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**DEVELOPMENT OF A TRANSFORMATION  
PROTOCOL AND CELL CULTURE SYSTEM FOR THE  
COMMERCIALY IMPORTANT SPECIES OF RED  
MACROALGA, *GRACILARIA GRACILIS***

**by**

**Suzanne Margaret Huddy**

A thesis submitted for the degree of Doctor of Philosophy  
in the Department of Molecular and Cell Biology,  
Faculty of Science, University of Cape Town,  
South Africa.

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**ABSTRACT**

*Gracilaria gracilis* belongs to a commercially important genus of red macroalgae that is used in the production of two commercially important grades of agar. In South Africa, the *Gracilaria* industry used to depend solely on the natural *G. gracilis* resource growing in Saldanha Bay, a resource which has been unreliable for commercial collections due to numerous population collapses over the past few years. Suspended cultivation has been suggested as the only means of establishing a reliable *Gracilaria* industry in South Africa. This type of cultivation however, is intensive and often leads to increased disease burden and stress. A better understanding of how this commercially important seaweed responds at a genetic level to stresses faced in the aquaculture environment would not only be advantageous to the South African industry, but this knowledge is essential for selecting and/or engineering macroalgal strains that are either more tolerant or resistant to these stresses. This requires *in vivo* analysis of *G. gracilis* gene function and regulation in order to introduce new or improved genes into *G. gracilis*, and for this to be possible, a protocol for transformation of recombinant DNA into *G. gracilis* is required.

In this study a transformation and tissue culture system for *G. gracilis* was developed. These tools provide the necessary groundwork for future genetic manipulation studies that are essential for improving our understanding of the role that various genes play in stress response and tolerance in *G. gracilis*.

Microparticle bombardment of *G. gracilis* thalli was investigated and optimized as a means to deliver foreign DNA into the macroalga. This technique proved successful for transformation of *G. gracilis* thalli with the *lacZ* reporter gene under the influence of the Simian virus 40 (SV40) promoter. Transient expression of the *lacZ* reporter gene was investigated further by comparing the effectiveness of the cauliflower mosaic virus 35S (CaMV 35S) and the Cytomegalovirus (CMV) promoters. The SV40 promoter was identified as the best promoter and selected for further transformation studies in this investigation.

In addition to an effective foreign DNA delivery method, stable transformation of macroalgae requires clonal seaweed culture and techniques for plant regeneration from single cells. The isolation of macroalgal protoplasts and their regeneration into whole plants has been more successful when compared to callus induction and subsequent whole plant regeneration. In this study, *G. gracilis* protoplasts were investigated as a suitable cell culture system. A protocol for protoplast isolation and purification was developed and optimized in order to ensure that large quantities of viable protoplasts could consistently be isolated from *G. gracilis* thalli. Cell wall re-synthesis of *G. gracilis* protoplasts, the first step towards whole plant regeneration, was shown to occur over the first 24 hrs of culture using calcofluor staining and scanning electron microscopy. Furthermore, the effect of light intensity and incubation temperature on whole plant regeneration from *G. gracilis* protoplasts was investigated. Under conditions of low light intensity ( $5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and high incubation temperature (18-19 °C), *G. gracilis* protoplasts underwent cell division that resulted in the formation of cell clumps. However, under conditions of higher light intensity ( $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and either high (18-19 °C) or low (14-15 °C) culture temperatures, whole plants were regenerated from protoplasts. These either regenerated slowly over a period of 4-6 months to produce plants which resembled the parental plants, exhibiting slender, branched thalli, or regenerated rapidly over a period of 1-3 months to produce plants which remained small with thalli that were thick and unbranched. To the best of our knowledge, this study is the first to describe whole plant regeneration from protoplasts of the commercially important agarophyte *G. gracilis*.

