ASWT used in these studies, but with citrate buffer instead of glycylglycine (gly-gly), and with glucose as the sole carbon source, rather than tryptone. Thus, Hemmingsen's work used a defined medium, while this work relied on nutrient rich medium for initial cell culturing. Indeed, later studies indicate that the medium used for initial growth of *N. alba* cultures did not have a significant impact on protoplasting efficiency as Hemmingsen postulated, if certain conditions were applied to the cells after transfer to hypotonic medium.

Of all the factors considered in producing protoplasts, mechanical stress seemed to be the most uniformly critical factor in all experiments conducted. No single factor was observed to affect the success of the

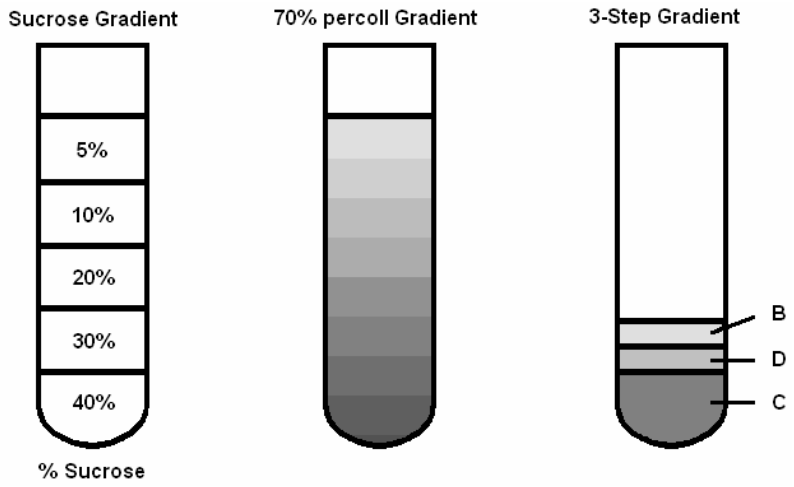
protoplasting incubation in hypotonic medium as much as vigorous stirring. The supposed reason for this is the simple explanation that beating the frustules helped the cells to shed the silica cell wall; perhaps even aided in damaging the organic cell wall in some way. Similar findings were reported by Hemmingsen.

2.5.1.2 Development of Density Gradients for Viable Protoplast Purfication

Microscopic studies were often hampered by contaminants. In some cases, bacterial contaminants created background fluorescence which made identification of regenerating cells impractical. In the same way, the presence of an abundance of whole cells and frustules inhibited the observation of regenerating cells according the 'needle-in-a-haystack' principle. Preliminary studies were attempted on cultures of *N. alba* protoplasts separated from cellular debris in 5-layer sucrose gradients with the composition pictured in scheme 2.2.

Part of the difficulty of separating the protoplasts on these gradients was due to the fact that whole cells of *N. alba* are nearly transparent in culture. Protoplasts of *N. alba* are invisible; and the liquid-liquid boundaries in sucrose gradients are likewise invisible, observed only by slight variations in refraction of each layer. Ordinarily, these would be minor drawbacks, since observing successive fractions should reveal the desired region. However, much of the

cellular debris observed in cultures of protoplasts tended to co-migrate in the density gradients along with the protoplasts, being similar in composition. The



Scheme 2.2: Side-by-side comparison of density gradients for centrifugation used in these studies. The composition of phases C,B, and D in the 3-step gradient are described in section 2.3.2; "Method 2"

sucrose gradient, having only five phases with no specificity for protoplast separation, probably failed to adequately produce a pure fraction of protoplasts, even if one could have be isolated. Ideally, cells would be visible in a band in the gradient. However, being an apochlorotic species, *N. alba* lacks all pigmentation commonly found in other species of diatoms, including *Thalassiosira pseudonana*. Therefore, serious attempts were made early on to induce protoplast formation in *T. pseudonana* (data not shown). When the feasibility of these attempts seemed untenable, alternative methods of protoplast purification from *N. alba* were considered.

Eventually, a method for purification of *N. alba* protoplasts using a percoll density gradient was devised. The gradient showed excellent separation of protoplasts from cellular debris. Although many whole-cells or whole-cell-like particles were visible in DIC microscopy of gradient fractions, the samples were significantly enriched in protoplasts and allowed easy viewing of up to ten protoplasts in a single field of view at 40x magnification. However, early studies using the percoll gradient failed to produce viable protoplasts; cells would quickly burst upon addition to the top of the gradient. The cause was quickly attributed to osmotic stress, and subsequent studies were carried out with an isotonic solution of 3% (w/v) NaCl, and 70% (v/v) percoll (determined as optimum for protoplast separation by successive experiments; data not shown). Although very successful, the percoll stock was prone to contamination which was the impetus for devising a new way to purify protoplasts since the bacterial contaminants significantly hampered confinement studies which were naturally prone to contamination.

A 3-step gradient was created, based on a gradient with similar composition designed to purify protoplasts of higher plants (Robinson, 1987; Barsby et al., 1986; Robinson et al., 1979; Edwards et al., 1978; Huber and Edwards, 1975). Gradient phases were designed so that the composition of the gradient was isotonic to protoplasting medium and buffered with Tris·Cl pH 8.0. The gradient relies on the differential osmolarities of sugar monomers and dimers in solution, rather than on differences in concentration. To

establish the gradient, layers of solutions containing mannose, sucrose, and a mixture of both were used to create a flotation column. This type of gradient has the advantage of avoiding entrapment of large particles migrating through the gradient resulting in overload because the sample begins in the lowest phase; less dense particles rise through the gradient during low-speed centrifugation. In case of overload, large particles still remain at the bottom, although overall yield is reduced. Using this gradient, 750 mL of liquid culture of *N. alba* cells were protoplasted, pelleted and resuspended in 1 mL of the lower fraction of the gradient, and upon centrifugation, yielded approximately 1.0-2.5 x 10^5 purified protoplasts in 200 µL of liquid growth medium after final washing.

2.5.1.3 Protoplast Viability Observed

The production of whole, viable protoplasts from *N. alba* enabled study of nascent valve formation in these cells. Hemmingsen (1971) had characterized the time-course of protoplast formation using transmission-electron microscopy (TEM), but had been unable to observe the regeneration of the cell walls. The studies here used silicic acid tracing dyes for epifluorescence microscopic observations of cell wall reformation. Previous work by Coombs et al. (1968), Lauritis et al. (1968), Li and Volcani (1984), Pickett-Heaps et al. (1990), Gordon and Drum (1994), Kröger et al. (2002; 2001; 2000; 1999), Frigeri et al. (2006) and many other research groups had

produced a wealth of information relating to the morphological, proteomic, metabolic, and genetic aspects of nascent valve formation in diatoms. However, this work is a novel study in *Nitzschia alba* using microscopy to study nascent valve formation from *protoplasts*. Microscopic images of the regenerating cell wall provide intriguing insights into the possible morphological processes of valve formation in *N. alba* protoplasts, and may represent the first steps in research that could add to new discoveries in biosilicification of marine diatoms.

Staining with PDMPO has been previously characterized as a useful method of observing biosilicification since PDMPO was found to be a novel tracer of silicic acid (Shimizu et al., 2001), and was subsequently used in studies of nascent valve formation in *Navicula salinarum*. Significantly, PDMPO was observed to be rapidly sequestered to the silica deposition vesicle (SDV) in this species (Hazelaar et al., 2005). Silicic acid is required for both DNA synthesis (Darley and Volcani, 1969), and valve formation (Lewin, 1955) in diatoms. Indeed, PDMPO localizes to all acidic organelles within the cell, but the bulk of sequestered silicic acid is clearly concentrated within the SDV when viewed with a short exposure time, and saved for generating the new valve of the daughter cells following mitosis. The work presented here demonstrates that PDMPO can be used to trace the deposition of silica within the SDV, presumably after the silicalemma has extended and encircled the entire protoplast.

2.5.2 Confinement Summarized

Immunoisolation chambers for sequestering of blood contaminants, microscopic biosensor arrays composed of compustats, microfabrication, and nanodesign are ideas evocative of science-fiction novels and films which may be out of reach for current technology, but remain the holy-grails of the frontiers in nanotechnology (reviewed in: Parkinson and Gordon, 1999). A recent issue of the Journal of Nanoscience and Nanotechnology was completely devoted to Diatom nanotechnology. Of all features of the diatom, the intricate nano-patterned silica frustule displaying species-specific morphology (Gordon and Drum, 1994; Mann, 1993; Pickett-Heaps et al., 1990) remains the most enigmatic and intriguing structure. Numerous studies have been dedicated to researching aspects of the biomolecular (Hazelaar et al., 2005; Sumper and Kröger, 2004), proteomic (Kröger et al., 2002; 2001; 2000; 1999), and genetic (Frigeri et al., 2006) controls of the silicification process in the hopes of one day harnessing the information to manipulate diatoms for the creation of nano-structures.

At the same time, research in 'green-chemistry;' biomimetic approaches to *in vitro* silicification under relatively gentle reaction conditions have yielded promising results in producing sheets and spheres of silica (Hazelaar et al., 2003). Indeed, Rajesh et al. (2003) succeeded in altering the morphology of silicifying aggregates of tetramethoxysilane (TMOS) *in vitro* through application of shear stress, or introduction of nitrogen gas bubbled through a

solution of 1M TMOS containing the R5 peptide derived from sil1p; a gene encoding the Silaffin-1A proteins which have been implicated in self-assembly of silica aggregates under slightly acidic conditions *in vitro* (e.g. pH 5). Together, these works comprise a promising set of tools for biomimetic silicification. However, diatoms themselves remain the mechanism of choice for *biosilicification* as inexpensive, low-maintenance biofactories for production of nanodevices.

This study presents a novel method for shaping diatom frustules from protoplasts devised independently by Dr. Mark Hildebrand. Protoplasts of *N. alba* were drawn into microchambers on the surface of a custom-designed filter, created by Dr. Derek Hansford, which contained nanopores only in the bottom of microchambers. SEM revealed that the protoplasts had been drawn into the microchambers, but were sadly ablated either during vacuum filtration, or the critical point drying procedure. Numerous studies have prescribed gentler methods of cellular preparation for SEM. For example Rogerson et al. (1986) used a graded serious of acetone dehydrations to prepare “pseudoprotoplasts” of *Coscinodiscus asteromphalus* for SEM. However, for the purposes of this study, the previous methods were untenable because they could destroy the filter membrane (negating the purpose of the observation), or disturb the device, causing cells to come out of the microchambers before viewing. Nonetheless, the protoplast pancakes (or

waffles) were observed within the confines of microchambers in several instances.