Having demonstrated that *G. gracilis* protoplasts can develop into mature plants, we investigated the possibility of transforming *G. gracilis* protoplasts by PEG-mediated transfection. A transformation protocol for these protoplasts was successfully developed and

optimized. This was subsequently used to investigate the effects of 18S rDNA-targeted homologous recombination (HR) and tobacco Rb7 matrix attachment regions (MARs) on transgene expression using *egfp* as a test gene. This was done in an effort to identify a possible means of increasing transgene expression. A suite of vectors was designed, constructed and transfected into *G. gracilis* protoplasts after which EGFP levels and the presence of *egfp* were monitored for nine days post-transfection. The presence of tobacco Rb7 MARs and 18S rDNA regions on vector DNA resulted in significant ( $P<0.05$ ) increases in relative fluorescence and therefore EGFP levels at both 3 and 4 days post-transfection. Furthermore, targeted HR was also shown to have taken place in *G. gracilis* protoplasts transfected with vector DNA containing 18S rDNA homologous regions.

As successful transformation in plant systems requires the use of selectable markers, *G. gracilis* protoplasts were assessed for their sensitivity to the antibiotics kanamycin and chloramphenicol and the herbicide BASTA<sup>®</sup>. While *G. gracilis* protoplasts exhibited a high level of resistance to kanamycin and chloramphenicol, they were shown to be sensitive to BASTA<sup>®</sup>. Vectors containing the *bar* gene (which confers resistance to the herbicide) under the influence of the SV40 promoter/enhancer were constructed and transfected into protoplasts which were assessed for their ability to survive BASTA<sup>®</sup> selection. Transfected *G. gracilis* protoplasts exhibited significantly ( $P<0.01$ ) increased survival percentages in comparison to the negative control protoplasts which were not transformed with vector DNA. In addition, 4-5% of the protoplasts survived a second round of selection 21 days post-transfection.

This study has established some key genetic tools which are essential for future genetic manipulation studies in *G. gracilis*. These include effective methods to deliver foreign DNA into *G. gracilis* thalli and protoplasts, a screening mechanism to select *G. gracilis* transformants from a multitude of recipients and a method to regenerate whole plants from protoplasts. This work also adds to the current scientific knowledge of general macroalgal, and more substantially and specifically to *G. gracilis*, transformation systems.

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## TABLE OF CONTENTS

	Abstract	ii
	Nomenclature	v
<b>CHAPTER 1</b>	General Introduction	<b>1</b>
<b>CHAPTER 2</b>	Microparticle bombardment of <i>Gracilaria gracilis</i> thalli	<b>24</b>
<b>CHAPTER 3</b>	Regeneration of whole plants from <i>Gracilaria gracilis</i> protoplasts	<b>59</b>
<b>CHAPTER 4</b>	Transfection of <i>Gracilaria gracilis</i> protoplasts	<b>119</b>
<b>CHAPTER 5</b>	General discussion and future research	<b>174</b>
<b>APPENDIX A</b>	Media and Solutions	<b>180</b>
<b>APPENDIX B</b>	Standard Methods	<b>198</b>
<b>APPENDIX C</b>	Construction of vectors for microparticle bombardment	<b>229</b>
<b>APPENDIX D</b>	Construction of vectors for protoplast transfection	<b>236</b>
<b>LITERATURE CITED</b>		<b>247</b>

## NOMENCLATURE

### ABBREVIATIONS

A	adenine
Amp	Ampicillin
Amp <sup>r</sup>	Ampicillin resistant
ANOVA	analysis of variance
bp	Base pairs
BSA	Bovine serum albumin
C	cytosine
CFU	colony forming units
cm	centimeter(s)
Cm	Chloramphenicol
Cm <sup>r</sup>	Chloramphenicol resistant
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP)
EDTA	ethylenediaminetetra-acetic acid
g	gram(s)
G	guanine
hr(s)	hour(s)
k	kilo
K	thousand
kb	kilobase(s)
kDa	kilodalton(s)
Kg	kilogram(s)
KI	potassium iodide
Km	Kanamycin
Km <sup>r</sup>	Kanamycin resistance
km	kilometre(s)
l	liter(s)
LA	Luria agar
LB	Luria broth
m	meter
M	molar
mA	milli-Amperes
MA	marine agar
MB	marine broth
MCS	multiple cloning site
mg	milligram(s)