In a subsequent experiment, PDMPO was replaced with rhodamine 123 dye. R123, which localizes to all acidic areas in cells, has also been shown to localize to areas of silicic acid in diatoms in proportion to the amount of silica present (Brzezinsky and Conley, 1994). Observation of this sample revealed a number of very distinctly shaped cells with clearly delimitated angular features (i.e. corners), indicating morphological alterations resulting from confinement of the cells. However, no single cell was observed to display the hexagonal shape of the microchamber. A possible explanation is that the PDMS stopper which acted as the microchamber lid, did not perfectly seal the chamber. Indeed, applying the filter to the stopper, while attempting to minimize flexing and stretching of the filter, which could potentially dislodge the protoplasts from the inverted membrane, was a difficult task which typically resulted in warps or ridges in the filter membrane. Furthermore, upon sealing stopper and filter together in the 'clamping jig' and applying pressure by tightening the nuts and bolts, the membrane would often distend, producing stretch lines which could easily raise the filter a significant distance from the stopper at many points. Future work should focus on producing a static membrane, perhaps mounted on a more rigid filter, to aid in the confinement procedure. Nevertheless, the results provide promising evidence that if

properly sealed within the chambers, protoplasts can be induced to take on the morphology of the microchamber.

A third experiment was carried out to confine the cells within the microchambers, then view the resulting morphotypes on the surface of the confinement filter using SEM imaging. No percoll particles were observed in this or previous SEM of filtered protoplasts, indicative of the extent of dilution of the percoll in growth medium used in recovery. Additionally, a number of cells with regenerated cell walls of diverse morphology were observed. Of these, several exhibited the same angular features observed in fluorescent microscopy of the cells, indicative of the effects of the confinement procedure. Also, a number of additional observations were made which were suggestive of a morphological model of the process of cell wall regeneration in the protoplasts.

2.5.3 Morphological Model of Protoplast Valve Regeneration

SEM of confined protoplasts had the consequence of simultaneously providing clues regarding the stages of cell wall regeneration in *N. alba*. Chiappino et al. (1977) have previously produced outstanding evidence in the form of number TEMs showing the morphological process of nascent valve formation in daughter cells of mitotically dividing *N. alba* (Chiappino et al., 1977) and *Navicula pelliculosa* (Chiappino and Volcani, 1977). Their microscopic observations showed that valve formation begins from the SDV,

which may not be found in the center of the pervalvar axis of the cell on all occasions. Following formation of the SDV (or at least the first visible signs of the SDV), which immediately follows mitosis, a ‘central nodule’ is formed in the center of SDV which is observed as an area of high electron density within the silicalemma. From this point, the raphe fissure forms by joining and fusing of the primary central nodule and secondary arms; both of which extend along the apical axis of the cell toward the periphery, defined by the plasmalemma. Expansion of the raphe fissure coincides with expansion of the silicalemma, which encapsulates the new valve until formation is complete. Upon completion of the raphe, transapical ribs grow perpendicularly from the raphe, outward to the limits of the cell wall. Formation of the ribs is followed by the silicification of bridging between the ribs termed costae. Once fully formed, the valve is exocytosed by an unknown mechanism. Presumably, the silicalemma remains associated with the exterior surface of the valve as part of the organic cell wall.

In the confined cells observed with SEM, some appeared to have been shaped in the course of regeneration by the limits of the chamber; these cells are those that displayed abnormal, angular morphology. Other cells displayed raphe fissures which wrapped around the cell in a ‘hook’ or ‘6’ configuration; or else showed completely spherical morphology. These latter specimens are thought to represent examples of cell wall regeneration in the absence of

confinement, and have been used in part as evidence towards a model of morphological development of the nascent valves from protoplasts of *N. alba*.

2.5.3.1 PDMPO Staining Suggests a Time-Course of Regeneration

Prior to studies involving the confinement of protoplasts to chambers, a number of studies were carried out to establish the viability of protoplasts in liquid media following purification in 70% (v/v) percoll. Of these studies, one yielded the unexpected result of producing data that suggested a time-course of protoplast cell wall regeneration. A series of PDMPO stained cells having achieved what appeared to be successive stages of cell wall regeneration, were gathered together for examination. Viewed side-by-side, the images present compelling evidence that in the absence of a frustule to help delimitate the boundaries of the cell, the nascent raphe continues to expand until coming into contact with *itself* on the opposite side of the cell. This intriguing data suggests that mechanical stimulation is essential for signaling turn-around of the primary central nodule for fusion with the secondary arms. Unfortunately, a definitive study of the time-course of regeneration of a single protoplast valve has yet to be accomplished.

One drawback in attempting to study valve morphogenesis in *N. alba* protoplasts is that synchronously growing cultures of protoplasts cannot be achieved by any currently available method. In studies of valve morphogenesis, for example, Chiappino et al. (1977) obtained true time-

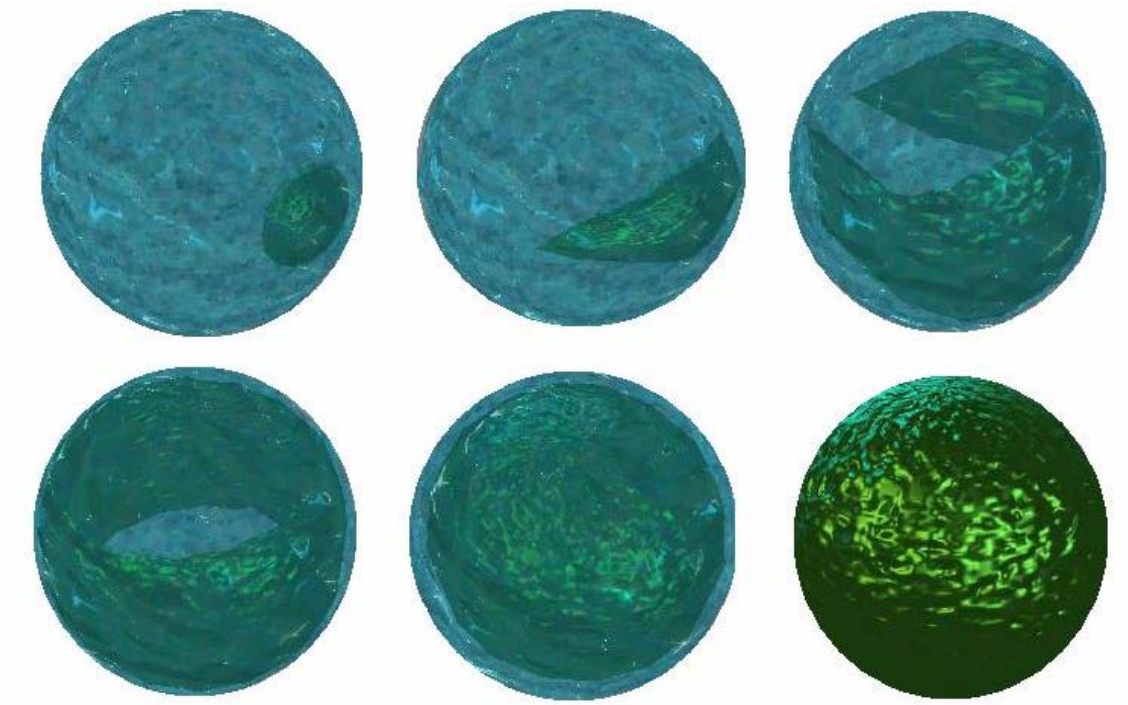
course series of TEMs from synchronously grown cultures of *N. pelliculosa* using alternating light and dark cycles of growth to synchronize the cultures as described by Darley and Volcani (1971). Unfortunately, *N. alba* is a chemoheterotroph, and probably could not be synchronized by this method. However, methods to synchronize cells by adding and subtracting silicic acid from the growth medium at regular intervals have been used to establish synchrony of cultures of *T. pseudonana* (Frigeri et al., 2006; Round et al., 1990). In any case, there is no sure evidence that cells will remain in synchrony after the protoplasting procedure. Perhaps if the incubation in L+3% medium served as one of the cycles of silica repletion, synchrony of *N. alba* protoplasts might be possible. Nevertheless, examination of partially regenerated cell walls have yielded clues to the time-course of nascent valve formation in protoplasts.

2.5.3.2 SEM Data Illustrate a Probable Model of Nascent Valve Formation

A number of *N. alba* protoplasts confined in microchambers failed to conform to the geometry of the wells. Instead, these cells appear to represent partially regenerated valves which might be observed in unconfined cells. Although the data are gathered from relatively few observations of unrepeated experiments, examination of these cells reveal some interesting features. However, further studies will need to be done to determine the exact mechanism of protoplast valve regeneration.

The raphe fissure is an especially prominent feature in the exterior of some cells examined; either encircling the cell in an equatorial manner, or spiraling around the circumference, producing fantastic shapes in the final silicified cell wall. In some cases, the raphe is even seen to spiral upward in a coil-like fashion; layer upon layer. The raphe is always flanked by perpendicular rows of striae. The raphe, flanked by the perpendicular aerolar rows of striae represents the standard native morphology of *N. alba* valves.

The remaining question is what mechanism may follow encirclement of the protoplast by the new valve? SEM data seem to suggest two possible mechanisms: 1) that in the case of whole cell encapsulation, valve encirclement is followed by amorphous silicification of the remaining space by lateral over-expansion of the valve to meet at the apical surfaces of the protoplast, forming a sphere (scheme 2.3), or 2) alternately, the raphe fails to meet itself (scheme 2.4), and continues on a tangential course until reaching the apex, at which point the single raphe arm coils back upon itself, and stimulates turnaround. In either case, once the raphe has formed, ribs extend outwards along the interior surface of the plasmalemma, within the SDV, until encountering resistance, at which point they too cease to expand, signaling subsequent stages of costae development, final silicification, and exocytosis of the complete valve.



Scheme 2.3: Proposed morphological model of nascent valve formation in *N. alba* by equatorial encompassment by valve form followed by lateral expansion and encapsulation and exocytosis

Scheme 2.4: Illustration of a cut-away cell showing a spiraling valve form. Failing to encompass the protoplast, the spiral will continue to the apical poles, eventually forming a "6" or "hook" shape at the apexes

At this point, two caveats should be introduced. First, according to the model of Pickett-Heaps et al. (1990), the periphery of the silicalemma is closely associated with microtubules of the cytoskeleton. Therefore, there could very well be vast differences in the cytoskeletal mechanism involved in formation of the equatorial or spiral shaped valve when compared to the process of forming a flat plate valve in dividing cells. Secondly, in the native cell, valve formation always follows mitosis, unless certain inhibitors are introduced into medium (Cohn et al., 1989; Coombs et al., 1968). The formation of the valve, followed by the expansion to encompass the protoplasm likely involves some genetic switch to prevent mitosis in the regenerating protoplast, unless, of course, the cell is willing to jettison half of all cellular components to survive.