## Nomenclature

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min	minute(s)
ml	milliliter(s)
mm	millimetre(s)
mM	millimolar
mol	mole(s)
mU	milliunit(s)
Nal	Nalidixic acid
Nal <sup>r</sup>	Nalidixic acid resistant
ng	Nanogram(s)
nm	Nanometer(s)
O/N	overnight
OD	optical density
p	plasmid
PCR	polymerase chain reaction
per. comm.	personal communication
psi	pounds per square inch
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
s	second(s)
SDS	sodium dodecyl sulphate
SEM	standard error of means
T	thymine
TAE	tris-acetate-EDTA buffer
TE	tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UV	ultraviolet
V	volts
v	volume
W	Watt(s)
w	weight

## SYMBOLS

$\alpha$	alpha
$\beta$	beta

## Nomenclature

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\$	dolar(s)
$\lambda$	lambda
$\mu$	micro
$\mu\text{g}$	microgram(s)
$\mu\text{l}$	microlitre(s)
$\mu\text{m}$	micrometer(s)
$\mu\text{M}$	micromolar(s)
$\mu\text{mol}$	micromole(s)
$M_r$	relative molecular mass
%	percentage
$\Psi$	psi
$^{\circ}\text{C}$	degrees Celcius

University of Cape Town

# **CHAPTER 1**

## **GENERAL INTRODUCTION**

## Contents

1.1	Aquaculture .....	3
1.2	Seaweed aquaculture .....	4
1.2.1	The uses of seaweeds .....	4
1.3	The genus <i>Gracilaria</i> .....	6
1.3.1	Classification.....	6
1.3.2	Life cycle .....	7
1.3.3	Habitat and general biology .....	9
1.4	<i>Gracilaria</i> farming .....	9
1.5	Uses of genetically engineered algae .....	13
1.6	Current status of macroalgal genetics .....	13
1.7	Transformation techniques.....	17
1.8	Algal tissue culture.....	18
1.8.1	Algal callus culture .....	18
1.8.2	Protoplasts.....	19
1.8.2.1	The uses of protoplasts.....	21
1.9	Concluding remarks .....	22
1.10	Aims and objectives of this study .....	23

## 1.1 Aquaculture

Aquaculture, defined as “the cultivation or rearing of aquatic animals and/or plants in a controlled environment for all or part of their lifecycle” (<http://www.siu.edu/~readi>), has been practiced for centuries dating back as early as the fifth century BC (Bardach *et al.*, 1972). Aquaculture is widely considered to be one of the fastest growing food-producing sectors in the world, estimated at 68.3 million tonnes and US\$ 106 billion in 2008 (FAO, 2011). Worldwide, the sector has grown at an average rate of 8.3% per year since 1970, compared with only 1.2% for capture fisheries and 2.7% for terrestrial farmed meat production systems over the same period (FAO, 2007; 2009 and 2011). The predicted population growth over the next two decades will mean that an additional 40 million tonnes of aquatic food will be required by 2030 to maintain the current per capita consumption and aquaculture has the greatest potential for meeting this growing demand (FAO, 2007).

Freshwater fish contribute 54.7% of the global aquaculture industry by weight and 41.2% by value. Aquatic plants, while only being the fourth most important in value, contribute the second largest quantity at 23.8%. Crustaceans make up 9.5% by quantity and 23% of the value, while molluscan aquaculture accounts for 24.9% by quantity and 13.3% by value (FAO, 2011).

Asia and the Pacific region supply 99.8% of cultured aquatic plants, 98% of cyprinids, 88% of penaeids and 95% of oysters. This accounted for 88.8% of the production quantity and 78.7% of the value of global aquaculture in 2008 (FAO, 2011). Sub-Saharan Africa however plays a minor role in aquaculture and accounted for only 0.5% of the global aquaculture production in 2008 (FAO, 2011). Currently, African countries import about 4.2 million tonnes of fishery products per annum (Brummett *et al.*, 2008). Although a substantial market exists and adequate land and water resources are available for use, there has been a lack of aquaculture development in Africa.