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CHAPTER III

DEVELOPMENT OF NOVEL TRANSFORMATION VECTORS IN THE MARINE DIATOMS *Thalassiosira pseudonana* AND *N. alba*

3.1 ABSTRACT

Cultured cells of *Thalassiosira pseudonana* and *Nitzschia alba* were harvested and used for transformation studies using the biolistic method of transformation. Both species were transformed with the vectors pTpACCNAT, pTpSVACCNAT, and pSVNAT. Putative transformants and non-transformed negative control samples were plated by the pour-plating method in selective media and allowed to grow until micro-colonies appeared in the medium. Micro-colonies, putative transformants, were selected for screening by further selection in liquid selective media before non-selective growth for DNA extraction. DNA extracted from putative transformants was screened by PCR for the presence of the NAT gene, and in some cases, the vector constructs. Results show the presence of the NAT gene in the genomic DNA of most putative transformants.

3.2 INTRODUCTION

Transformation of diatoms is still a relatively new field of study. Genetics studies of diatoms are hampered by the silica frustule which acts as

an armor which repulses traditional attempts to transform diatoms. The biolistic method of transformation used in diatoms utilizes a “gene-gun” to fire tungsten microparticles into a lawn of diatom cells, penetrating the frustules by physical force. This method has been successfully used in the development of a number of transformation systems in diatoms.

The first successful transformation of diatoms was reported by Dunahay et al. (1995) using *E. coli* neomycin phosphotransferase II (npt*II*) to create transformants resistant to G418 with the biolistic method. The constitutive expression of npt*II* was driven by the acetyl-CoA carboxylase (ACCase) promoter from *Cyclotella cryptica* T13L Reimann, Lewin, and Giullard. In the study by Dunahay et al., transformants of both species *Cyclotella cryptica* and *Navicula saprophila* were shown to be stable, and expression of npt*II* protein was proven by western blotting. Apt et al. (1996) soon after transformed the pennate diatom *Phaeodactylum tricornutum*. Using the *sh ble* gene from *Streptococcus hindustanus*, Apt et al. produced stable transformants resistant to the antibiotic zeocin. Moreover, the study showed that the *cat* reporter gene, encoding the enzyme chloramphenicol acetyl-transferase which catalyzed the digestion of ^{14}C-chloramphenicol could be used to report promoter efficiency. Evidence of the inefficacy of a heterologous fungal promoter fused to these two genes and introduced into *P. tricornutum* was interpreted as evidence that heterologous promoters may not be of use in diatoms.

Falciatore et al. (1999) produced concurrent evidence of stable transformation of *P. tricornutum* with the *sh ble* gene using constructs containing fucoxanthin, chlorophyll a/c-binding protein (*fcp*) promoter and terminator elements. Using these constructs, a vector was created for expression of the firefly luciferase gene (luc), which was said to be light-inducible. Cotransformation of the two genes using chimeric constructs with *fcp* promoter and the ACCase terminator amplified by Dunahay et al. (1995) were surprisingly successful, demonstrating the usefulness of heterologous promoter-terminator cassettes. Falciatore et al. noted that the salinity and plating density of cells on the agar medium had a large effect on wild-type resistance to antibiotic. Moreover, Falciatore et al. used PCR to determine whether the *sh ble* gene was present in the genome of transformants. This information, in combination with western and southern blots allowed the researchers to characterize the incorporation of the gene into transformant cells which was found to proceed by illegitimate recombination.

In another study of biolistic transformation of *P. tricornutum* which uses methods later published by Dr. Peter Kroth (2007), the pPha-T1 vector was used to transform this pennate diatom with *sh ble* and the *egfp* gene. In the case of the eGFP gene product, Zaslavskaia et al. (2000) found that codon bias affected the ability of the cells to properly express the gene. Fortunately, the eGFP gene was engineered to exhibit codon bias which allowed its expression and localization in these cells. Although successful in transforming

P. tricornutum, the *fcp* promoter used in the pPha-T1 vector was found to be nonfunctional in attempts to transform another diatom, *Cylindrotheca fusiformis* by Poulsen and Kröger (2005). Instead, native *fcp* promoter and terminator elements from *C. fusiformis* were used to cotransform cells with vectors containing the *ble* gene under constitutive control of the *fcp* promoter, with a construct utilizing native nitrate reductase promoter and terminator (*Pnr/Tnr*) elements for inducible expression of a *gfp* reporter gene. Inducible expression by addition of nitrates and subtraction of ammonium from growth medium presents a novel means of controlling gene expression in diatoms. Poulsen et al. (2006) subsequently transformed *Thalassiosira pseudonana* with a similar pair of systems, creating vectors pTpfcpGFP and pTpfcpNAT.

With the creation of a number of transformation systems in diverse species of diatoms, the way is opened to the production of further systems for specific control of expression of heterologous genes for study. The current work is an effort to expand the repertoire of transformation tools available for study of *T. pseudonana*, in addition to creating new vectors for the transformation of *N. alba*, a heretofore unstudied subject. In all, six individual vectors were created for these studies, three of which are reported here. Assays in the form of PCR of genomic DNA which amplify the foreign selectable marker gene nourseothricin acetyl-transferase (NAT) in putative transformants has provided evidence for the efficacy of vectors pTpACCNAT, pTpSVACCNAT, and pSVNAT in transient transformation of *T. pseudonana*.

Additionally, vector pTpACCNAT also produced putative transformants in *N. alba*.

3.3 MATERIALS AND METHODS

3.3.1 Cells and Culturing

N. alba species and culturing conditions were described in section 2.3.1. *Thalassiosira pseudonana* was obtained from the Provasoli-Guillard National Center for Culturing of Marine Phytoplankton, CCMP1335, and was maintained in 50 mL stock cultures of Northeast Pacific Culture (NEPC) medium with 1x penicillin/streptomycin mix (Cellgro®) at 18°C under a 12 hr light/dark cycle. Fresh stock cultures were inoculated approximately once a month. Cells were cultured in 1.0 L to 1.5 L batches in NEPC medium at 18°C under constant lighting (150 μmol/m²/sec) with stirring and aeration. Cultures typically reached a density of 2-3 x 10^6 cells/mL within 4 days.

3.3.2 Constructing the Vectors

3.3.2.1 General Methods for Plasmid Vector Construction

The three vectors, depicted in linear form in scheme 3.1, were constructed using the same general methods, except where otherwise noted. In general, three steps were employed for construction: 1) amplification of

desired promoter, terminator, and selectable marker sequence with chimeric primers conferring restriction sequence incorporated into 5' and 3' ends of products to aid in ligation, 2) stepwise construction of the vector by digestion of each fragment (promoter, NAT gene, terminator) with appropriate enzymes and ligation into digested pBluescript II SK(+) phagemid vector (Stratagene, La Jolla, CA), and 3) cloning of each construct in One Shot® Top10 Competent E. coli (Invitrogen, Carlsbad, CA) for amplification and storage.

Table 3.1: Primers Used in Vector Construction*

Project Stage	Oligo	RE site	Seq (5'-3')
NAT	pNATF1	HindIII	atttaagcttatgaccactcttacgacacgg
	pNATR1	EcoR1	tatatatagaattctcaggggcagggcatgctcatg
pTpACCNAT construct	TpACCpromF	Xho1	gtcactcgagatggaatgtcataggtgagttcg
	TpACCpromR	HindIII	gccccgaagcttggttattttgctattgattgg
	TpACCtermF	EcoR1	atccgaattcacgactttggcttatccaggaataaatg
	TpACCtermR	Xba1	tatatctagacttcatacttgtgtgcctcaagagtcag
pTpSVACCNAT/ pSVNAT SV40ep	SVepF	Xho1	atgtctcgagcagctgtggaatgtgtgtcag
	SVepR	HindIII	acgcaagctttttgcaaaagcctaggactcc
PolyA	SVpaF	EcoR1	agtcggcgaattctaacttgtttattgcagcttataatgg
	SVpaR	Xba1	aggctgctctagaagacatgataagatacattgatg
Sequencing	TpfcpGFP50F	--	cgaacaaatccattcaacctc
	TpfcpGFP50R	--	ttggatcttagctagccgaat

*the primers M13F/R and T3/T7 are commercially available; sequence was omitted
†i=inosine; A/T, C/G

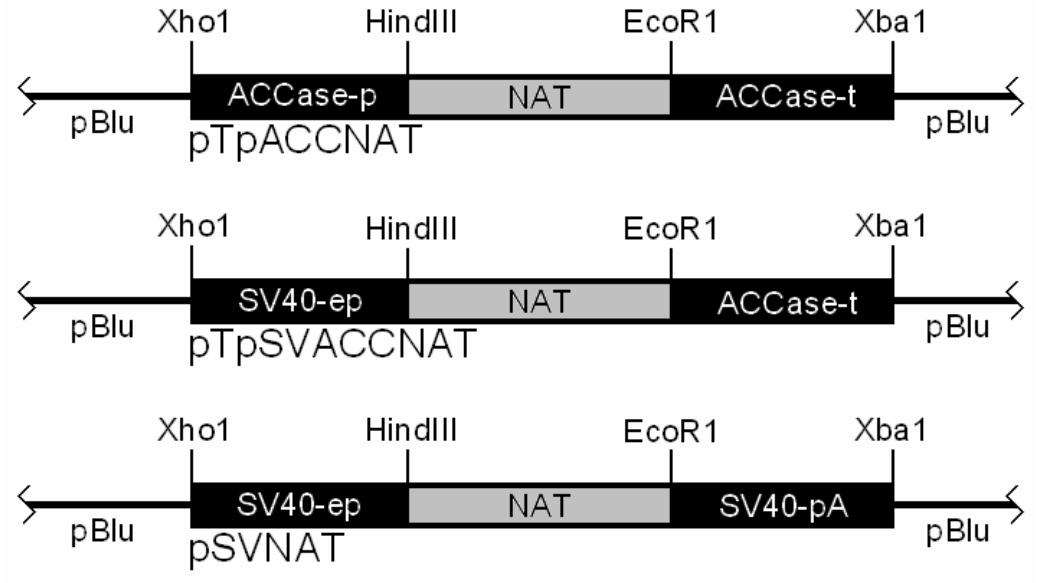
Fragments were amplified by PCR using custom primers (table 3.1). Following PCR in 50 µL volume, reaction mixtures were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). After cleanup, insert fragments were each digested with two appropriate enzymes to create sticky

ends by addition of 10-20 units of each enzyme (depending on total reaction volume), 1x Magic Buffer, and ddH_2O (i.e. autoclaved Milli-Q; Millipore, Billerica, MA) to a final volume of 20-40 µL. Reaction mixtures were left to digest overnight in a 37°C waterbath. Plasmids were similarly digested with appropriate enzymes targeting the multiple cloning sight to match the sticky ends of the fragment to be added. c.a. 1.5-2.0 µg plasmid DNA was digested with 20 units of each enzyme in 1x Magic buffer and ddH_2O to a total volume of 20 µL by incubation at 37°C for 3-4 hrs. Immediately following digestion, the plasmids were digested with 1 unit of Calf-Alkaline Phosphatase (CIP) (New England Biolabs, Ipswich, MA) to prevent self-ligation or concatomerization of the linearized plasmids. Briefly, to the 20 µL reaction, 1 µL 1:10 dilute CIP in 1x CIP Buffer "3" (New England Biolabs) was added at room temperature. ddH_2O and Buffer "3" were added to a final concentration of 1x Buffer "3" in a total reaction volume of 50 µL. The reaction was incubated at 37°C for 30-60 min.

After incubation, each digest was gel-purified using the Marligen Rapid Gel-Purification Kit (Marligen Biosciences, Ijamsville, MD), and quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Measured concentrations were used to calculate amounts of each fragment to use in ligation reactions. The equation of R.M. Cranenburgh (2004) was used to calculate volumes of each fragment needed to give a 1:3 ratio of vector:insert with a total DNA amount of at least

200 ng (although ligation was successfully carried out with as low as 100 ng). The total mixture of vector and insert, 10-25 µL, depending on the concentration of the components, was combined with 3 units of T4 DNA Ligase, 1x Ligase Buffer (New England Biolabs), and ddH_2O to a final volume of 30 µL. The reactions were left to incubate overnight at room temperature (c.a. 22°C).

The completed ligations were cloned in One Shot® Top10 Competent Cells, amplified, and analyzed by restriction digest with the same enzymes used in digestion of the fragments for ligation to ensure proper incorporation of each insert. Following successful assembly of the entire plasmid vector, each vector was sequenced by SeqxCel® (San Diego, CA), using the primers enumerated for each case, and proper construction was confirmed using AlignX in Vector NTI v. 10.0 (Invitrogen) to compare to expected sequence.



Scheme 3.1: Plasmid vectors created for this study. pBlu: pBluescript II SK(+)

3.3.2.2 Construction of pTpACCNAT

Acetyl-CoA carboxylase promoter and terminator sequence data were obtained from the Department of Energy Joint Genome Institute website (http://www.jgi.doe.gov). Acetyl-CoA carboxylase/biotin carboxylase is assigned the Protein ID: 6770, and is located on chromosome 7, base pairs 456734-463808. Chimeric primers TpACCpromF/R (sense/antisense) and TpACCtermF/R for amplification of promoter and terminator regions of the gene were designed to amplify regions from 1019 bp upstream of the start of ACCase, and from the 3' end of ACCase to 994 bp downstream; the regions found on chromosome 7; 455714-456733, and 463809-464803, respectively.

Promoter and terminator of ACCase were amplified from *T. pseudonana* genomic DNA generously provided by Dr. Luciano Frigeri using the following PCR reaction mixture: 5 µL *T. pseudonana* genomic DNA (1:10 dilution); 0.2 mM dNTP mix; 1 µM TpACCpromF and 1 µM TpACCpromR, or 1 µM TpACCtermF and 1 µM TpACCtermR; 1x Thermopol Buffer (New England Biolabs); 2.5 units Taq DNA Polymerase; and ddH_2O to 50 µL. PCR for both reactions was carried out in a Bio-Rad iCycler (Bio-Rad, Hercules, CA) with the following cycles: 1 cycle of 94°C for 4 min; 30 cycles of 94°C for 30 sec, 66°C for 30 sec, and 72°C for 1 min; 1 cycle of 72°C for 7 min; and a final dwell temperature of 4°C.

The NAT gene was amplified from 1 µL 1:1000 dilute pNAT vector stock using the chimeric primers pNATF1 (sense) and pNATR1 (antisense) with the same reaction mixture listed above, and the following cycles in an iCycler Thermal Cycler (Bio-Rad): one cycle of 95°C for 4 min; 30 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 90 sec; one cycle of 72°C for 7 min, and a final dwell temperature of 4°C. Both the resulting fragment and vector pBluescript SK(+) were digested with HindIII and EcoR1, and ligated as described in section 3.3.2.1.

The ACCase promoter fragment and vector pBluescript II SK(+) incorporating NAT gene were prepared for ligation by digestion with Xho1 and HindIII. The promoter and vector were ligated, and following successful ligation of the promoter fragment, the ACCase terminator was similarly ligated after digestion of the fragment and vector incorporating the ACCase promoter with EcoR1 and Xba1. The completed construct was sequenced using primers M13F/R to confirm proper incorporation of ACCase promoter and terminator elements.

3.3.2.3 Construction of Vectors Incorporating SV40 early promoter

SV40 early promoter and poly-A signal have been previously used by Dr. Paul Roessler to successfully drive constitutive expression of genes in diatom transformants (unpublished). SV40 promoter and terminator sequence data was gathered from multiple sequence alignment of existing vectors

incorporating SV40 early promoter and poly-A signal. Early promoter sequences and poly-A sequences were aligned independently with Clustal-W (Thompson et al., 1994) to determine consensus regions for construction of the vectors. A 325 bp SV40 early promoter consensus sequence was selected for amplification with the chimeric primers SvepF/R (sense/antisense), and a 131 bp poly-A consensus region was selected for amplification with the chimeric primers SVpaF/R. These consensus regions are herein referred to as the "SV40 early promoter" and "SV40 poly-A signal."

3.3.2.3.1 Construction of pTpSVACCNAT

SV40 early promoter (section 3.3.2.3) was amplified from 1 µL vector pSV_2CAT (Gilbert, 1991; unpublished) stock using the reaction mixture from section 3.3.2.2 with primers SVepF and SVepR. The PCR reaction was run in a Bio-Rad iCycler with the following parameters: 1 cycle of 95°C for 4 min; 30 cycles of 95°C for 30 sec, 67°C for 30 sec, 72°C for 25 sec; 1 cycle of 72°C for 7 min; and a final dwell temperature of 4°C. The product was prepared for ligation with vector pTpACCNAT; both vector and insert were digested with Xho1 and HindIII and ligated as described in section 3.3.2.1. The resulting vector having SV40 early promoter and native ACCase terminator elements, was sequenced with primers T3 and T7 to confirm proper assembly.

3.3.2.3.2 Construction of pSVNAT

Vector pSVNAT was created by replacing the native ACCase terminator fragment in vector pTpSVACCNAT with the SV40 poly-A signal to yield a vector having both SV40 early promoter and terminator elements. SV40 poly-A signal was amplified from vector pSV_2CAT using the same reaction mixture described in section 3.3.2.2 with the primers pSVepF and pSVepR. The PCR reaction was carried out in a Bio-Rad iCycler with the following settings: 1 cycle of 95°C for 4 min; 30 cycles of 95°C for 30 sec, 63°C for 30 sec, 72°C for 10 sec; 72°C for 7 min; and a final dwell temperature of 4°C. The product was prepared for ligation, along with vector pTpSVACCNAT, both of which were digested with EcoR1 and Xba1 and ligated using the methods described in section 3.3.2.1. The completed vector, pSVNAT, was analyzed for proper assembly using the sequencing primers M13F/R.

3.3.3 Microparticle Bombardment (i.e. Biolistic Transformation)

N. alba and *T. pseudonana* were transformed with vectors pTpACCNAT, pTpSVACCNAT, and pSVNAT. Transformation was carried out using the biolistic transformation method previously described by Poulsen et. al. (2006) with modification. Cultures of sessile *N. alba* strain cc3 were grown to densities of at least 0.5×10^6 cells/mL in 1.5 L of ASWT, harvested and approximately 1.0×10^7 cells were spread on each plate to a diameter of 2.5 cm on the center of ASWT plates containing 1x penicillin/streptomycin mix. *T.*

pseudonana was grown in 1.0-1.5 L NEPC medium to a minimum density of 1.0 x 10^6 cells/mL, harvested and approximately 1.0 x 10^8 cells/plate were spread on each NEPC plate as previously described. Transformation plates were allowed to dry in a sterile hood.

Tungsten M17 microparticles separated into 3.33 mg aliquots in 50 µL ddH_2O were coated with 5 µg of one of the vectors as described by Poulsen et al. (see also: Kroth, 2007). Aliquots of 10 µL of coated microparticles were spread on macrocarriers and allowed to dry in a sterile hood until transformation. Cells on transformation plates were bombarded with coated microparticles using the Bio-Rad biolistic PDS-1000/He unit according to manufacturer instructions using the following settings: 25 psi; 7 cm shot distance (macrocarrier to plate distance); 1350 psi rupture disc. Rupture disc pressure was selected on the basis of several experiments in *N. alba* where a range of pressures were used in transformation, including 1800, 1550, 1350, and 900 psi rupture discs. None of the values appeared to be significantly more successful in transformation, although 900 psi seemed slightly less effective (data not shown).

For *T. pseudonana*, 1350 psi was selected from the three pressures recommended in the protocol provided by Pouslen et. al. For uniformity, 1350 psi was used in all subsequent transformations in both species. Rupture discs were sterilized with isopropanol and allowed to air-dry prior to bombardment. Macrocarriers and holders, and stopping screens were autoclaved prior to

loading. The firing chamber and components were sanitized with 70% (v/v) ethanol and allowed to air-dry approximately 15 min prior to shooting. Immediately following bombardment of *T. pseudonana* and *N. alba* cells with the plasmid vectors pTpACCNAT, pTpSVACCNAT, or pSVNAT, 10 mL of appropriate growth medium was added to each plate, including wild-type negative controls, for recovery; plates were wrapped with parafilm. *N. alba* were allowed to recover 24 hrs in darkness at 31°C; *T. pseudonana* were incubated 24 hrs with light (150 µmol/m²/sec) at 18°C. After recovery, cells were plated by the pour-plating method for selection with 100 µg/mL clonNAT in 0.8% (w/v) agar medium: ASWT with 1x penicillin/streptomycin mix for *N. alba*, and NEPC for *T. pseudonana*. clonNAT selection levels were determined for *N. alba* by titration of wild-type cells of on plates containing 0, 25, 50, 75, 100, 125, 150, 175, or 200 µg/mL clonNAT which showed complete inhibition of growth on agar plates at the concentration of 100 µg/mL clonNAT (data not shown).

3.3.4 Screening Putative Transformants

Incubation conditions were uniform throughout selection and growth (except where noted). Transformed cells of *N. alba* were incubated at 31°C in darkness; transformed *T. pseudonana* were incubated at 18°C with moderate lighting as described in section 3.3.3. After 2-4 wks of incubation of pour-plated cells bombarded with NAT-containing constructs, microcolonies were

visible on transformed, but not wild-type control plates. Putative transformant clones were transferred by pipet to 24-well plates containing 1.5 mL liquid growth medium and 100 µg/mL clonNAT for a second round of selection. Following 2 wks of additional growth under the uniform incubation conditions, *T. pseudonana* cells were subcultured in a second 24-well plate for further selection under the same conditions. Following 4 wks of additional growth *N. alba* were subcultured in 75 mL of ASWT with 1x penicillin/streptomycin mix in 125 mL Erlenmeyer flasks to grow for DNA extraction. Following the second round of selection, *T. pseudonana* cells were also subcultured for DNA extraction in 75 mL of NEPC medium.