In developing countries, aquaculture could serve as a valuable resource for earning foreign currency and boosting economic growth through job creation. It is estimated that 8% of the world’s population is dependent on this sector in terms of work (FAO, 2009). Furthermore, aquaculture provides a means of supporting and supplementing local diets adding to food security, while enhancing conservation of natural populations.

Despite the minor role South Africa plays in global aquaculture, the aquaculture of marine organisms (mariculture) is becoming a rapidly developing sector in this country. The mariculture industry focuses on high-value niche-market species including seaweeds, mussels, oysters, abalone and prawns ([http://www.fao.org/fishery/countrysector/FI-CP\\_ZA/1/en](http://www.fao.org/fishery/countrysector/FI-CP_ZA/1/en)).

### 1.2 Seaweed aquaculture

Mariculture accounts for 50.9% of global aquaculture production, and is estimated at 30.2 million tonnes and US\$ 28.1 billion (Chopin and Sawhney, 2009). Aquatic plants represent 45.9% of the tonnage and 24.2% of the value of the global mariculture industry. The increase in demand for seaweeds over the past fifty years has surpassed the amount that can be supplied by natural stocks (FAO, 2003). However, research and improved understanding of the life cycles of the commercially important seaweeds has led to the development of a cultivation industry which now supplies the majority of the world's seaweed requirements (93.8%) (FAO, 2011). This is estimated at 11.3 million tonnes and US\$ 5.7 billion (Chopin and Sawhney, 2009).

Although 220 species of algae are cultivated worldwide, only six genera contribute 94.8% of the seaweed aquaculture production. These six genera include: *Laminaria*, commonly referred to as kombu; (40.1%), *Undaria* or wakame, (22.3%), *Porphyra* or nori; (12.4%), *Eucheuma/Kappaphycus*, (11.6%) and *Gracilaria*, (8.4%). Four of these genera, *Gracilaria*, *Laminaria*, *Porphyra*, and *Undaria* provide 95.6% of the seaweed aquaculture value (Chopin and Sawhney, 2009).

#### 1.2.1 The uses of seaweeds

The use of seaweeds as a human food source has been traced back to as early as the fourth century in Japan and the sixth century in China (FAO, 2003). Today the largest use of seaweeds is still as a food source. Approximately 76.1% of the tonnage and 88.3% of the value of the seaweed industry is allocated to the sea-vegetable sector (Chopin and Sawhney, 2009). About 5 million wet tonnes of edible seaweed is harvested in China every year (FAO, 2003). *Laminaria japonica* (kombu), *Undaria pinnatifida* (wakame) and *Porphyra* (nori) are

the most prominent kinds. Of these, nori is the most valuable fetching approximately US\$ 16,000 per dry tonne (FAO, 2003).

Red seaweeds (Rhodophyta) and brown seaweeds (Phaeophyta) are used in the production of three hydrocolloids: agar, carrageenan and alginate (Wikfors and Ohno, 2001). A hydrocolloid can be broadly defined as a non-crystalline substance that dissolves in water to yield a viscous solution and as such, hydrocolloids are used to stabilize many products (FAO, 2003). The use of seaweeds for their hydrocolloids dates back to the late 1650s, but commercial production only began in the 1930s (FAO, 2003). Today the phycocolloid sector accounts for 11.2% of the tonnage and 10.8% of the value of the seaweed industry (Chopin and Sawhney, 2009).

Alginate is extracted from brown seaweeds and is generally used as a gelling, emulsifying and stabilizing agent in the textile, food, paper, welding and pharmaceutical industries. It is also used as a binder in fish feeds, as a releasing agent in moulds and in the immobilization of biocatalysts (Anderson *et al.*, 1989; Renn, 1997; FAO, 2003; Bixler and Porse, 2010).