3.3.5 PCR Assay of Putative Transformants

Specific primers were designed for amplification of fragments of DNA of either the NAT gene (NATscreenF/R; sense/antisense), or a combination of promoter and gene elements to demonstrate incorporation of the intact construct in gDNA of putative transformants. Primers used in this study are listed in table 3.2.

Table 3.2: Primers Used in Putative Transformant Screening Assays

Assay	Oligo	Sequence (5'-3')
NATscreen	NATscreenF	accactcttgacgacacggcttacc
	NATscreenR	cgtacagggcggtgtccagcc
ACCNATscreen2	ACCNATscreenF2	tgtcggagagtgccaacaacc
	ACCNATscreenR2	ttgacgttggtgacctccagc

3.3.5.1 Assay of *N. alba* Transformed with pTpACCNAT

Screened putative transformants of *N. alba* (section 3.3.4), transformed with pTpACCNAT were analyzed by PCR after extraction of DNA from 75 mL cultures of putative transformants using an alkaline lysis and protein precipitation protocol provided by Nicole Poulsen, utilizing reagents from Promega Corp. (Madison, WI). Extracted DNA was analyzed for concentration using a NanoDrop ND-1000 UV/Vis spectrophotometer, and approximately 60 ng of each sample were used as templates in the PCR assay. The assay reaction mixture was identical to that described in section 3.3.2.2, except that primers NATscreenF and NATscreenR are were used. Also, each template was mixed with Tris-HCl pH 8.0 to a volume of 10 µL, and this mixture used as the template for each reaction. The PCR reactions were run in an Eppendorf Mastercycler® ep (Eppindorf AG, Hamburg, Germany). The ideal annealing temperature for the assay was determined using the Thermal Gradient function to test a range of temperatures using genomic DNA from *T. pseudonana* cotransformed with the vectors pTpfcpGFP and pTpfcpNAT, supplied by Nicole Poulsen, as the positive control template (Poulsen et al., 2006). Based on the results of Thermal gradient PCR, the program titled "NATscreen" was created: 1 cycle of 95°C for 4 min; 35 cycles of 95°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec; 1 cycle of 72°C for 7 min; and a final dwell temperature of 4°C. The PCR products were analyzed in 0.7% (w/v) agarose gel.

3.3.5.2 Promoter Assay of Putative Transformant of *N. alba* Transformed with pTpACCNAT

A putative transformant of *N. alba* transformed with pTpACCNAT was also assayed with primers ACCNATscreenF2/R2 (sense/antisense) which amplify a fragment comprising a segment of the acetyl-CoA carboxylase promoter from *T. pseudonana* upstream from the NAT gene, spanning into the NAT gene. Thermal gradient PCR was used to determine the ideal annealing temperature for the primers using *N. alba* genomic DNA extracted from a putative transformant transformed with vector pTpACCNAT, named clone "NA2." Clone "NA2" was the template in a reaction mixture with the following composition: 0.8 µL template DNA "NA2;" 0.2 mM dNTP mix; 1 µM ACCNATscreenF2; 1 µM ACCNAT screenR2; 1x Thermopol buffer; 1 unit Taq DNA Polymerase; and ddH_2O to 20 µL total volume. 12 reactions were run using a thermal gradient PCR protocol in an Eppendorf Mastercycler® ep with the following settings: one cycle of 95°C for 4 min; 35 cycles of 95°C for 30 sec followed by a thermal gradient from 60°C-40°C for 30 sec, ending with 72°C for 1 min; 1 cycle of 72°C for 7 min; and a final dwell temperature of 4°C. The resulting PCR products were electrophoresed in 0.7% (w/v) agarose gel.

3.3.5.3 Assay of *T. pseudonana* Transformed with pTpACCNAT, pTpSVACCNAT, and pSVNAT

Putative transformants of *T. pseudonana* were screened as described in section 3.3.4. All other methods for PCR assay of putative transformants of *T. pseudonana* transformed with pTpACCNAT, pTpSVACCNAT, and pSVNAT are identical to those described for assay of putative transformants of *N. alba* in section 3.3.5.1, except that 3 µL of genomic DNA were used as templates for each putative transformant assayed, from wild-type *T. pseudonana* (negative control), and from *T. pseudonana* transformed with vectors pTpfcpGFP and pTpfcpNAT by Nicole Poulsen (positive control). Additionally, a putative transformant of *T. pseudonana* transformed with vector pTpACCNAT was screened as described in section 3.3.5.2 using clone "A3" as the template for thermal gradient PCR. Following thermal gradient PCR, additional clones were assayed using the same 20 µL reaction mixture and a PCR protocol with a 58°C annealing temperature. The resulting products were electrophoresed on 0.7% (w/v) agarose gel.

3.4 RESULTS

3.4.1 Colony Counts of Putative Transformants of *Thalassiosira pseudonana*

As part of the development of new systems for diatom transformation, the promoter of the gene encoding acetyl-CoA carboxylase (ACCase) (biotin

carboxylase) was studied as a potential candidate for use as a universal promoter to drive constitutive expression of genes in transformed cells. Dunahay et al. (1995) have previously shown this promoter to work in the first successful report of diatom transformation in evolutionarily diverse species. Although the factors controlling expression of this gene are not yet understood in diatoms (Wilhelm et al., 2006), a number of factors have been found to inhibit its expression. Also, ACCase has been implicated in a key step of lipid synthesis in diatoms, and as such, is likely to be highly expressed in most species (Roessler et al., 1994).

Two additional vector constructs were created to study the possibility of utilizing the SV40 early promoter to drive constitutive expression in *Thalassiosira pseudonana*. Dr. Paul Roessler has previously shown that the SV40 early promoter can be used to drive constitutive expression of genes in diatoms (unpublished). As a mammalian viral promoter, the SV40 early promoter drives high levels of constitutive expression of diverse genes, yet has enhancers which have worked in a wide variety of organisms (Gluzman and Shenk, 1983; Khoury and Gruss, 1983; Laimins et al., 1982; Levinson et al., 1982; de Villiers et al., 1981, 1982). Components of the SV40 early promoter and poly-A signal were amplified from vector pSV_2CAT (Gilbert, 1991; unpublished). Table 3.3 compares resistance conferred by the three vector constructs: pTpACCNAT, pSVNAT, and pTpSVACCNAT. The latter two constructs contain the NAT gene under the transcriptional control of the

SV40 early promoter. Vector pSVNAT also contains poly-A signal derived from SV40 genomic DNA inserted immediately downstream of the NAT gene. Construct pTpSVACCNAT utilizes the native ACCase terminator from vector pTpACCNAT. Of note in the colony counts is the great difference in apparent transformants produced by introduction of vector pSVNAT, compared to

Table 3.3
Growth in Selective Media Containing 100µg/mL clonNAT in 0.8% (w/v) NEPC Agar of Putative Transformants of *Thalassiosira pseudonana* Transformed with Various Constructs

Vector Construct used in Transformation	avg. cfu's/plate*
pTpACCNAT	150
pTpSVACCNAT	30
pSVNAT	250
--	
wild-type cells	0

* data represent the average of at least 3 independent experiments. Cells were grown in selective media prepared by the pour-plating method

transformation with vector pTpACCNAT. The data suggest either more efficient transformation using the viral promoter, or else greater levels of expression of the NAT gene product using the SV40 early promoter to drive constitutive expression. The data may also suggest the difference of control mechanisms associated with each promoter, hinting at native control mechanisms which may inhibit over-expression of the NAT gene when under the control of the ACCase promoter, which do not act on the foreign SV40 promoter. Also, both constructs show dramatically greater transformation success when compared to the chimeric vector pTpSVACCNAT. The construct was created with the hope of increasing expression of the NAT gene

product by using the native terminator region of ACCase to improve post-translational processing of the gene transcripts. In contrast, the data suggest more efficient processing with the homologous promoter-terminator cassettes.

3.4.2 PCR Assay of Putative Transformants of *T. pseudonana* and *N. alba* Transformed with Vector pTpACCNAT

A construct containing the NAT gene under control of ACCase promoter and terminator elements from *T. pseudonana* was used to biolistically transform cells of both *T. pseudonana* and *N. alba*. Transformed cells were selected by growth in 0.8% (w/v) ASWT with 100 IU/mL penicillin and 100 µg/mL streptomycin; or 0.8% (w/v) NEPC agar, both with 100 µg/mL clonNAT. After 2-3 wks, transformants, visible as microcolonies within the agar plates, were transferred to 1.5 mL of the appropriate liquid medium for further selection. After successive rounds of selection, cells were grown to density in 75 mL liquid medium without clonNAT for DNA extraction by alkaline lysis, protein precipitation, centrifugation, and separation of the liquid supernatant containing DNA.

3.4.2.1 PCR of gDNA from *T. pseudonana* and *N. alba* Transformed with pTpACCNAT Detects the NAT Gene

Genomic DNA extracted from putative transformants of *N. alba* and *T. pseudonana* was assayed for incorporation of the NAT gene using the

NATscreen (section 3.3.5.1). Figure 3.1 shows the presence of the foreign NAT gene in all six clones of *N. alba* assayed, and all eight clones of *T. pseudonana* assayed. The 'negative controls' applied in studies of putative transformants of *T. pseudonana* clones were amplified from gDNA from stock-cultures of wild-type *T. pseudonana*. Each selection study was accompanied by culturing of, and DNA extraction from the wild-type cells used in transformation, to ensure that no cross-contamination event had occurred; yielding false positives in clones. The 'water blanks' ensured the absence of contaminants in PCR reagents. Further PCR studies were conducted

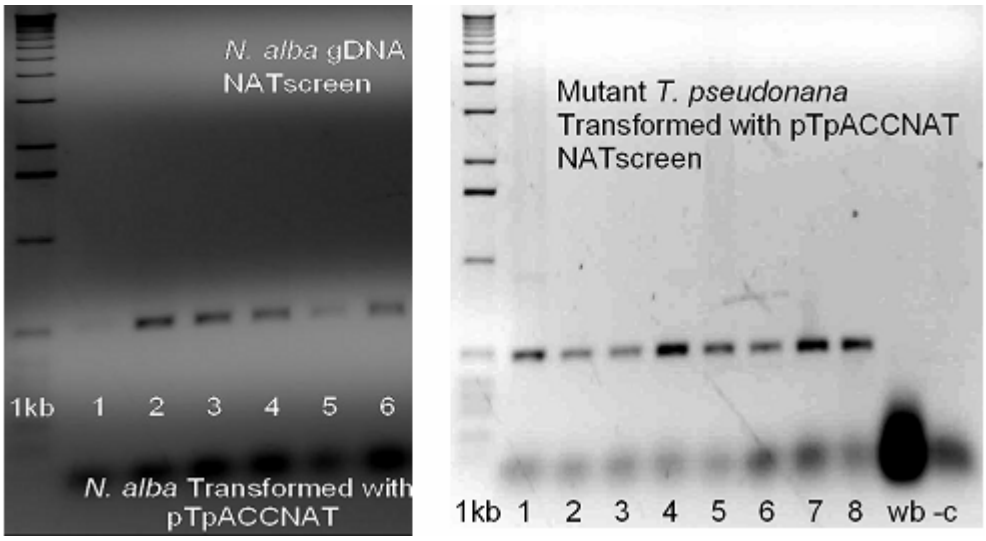


Figure 3.1: 0.7% (w/v) agarose gel elecrophoresis showing putative transformants of *N. alba* and *T. pseudonana* transformed with vector pTpACCNAT. The image to the left shows the screening of putative transformants of *N. alba*. Two control samples were included in the *T. pseudonana* assay: 'wb'—water blank; '–c'—negative control (genomic DNA from wild-type cells)

to determine whether the pTpACCNAT construct remained intact within the genome of putative transformants.