Red seaweeds are used as a source of agar and carrageenan (Renn, 1997). Carrageenan is used mainly as a thickening and stabilizing agent in the food industry. It is also used to immobilize biocatalysts (Renn, 1997; FAO, 2003). Agar is largely extracted from two genera of seaweeds, *Gelidium* and *Gracilaria*, where *Gracilaria* comprises approximately 80% of the agar market (McHugh, 2002; Bixler and Porse, 2010). *Gracilaria* species were once thought to be unsuitable for agar extraction because of the poor gelling strength of their agar. However, in the late 1950s it was found that an alkali pre-treatment of the seaweed lead to the yield of a better quality agar. *Gracilaria chilensis* and *Gracilaria gracilis* are the main species of economic importance based on agar yield and quality (De Oliveira *et al.*, 2000).

Approximately 90% of the agar harvested every year is used in the food industry as a stabilizer and thickener in foods such as jellies, mayonnaise, processed cheese and sweets (Armisen, 1995; Renn, 1997; FAO, 2003). The other 10% is used for bacteriological and other biotechnological purposes. Bacteriological agar can only be made from species of *Gelidium*; this is because of the lower gelling temperature of the agar (FAO, 2003).

Seaweeds are also used to produce seaweed meal which is used as an additive in animal foods or as a direct feed for other aquaculture species such as abalone and sea urchins (Phillips, 2009). They are used in the cosmetics industry in products containing “seaweed extract”, as fertilizers, especially as liquid extracts (Dhargalkar and Pereira, 2005). Seaweeds can also potentially be used in the management of wastewater, such as water polluted with heavy metals or wastewater from fish and abalone farms (Robertson-Andersson *et al.*, 2008). A large amount of research has also been focused on the use of seaweeds as an indirect source of fuel (FAO, 2003).

### 1.3 The genus *Gracilaria*

#### 1.3.1 Classification

Rhodophyta (red algae) is a morphologically diverse group of macroalgae consisting of more than 700 genera and 6,000 species (Chapman *et al.*, 1998). Although termed red algae, these algae occur in a variety of colours (red, pink, violet, blue, brown, black, yellow and green) depending on the varying proportions of the pigments they contain (Woelkerling, 1990). Rhodophyta are classified on the basis of several characteristics:

- i) the lack of flagellae at any stage of their life history
- ii) the storage of floridean starch in the cytoplasm
- iii) unstacked thylakoid membranes in the plastids and lack of an encircling endoplasmic reticulum membrane around them
- iv) the presence of red/blue phycobilin accessory pigments (Maggs *et al.*, 2007)

Gracilarioid algae, belonging to the phylum Rhodophyta, are generally described as macroscopic algae which can range from 0.1 to 5 m in length (<http://www.algaebase.org>). *Gracilaria* species are classified based on several morphological features: shape of the thallus (terete, compressed, or flattened to foliose), branching mode (dichotomous, alternative to irregular, little to extensively branched), grade of constriction along the thallus and reproductive structures (morphology of the cystocarp, patterns of spermatangia and tetrasporangial distribution and size) (Fredericq and Hommersand, 1990; De Oliveira and Plastino, 1994). Species distinction based on these characteristics is however problematic due to the fact that morphologies are shared by many species and they are subject to influence

by external factors (Bird, 1995). Traditional taxonomic approaches have been more recently supplemented with studies on chromosome number, characterization of cell wall polysaccharides, carotenoids and fatty acids. These supplemental methods however, were said to be restricted in use and not practical. The use of molecular techniques such as random amplification of polymorphic DNA (RAPD) and sequence analysis of the 18S rRNA and Rubisco genes as well as the Rubisco spacer region, have consequently been employed with great success, not only enabling relatively easy distinction between similar looking species but also inferring phylogenetic relationships between species (De Oliveira and Plastino, 1994; Lim *et al.*, 2001; Byrne *et al.*, 2002; Iyer *et al.*, 2005).

*Gracilaria* species are classified as follows (Bird and Kain, 1995):

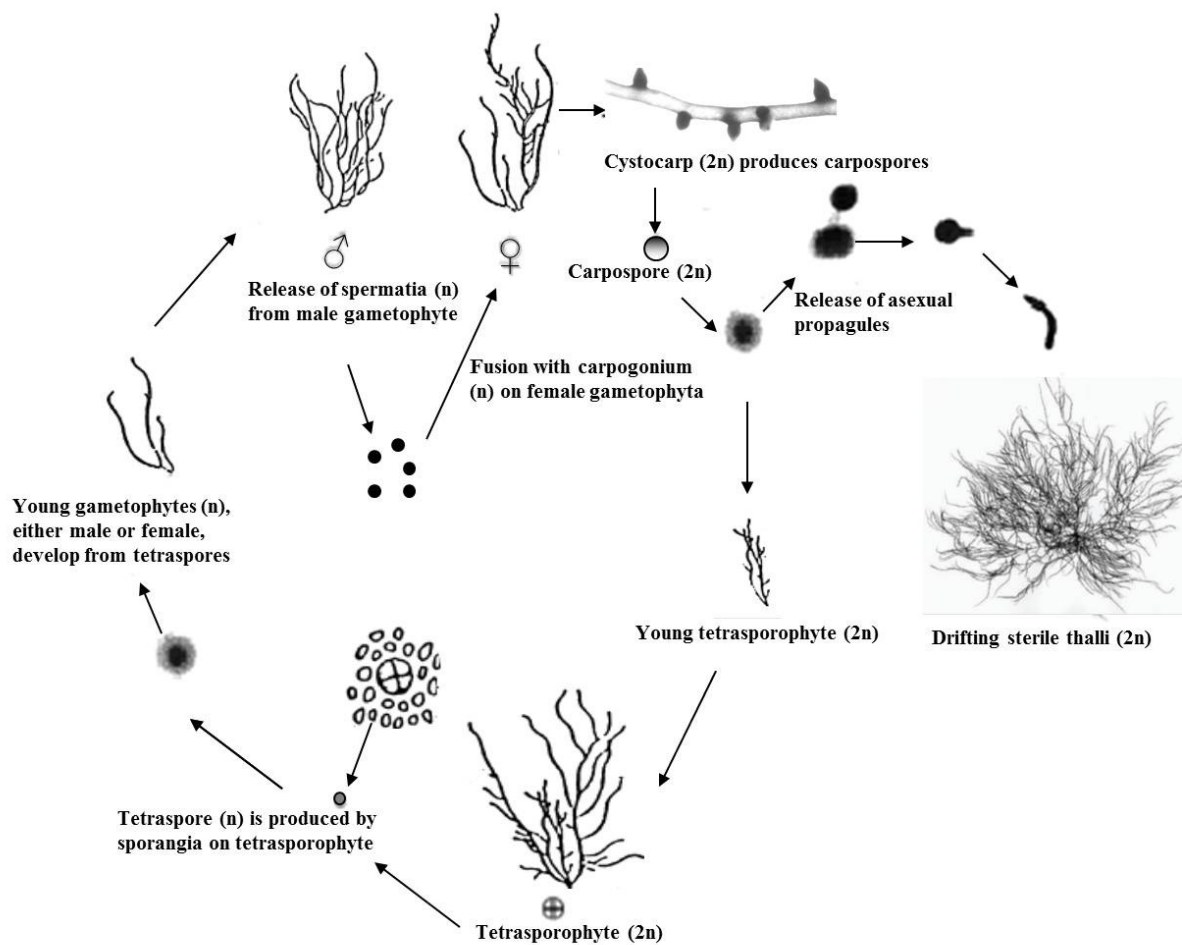
Division	Rhodophyta
Class	Florideophyceae
Order	Gracilariales
Family	Gracilariaceae

### 1.3.2 Life cycle

*Gracilaria* species reproduce sexually by a typical *Polysiphonia*-type life history (Fig. 1.1) (De Oliveira and Plastino, 1994; Kain and Destombe, 1995). This life cycle is referred to as triphasic since there are three phases present during the life cycle. These are: the diploid (tetrasporophyte) phase, the haploid (gametophyte) phase and the additional diploid (carposporophyte) phase (Engel *et al.*, 2001). Fertilization of a female gamete gives rise to a diploid zygote which germinates within the mother plant and gives rise, by mitosis, to diploid spores (carpospores). The carpospores, once released, attach to the substratum and give rise to a tetrasporophytic plant which produces meiotic sporangia. The sporangia produce haploid spores (tetraspores) which give rise to either male or female plants (De Oliveira and Plastino, 2000). The diploid tetrasporophyte and haploid gametophyte phases are morphologically identical and can only be distinguished under a microscope. Fertilized female plants can, however, be identified without the use of a microscope when cystocarps are present.