3.4.2.2 Thermal Gradient PCR to Calibrate Primers ACCNATscreenF2/R2 in a Putative Transformant of *N. alba*

In order to show that the pTpACCNAT construct remains intact in the genome of putative transformants, PCR was used to amplify a fragment comprising the *C*-terminal domain of the ACCase promoter, spanning most of the length of the NAT gene. The amplification target is a 513 bp region depicted in scheme 3.2. Primers ACCNATscreenF2/R2 (sense/antisense) were designed to anneal to the target. Thermal gradient

Fragment Amplified from Mutant *T. pseudonana* and *N. alba* Transformed with pTpACCNAT

513 bp

Scheme 3.2: Amplification fragment inclusive of the *C*-terminal domain of the ACCase promoter, into the 3' region of the NAT gene. Primers ACCNATscreenF2 (sense) and ACCNATscreenR2 (antisense) are depicted as half-arrows at either end of the fragment

PCR was used to amplify the fragment in *N. alba* genomic DNA. Figure 3.2 shows the results of the thermal gradient PCR in 0.7% (w/v) agarose gel electrophoresis. The band roughly corresponds to the size of the expected 513 bp sequence. Also, no spurious bands are present. The high specificity of this assay was not unexpected since neither the NAT gene, or the ACCase promoter were native to the *N. alba* cells; making mispriming less likely. The results suggest that the NAT gene remains under control of the ACCase promoter from *T. pseudonana* in the putative transformant of *N. alba*.

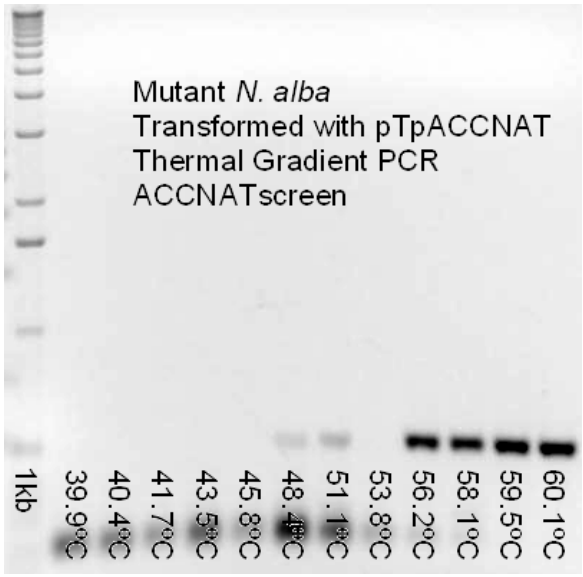


Figure 3.2: 0.7% (w/v) agarose gel electrophoresis showing results of thermal gradient PCR used to amplify a 513 bp fragment from a putative transformant of *N. alba* heterologously transformed with vector pTpACCNAT. Temperatures indicate annealing temperatures for samples in each lane. The expected product is visible in the far right four lanes

Experiments such as sequencing of the band, or reamplification of the NAT gene portion using PCR could determine whether a portion of the NAT gene is actually present in these bands.

3.4.2.3 ACCNATscreen of a Putative Transformant of *T. pseudonana* Transformed with pTpACCNAT

Screening for incorporation of the construct pTpACCNAT in a putative transformant of *T. pseudonana* was also carried out using primers ACCNATscreenF2/R2. Just as in the screening of the putative transformant of *N. alba* (section 3.4.2.2), thermal gradient PCR was used in an attempt to

amplify the target. The gradient failed to appreciably amplify any single band, but yielded clues to the optimum annealing temperature needed for the assay (data not shown). Subsequently, clone "A3," selected for resistance to the NAT gene, was screened using these primers. Figure 3.3 shows the results of the PCR assay in clone "A3."

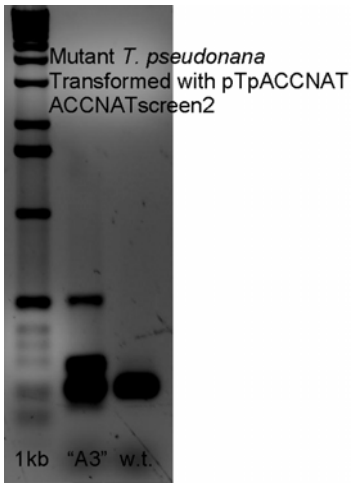


Figure 3.3: 0.7% (w/v) agarose gel electrophoresis showing amplification of a fragment from vector construct pTpACCNAT incorporated into genomic DNA of a putative transformant of *T. pseudonana*. Lane "A3," putative transformant showing the expected band of 513 bp size; two additional bands are amplified; one is shown to be spurious by comparison to the wild-type genomic template in the right lane

The relative difficulty of amplifying the construct in *T. pseudonana*, as opposed to *N. alba*, and the presence of spurious bands in the amplification, can be explained by the fact that the ACCase promoter sequence recognized by the screening primers is also present in wild-type cells. Mispriming and spurious products are to be expected when assaying this putative transformant with these primers. The problem could likely be solved by creating a set of primers which spanned the junction of the ACCase promoter

and terminator elements and the 5' and 3' ends of the NAT gene, limiting mispriming. Amplification of the product of the expected size suggests that the NAT gene remains under control of the native ACCase promoter in transformed *T. pseudonana*.

3.4.3 PCR of gDNA from *T. pseudonana* Transformed with Constructs Under the Control of the SV40 early promoter Detects the NAT Gene

Genomic DNA was extracted from putative transformants of *T. pseudonana* transformed with either vector pTpSVACCNAT, or pSVNAT. The gDNA was assayed by NATscreening, and the results, depicted in figure 3.4,

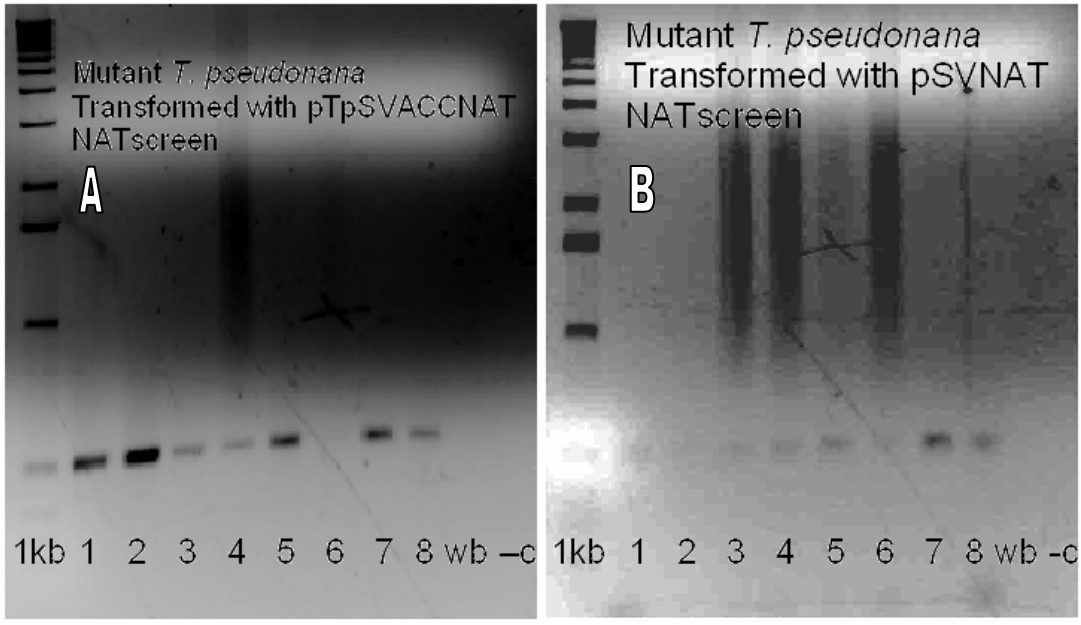


Figure 3.4: 0.7% (w/v) agarose gel electrophoresis showing results of NATscreening of putative transformants of *T. pseudonana*. All clones show bands of approximately 500 bp size, corresponding to the NAT gene. A. Clones transformed with vector pTpSVACCNAT. B. Clones transformed with vector pSVNAT; lanes 1,2 show product bands which are barely discernable. 'wb'—water blank; '-c'—negative control (wild-type gDNA template)

show that all resistant clones contain NAT gene integrated into the genomic DNA. Further work will need to be carried out to determine whether the NAT gene is still associated with flanking promoter elements in these constructs.

3.5 DISCUSSION

3.5.1 Novel Vectors for Transformation of *T. pseudonana*

The diploid genome of *T. pseudonana* comprises 24 chromosomes which have been completely sequenced. The genomic data is available at the DOE Joint Genome Institute website, and is now in a third iteration (v 3.0) (Armbrust et al., 2004). Frigeri et al. (2006) have demonstrated the utility of the genomic data in studies of proteins which are likely to be associated with the SDV in diatoms. Having a sequenced genome, *T. pseudonana* is emerging as a model species for diatom research (Chepurnov, 2005), and the need for tools to genetically transform the cells is growing. The results of Frigeri et al. demonstrate the utility of genetics in understanding complex cellular processes in the context of diverse cellular changes, but highlight the need for genetic transformation tools to study and characterize the 31 protein genes identified.