Deviations from this typical life history strategy, such as the presence of mixed reproductive phases, have been described for many species of *Gracilaria* (Plastino and De Oliveira, 1988; Destombe *et al.*, 1989; De Oliveira and Plastino, 1994). *Gracilaria* can also propagate

vegetatively through fragmentation of thalli (Goldstein, 1973; Marinho-Soriano *et al.*, 1998) and in some cases entire populations have been reported to be sterile and propagate by thallus fragmentation alone (Engeldow and Bolton, 1992). This is said to often be the case with unattached and farmed populations (Santelices and Doty, 1989; Dawes, 1995). Although not a common occurrence, vegetative propagation through the formation and release of propagules has also been recorded in *Gracilaria* (Fig. 1.1) (Plastino and De Oliveira, 1988; Yan and Wang, 1993; Polifrone *et al.*, 2006).



**Figure 1.1.** Model of the typical *Polysiphonia*-type life-cycle of *Gracilaria* species with the deviation of the release of asexual propagules included (adapted from Polifrone *et al.*, 2006).

### 1.3.3 Habitat and general biology

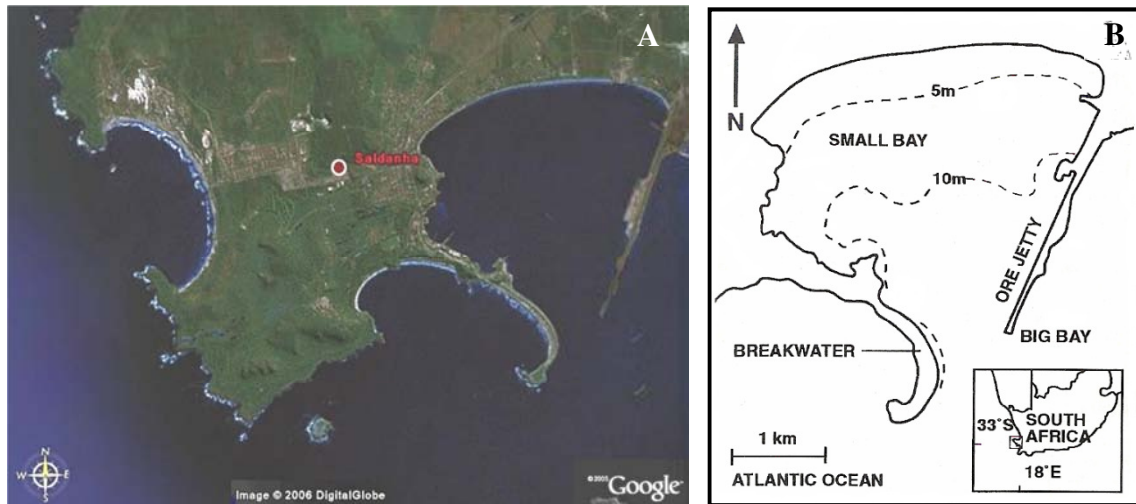
Gracilarioid algae are distributed throughout the world with most species being found in the warmer waters of the northern hemisphere. However, there are a few species, such as *G. gracilis* (previously known as *G. verrucosa* prior to 1995), that extend their distribution to temperate waters (De Oliveira *et al.*, 2000).