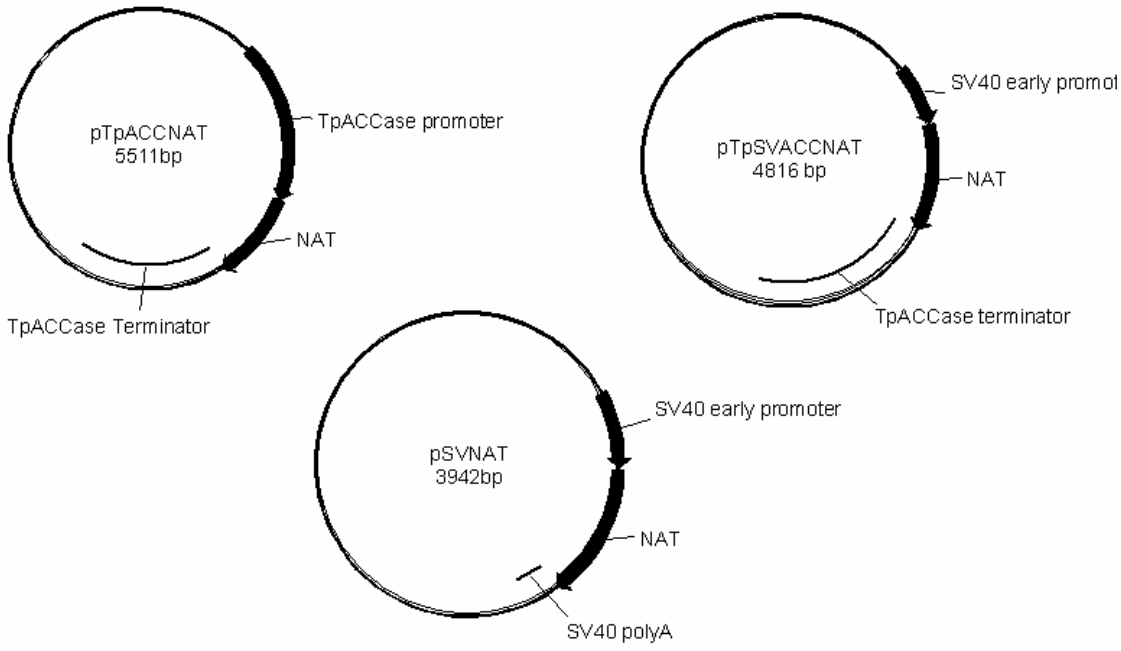
Previous studies by Poulsen and Kröger et al. (2007, 2006, 2005) have yielded a number of transformation systems in *T. pseudonana* which drive expression of the NAT gene under constitutive control of the fucoxanthin,

chlorophyll a/c-binding protein (*fcp*) promoter, LHCF9; or drive inducible expression of the eGFP gene using metabolic switching of the nitrate reductase (NR) gene promoter by addition or repletion of ammonium from medium. Poulsen et al. (2006) have further demonstrated that *T. pseudonana* is sensitive to clonNAT, showing complete growth suppression in 100 µg/mL clonNAT in medium, and have used cotransformation to simultaneously transform *T. pseudonana* with this selectable marker, and eGFP. The vectors designed for this study may present alternate vectors to drive constitutive expression in *T. pseudonana* and heterologous species, which could aid in gene characterization.

3.5.2 Towards the Creation of Universal Transformation Vectors

Although extremely useful in species-specific studies, vectors bearing native promoter-terminator cassettes may not be useful for comparative studies in heterologous species, and in any case require the investment of time and resources to evaluate their effectiveness. In a forward-looking effort to improve research in the area of diatom genetic transformation, attempts were made to design potential candidate vectors for use as universal transformation vectors in diatoms. As a first step in this process, several vectors were used to biolistically transform cells of both *T. pseudonana* and *N. alba*. These two species represent diverse types of diatoms; *T. pseudonana* being a centric photoautotroph, and *N. alba* being a pennate

chemoheterotroph. Therefore, any vector construct capable of inducing resistance to clonNAT in both species could be considered a candidate for further study. Scheme 3.3 depicts three of the constructs tested which are reported in this work.



Scheme 3.3: Candidates for use as universal transformation vectors in diatoms. p—plasmid vector pBluescriptSKII+; Tp—*Thalassiosira pseudonana*; SV—simian virus SV40 early promoter (and poly-A signal); ACC—Acetyl-CoA carboxylase promoter (and terminator); NAT—nourseothricin *N*-acetyltransferase encoding gene

All three vector constructs were shown to generate resistant colonies on selective plates in both species tested (data not shown), but only the clones from *T. pseudonana* were proven to incorporate the NAT gene into genomic DNA in cells transformed with the constructs under constitutive control of the SV40 early promoter. On the other hand pTpACCNAT has been demonstrated to produce transformants in both *T. pseudonana* and *N. alba*.

which incorporate the NAT gene into genomic DNA. Furthermore, analysis by PCR suggests that the vector construct remains intact in transformants of both species, and that the NAT gene remains under constitutive control of the ACCase promoter from *T. pseudonana*. Based on counting of cfu's on selective plates, the SV40 early viral promoter appeared to drive the highest expression levels of NAT in *T. pseudonana*.

3.5.3 Novel PCR Assays of Putative Transformants

NATscreening was devised as a rapid PCR assay of NAT integration into genomic DNA of putative transformants using a gene-specific primers and defined reaction parameters. An additional type of PCR assay was developed for a number of specific clones in an effort to determine whether constructs remained intact after incorporation into genomic DNA. For reasons mentioned earlier, these assays were largely unsuccessful, and only single clones of both *T. pseudonana* and *N. alba* were identified to display fragments of the proper length indicating the presence of the complete construct (i.e. promoter-NAT-terminator) in genomic DNA.

The studies conducted here represent preliminary investigations into the usefulness of the various promoters and terminators for transformation of the diatoms *T. pseudonana* and *N. alba* using proof-of-concept models. As such, the vector constructs were assembled without conservation of multiple cloning sites upstream or downstream of selectable marker genes. Once

shown to work in repeated experiments, the vectors may be reengineered for use by incorporation of new multiple-cloning sites or direct insertion of genes of interest to generate specific vectors for study.

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APPENDIX

NOVEL VECTOR SEQUENCES

A.1 Vector pTpfcpL44mut

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  51 AATGTATCCG ACAACGGCGA GCATAGCAAC ACGTCCGTCT TTGGAGTAGA
 101 ATCATCATGT TGTGGATGAA TACACAGATG AATGACATTA AAAGCATGAA
 151 CATGTTAGAG AGTAGGAGGT AGAGATTGAT ATGGTAGCAT TGCGATGTTT
 201 GTTTTTGGTC AGCATATGAT GAGTGGATAC CAATATGATG AAAGTTGAAT
 251 CTCGCGTTTG AGCTCAGCGG TACGTTATTG ATCGAAAGTA GCCTGATCAA
 301 AATCCTTGGA GAGTACAAGA GGATCAAAGA ATCCAGTGGG GGCGATAACT
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 501 GTTGAACCGC TACCTACAAT GACAAAGTAT CGTATCGATT TTGATGTTGG
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 601 AACGAACACA TCTTTCCGAT CATTCACCTC CACACTGCAA CAACACGGTA
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4651 TTCCCAGTCA CGACGTTGTA AAACGACGGC CAGTGAGCGC GCGTAATACG
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4751 CGGTATCGAT A
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TpampL41F; TpampL41R: underlined
ACC65I; Not 1: dbl underlined

p.P56>Q: lowercase; emphasized
Region 990-1307 bp is the NAT gene

A.2 Vector pNarpL41NAT

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 251 TTTTTGGGGT CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG
 301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG
 351 AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG
 401 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA
 451 GGGCGCGTCC CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT
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4651 C
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NATscreenF; NATscreenR: underlined
Sal 1, HindIII, EcoR1, Xba1: dbl underlined

Region 680-1581 bp is the RL41 promoter
Region 1588-2154 bp is the NAT gene
Region 2164-2420 bp is the RP41 terminator

A.3 Vector pTpACCNAT

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3801 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
3851 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
3901 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
3951 CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
4001 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
4051 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
4101 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT
4151 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
4201 GAAGAACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA
4251 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
4301 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
4351 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
4401 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC
4451 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
4501 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
4551 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
4601 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG
4651 CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
4701 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC
4751 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT
4801 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG
4851 GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
4901 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT
4951 CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
5001 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT
5051 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
5101 AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT
5151 ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC

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5201 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
5251 TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC
5301 AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG
5351 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
5401 ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
5451 GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
5501 AAAAGTGCCA C
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NATscreenF; NATscreenR: underlined

ACCNATscreenF2; ACCNATscreenR2: bold

Xho 1, HindIII, EcoR1, Xba1: dbl underlined

Region 674-1693 bp is the ACCase promoter
Region 1700-2269 bp is the NAT gene
Region 2276-3280 bp is the ACCase terminator

A.4 Vector pTpSVACCNAT

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   1 CTAAATTGTA AGCGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT
  51 AAATCAGCTC ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT
 101 AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA
 151 CAAGAGTCCA CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA
 201 CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC CTAATCAAGT
 251 TTTTTGGGGT CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG
 301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG
 351 AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG
 401 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA
 451 GGGCGCGTCC CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT
 501 CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT
 551 GCAAGGCGAT TAAGTTGGGT AACGCCAGGG TTTTCCCAGT CACGACGTTG
 601 TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCACTA TAGGGCGAAT
 651 TGGGTACCGG GCCCCCCCTC GAGCAGCTGT GGAATGTGTG TCAGTTAGGG
 701 TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCATGCA
 751 TCTCAATTAG TCAGCAACCA GGCTCCCCAG CAGGCAGAAG TATGCAAAGC
 801 ATGCATCTCA ATTAGTCAGC AACCATAGTC CCGCCCCTAA CTCCGCCCAT
 851 CCCGCCCCTA ACTCCGCCCA GTTCCGCCCA TTCTCCGCCC CATGGCTGAC
 901 TAATTTTTTT TATTTATGCA GAGGCCGAGG CCGCCTCGGC CTCTGAGCTA
 951 TTCCAGAAGT AGTGAGGAGG CTTTTTTGGA GGCCTAGGCT TTTGCAAAAA
1001 GCTTATGACC ACTCTTGACG ACACGGCTTA CCGGTACCGC ACCAGTGTCC
1051 CGGGGGACGC CGAGGCCATC GAGGCACTGG ATGGGTCCTT CACCACCGAC
1101 ACCGTCTTCC GCGTCACCGC CACCGGGGAC GGCTTCACCC TGCGGGAGGT
1151 GCCGGTGGAC CCGCCCCTGA CCAAGGTGTT CCCCGACGAC GAATCGGACG
1201 ACGAATCGGA CGCCGGGGAG GACGGCGACC CGGACTCCCG GACGTTCGTC
1251 GCGTACGGGG ACGACGGCGA CCTGGCGGGC TTCGTGGTCG TCTCGTACTC
1301 CGGCTGGAAC CGCCGGCTGA CCGTCGAGGA CATCGAGGTC GCCCCGGAGC
1351 ACCGGGGGCA CGGGGTCGGG CGCGCGTTGA TGGGGCTCGC GACGGAGTTC
1401 GCCCGCGAGC GGGGCGCCGG GCACCTCTGG CTGGAGGTCA CCAACGTCAA
1451 CGCACCGGCG ATCCACGCGT ACCGGCGGAT GGGGTTCACC CTCTGCGGCC
1501 TGGACACCGC CCTGTACGAC GGCACCGCCT CGGACGGCGA GCAGGCGCTC
1551 TACATGAGCA TGCCCTGCCC CTGAGAATTC ACGACTTTGG CTTATCCAGG
1601 AATAAATGTA GAAACTATTG GCTAAACAAG AAGTGAATCA TCTTTCTATT
1651 GTTGCTGTGA ACATGATGTG TGTAGGTAGA AGAGAGGGAG CTCTGTAACT
1701 TGCAGTTATG TTGATGTCTC CCGGATCTTT GACACCAACC TTGGCATCGT
1751 CTTTCTGATG GGCGCTTGCG GTTCTGCAAA TGTCTTCCGG GTATTTTTTT
1801 TATCTCTACT CGTAAAAACT GTGTAGACAG CTGACCAGGG TCTTATCCTT
1851 GTCTGCCAAT CACCCACACT TCTCTAAACA TGCAATGTAC CAGCAAAAGG
1901 CACCCTACTC AACGGCAAAG ACAATATAAG ATGTTGATGT CAGAGCTAGG
1951 GCTAGTCCTC ATCTGCTGCT GCTGTCGAGT CGAAGAAGTT CATCAGTGTC
2001 CTCAACCTTG ACACCAGAAG TCTTCAGATG AGTAGAGGTA TGCTTTACGA
2051 TGCCAGAGTA AAGAAGCCTA AAGACATGCC AAAGCGACCT CTGAGGTAAT
2101 GCAGATCGTG TCTTTGTTGT TGTTGTTGTT GTTGTAGACG AGTTGATGAC
2151 GACGATCCTT CGCAGCGTTG CTACTTGATG CATGACACTG CTGAACTTAC
2201 ACTATCATCT ATCAACTTAA TCCTCTTCTT AATCCGCTCT ACAATGTCTC
2251 CTTCAAGGAA GAACGACTGA AAATACTTTC CGCTACCAAC AAGGAAGAGG
2301 AGGAATGCAT ATTCCAACAA CACAAAACCA GTGTTACAAT CGAATGGCAA
2351 ACGTTTCTTG ATAAGGGGAT CTTTATGTGC GACAAATGCT ACTTTGATCG
2401 GTAAAGATAT TTAGGATTGG AATCGTATGC TACTGACGAT GATGACGAGC
2451 TTTGAGGAGA TGAGTACTCA TTGTGTACTG ACGTATTGTA CTGGAGCGAA
2501 ACGAACAAAT GCGATGTAAT GAGTTAACAG ATAACAACAA CCGATCTACA
2551 TTGATACTGA CTCTTGAGGC ACACAAGTAT GAAGCTCTAG AGCGGCCGCC
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2601 ACCGCGGTGG AGCTCCAGCT TTTGTTCCCT TTAGTGAGGG TTAATTGCGC
2651 GCTTGGCGTA ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG
2701 CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT GTAAAGCCTG
2751 GGGTGCCTAA TGAGTGAGCT AACTCACATT AATTGCGTTG CGCTCACTGC
2801 CCGCTTTCCA GTCGGGAAAC CTGTCGTGCC AGCTGCATTA ATGAATCGGC
2851 CAACGCGCGG GGAGAGGCGG TTTGCGTATT GGGCGCTCTT CCGCTTCCTC
2901 GCTCACTGAC TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG
2951 CTCACTCAAA GGCGGTAATA CGGTTATCCA CAGAATCAGG GGATAACGCA
3001 GGAAAGAACA TGTGAGCAAA AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA
3051 GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC
3101 ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA
3151 AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC
3201 GACCCTGCCG CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG
3251 TGGCGCTTTC TCATAGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC
3301 GTTCGCTCCA AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG
3351 CTGCGCCTTA TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG
3401 ACTTATCGCC ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG
3451 TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA
3501 CACTAGAAGA ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT
3551 TCGGAAAAAG AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT
3601 AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG
3651 ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA
3701 ACGAAAACTC ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC
3751 TTCACCTAGA TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG
3801 TATATATGAG TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG
3851 CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC
3901 CCCGTCGTGT AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG
3951 TGCTGCAATG ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG
4001 CAATAAACCA GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT
4051 TTATCCGCCT CCATCCAGTC TATTAATTGT TGCCGGGAAG CTAGAGTAAG
4101 TAGTTCGCCA GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA
4151 TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC
4201 CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT
4251 TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT
4301 TATCACTCAT GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA
4351 TCCGTAAGAT GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG
4401 AGAATAGTGT ATGCGGCGAC CGAGTTGCTC TTGCCCGGCG TCAATACGGG
4451 ATAATACCGC GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA
4501 CGTTCTTCGG GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG
4551 TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT
4601 TCACCAGCGT TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA
4651 AAGGGAATAA GGGCGACACG GAAATGTTGA ATACTCATAC TCTTCCTTTT
4701 TCAATATTAT TGAAGCATTT ATCAGGGTTA TTGTCTCATG AGCGGATACA
4751 TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT
4801 CCCCGAAAAG TGCCAC
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NATscreenF; NATscreenR: underlined

Xho 1, HindIII, EcoR1, Xba1: dbl underlined

Region 674-998 bp is the SV40 early promoter
Region 1005-1574 bp is the NAT gene
Region 1581-2585 bp is the ACCase terminator

A.5 Vector pSVNAT

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   1 CTAAATTGTA AGCGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT
  51 AAATCAGCTC ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT
 101 AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA
 151 CAAGAGTCCA CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA
 201 CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC CTAATCAAGT
 251 TTTTTGGGGT CGAGGTGCCG TAAAGCACTA ATCGGAACC CTAAAGGGAG
 301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG
 351 AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG
 401 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA
 451 GGGCGCGTCC CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT
 501 CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT
 551 GCAAGGCGAT TAAGTTGGGT AACGCCAGGG TTTTCCCAGT CACGACGTTG
 601 TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCACTA TAGGGCGAAT
 651 TGGGTACCGG GCCCCCCCTC GAGCAGCTGT GGAATGTGTG TCAGTTAGGG
 701 TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCATGCA
 751 TCTCAATTAG TCAGCAACCA GGCTCCCCAG CAGGCAGAAG TATGCAAAGC
 801 ATGCATCTCA ATTAGTCAGC AACCATAGTC CCGCCCCTAA CTCCGCCCAT
 851 CCCGCCCCTA ACTCCGCCCA GTTCCGCCCA TTCTCCGCCC CATGGCTGAC
 901 TAATTTTTTT TATTTATGCA GAGGCCGAGG CCGCCTCGGC CTCTGAGCTA
 951 TTCCAGAAGT AGTGAGGAGG CTTTTTTGGA GGCCTAGGCT TTTGCAAAAA
1001 GCTTATGACC ACTCTTGACG ACACGGCTTA CCGGTACCGC ACCAGTGTCC
1051 CGGGGGACGC CGAGGCCATC GAGGCACTGG ATGGGTCCTT CACCACCGAC
1101 ACCGTCTTCC GCGTCACCGC CACCGGGGAC GGCTTCACCC TGCGGGAGGT
1151 GCCGGTGGAC CCGCCCCTGA CCAAGGTGTT CCCCGACGAC GAATCGGACG
1201 ACGAATCGGA CGCCGGGGAG GACGGCGACC CGGACTCCCG GACGTTCGTC
1251 GCGTACGGGG ACGACGGCGA CCTGGCGGGC TTCGTGGTCG TCTCGTACTC
1301 CGGCTGGAAC CGCCGGCTGA CCGTCGAGGA CATCGAGGTC GCCCCGGAGC
1351 ACCGGGGGCA CGGGGTCGGG CGCGCGTTGA TGGGGCTCGC GACGGAGTTC
1401 GCCCGCGAGC GGGGCGCCGG GCACCTCTGG CTGGAGGTCA CCAACGTCAA
1451 CGCACCGGCG ATCCACGCGT ACCGGCGGAT GGGGTTCACC CTCTGCGGCC
1501 TGGACACCGC CCTGTACGAC GGCACCGCCT CGGACGGCGA GCAGGCGCTC
1551 TACATGAGCA TGCCCTGCCC CTGAGAATTC TAACTTGTTT ATTGCAGCTT
1601 ATAATGGTTA CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA
1651 TTTTTTTCAC TGCATTCTAG TTGTGGTTTG TCCAAACTCA TCAATGTATC
1701 TTATCATGTC TTCTAGAGCG GCCGCCACCG CGGTGGAGCT CCAGCTTTTG
1751 TTCCCTTTAG TGAGGGTTAA TTGCGCGCTT GGCGTAATCA TGGTCATAGC
1801 TGTTTCCTGT GTGAAATTGT TATCCGCTCA CAATTCCACA CAACATACGA
1851 GCCGGAAGCA TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT
1901 CACATTAATT GCGTTGCGCT CACTGCCCGC TTTCCAGTCG GGAAACCTGT
1951 CGTGCCAGCT GCATTAATGA ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG
2001 CGTATTGGGC GCTCTTCCGC TTCCTCGCTC ACTGACTCGC TGCGCTCGGT
2051 CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT
2101 TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC
2151 CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA
2201 TAGGCTCCGC CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA
2251 GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA
2301 AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA CCGGATACCT
2351 GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT AGCTCACGCT
2401 GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG
2451 CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG
2501 TCTTGAGTCC AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA
2551 CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC
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2601 TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGAACAG TATTTGGTAT
2651 CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT
2701 GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG
2751 CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT
2801 TTCTACGGGG TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT
2851 TGGTCATGAG ATTATCAAAA AGGATCTTCA CCTAGATCCT TTTAAATTAA
2901 AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA CTTGGTCTGA
2951 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT
3001 TTCGTTCATC CATAGTTGCC TGACTCCCCG TCGTGTAGAT AACTACGATA
3051 CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC
3101 ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG
3151 CCGAGCGCAG AAGTGGTCCT GCAACTTTAT CCGCCTCCAT CCAGTCTATT
3201 AATTGTTGCC GGGAAGCTAG AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG
3251 CAACGTTGTT GCCATTGCTA CAGGCATCGT GGTGTCACGC TCGTCGTTTG
3301 GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA
3351 TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT
3401 TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC
3451 TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT
3501 GGTGAGTACT CAACCAAGTC ATTCTGAGAA TAGTGTATGC GGCGACCGAG
3551 TTGCTCTTGC CCGGCGTCAA TACGGGATAA TACCGCGCCA CATAGCAGAA
3601 CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG AAAACTCTCA
3651 AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC
3701 CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA
3751 AAACAGGAAG GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGGAAA
3801 TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA
3851 GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA
3901 AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC AC
```

NATscreenF; NATscreenR: underlined

Xho 1, HindIII, EcoR1, Xba1: dbl underlined

Region 674-998 bp is the SV40 early promoter
Region 1005-1574 bp is the NAT gene
Region 1581-1711 bp is the SV40 polyA