*G. gracilis* normally occurs in the lower intertidal and upper subtidal regions of the ocean where it is generally found attached to the substratum by a holdfast. Erect terete thalli with lateral branches of varying lengths develop from these holdfasts. A cross-section through the thallus shows large unpigmented, medullary cells positioned at the centre, with a gradual decrease in cell size towards the subcortical and highly pigmented cortical cells situated closer to the outer cuticle of the thallus (De Oliveira and Plastino, 1994; Jaffray, 1998).

### 1.4 *Gracilaria* farming

*Gracilaria* species are currently considered to be the most important algae for use in food grade agar production (De Oliveira *et al.*, 2000). The growing demand for agar from *Gracilaria* species over the years put an increasing strain on the natural stocks which as a result were depleted in many countries (Santelices and Doty, 1989; De Oliveira *et al.*, 2000). *Gracilaria* species are now cultivated in ponds and protected bays with great success in countries such as Chile, China and Taiwan (Armisen, 1995; Buschmann *et al.*, 1995; Friedlander and Levy, 1995; Alveal *et al.*, 1996; De Oliveira *et al.*, 2000; Buschmann *et al.*, 2001).

The South African *Gracilaria* industry began in the early 1950s with commercial collections of beach-cast *G. gracilis* in Saldanha Bay (Fig. 1.2), shortly after agar supplies from Japan became limited during World War 2 (Fox and Stephens, 1943; Anderson *et al.*, 1989; Rotmann, 1990). The local agar production ceased shortly after this for economic reasons, but it was re-established in the 1960s with two factories functioning until 1974 when the supply of beach-cast *Gracilaria* collapsed due to the dredging and construction of a large ore-loading jetty (Fig. 1.2) (Anderson *et al.*, 1989).



**Figure 1.2** Saldanha Bay, South Africa. A) Google Earth Satellite Image; B) diagram of Saldanha Bay (Anderson *et al.*, 1999).

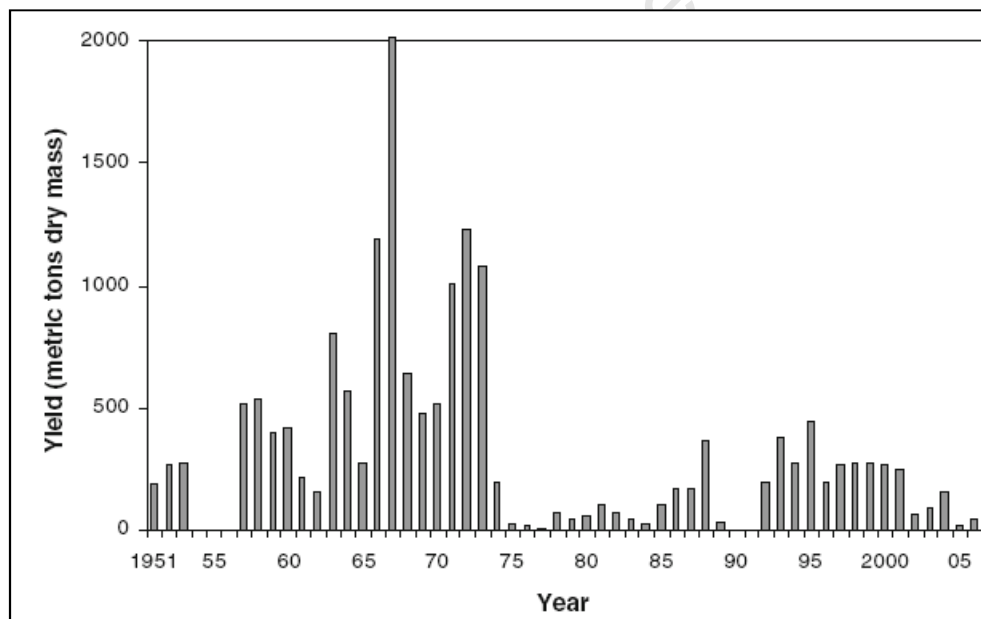
Over the next few years the beach-cast resource (Fig. 1.3) slowly increased to approximately 170 tonnes (dry mass) in 1987, but this was no comparison to the 2,000 tonnes (dry weight) collected in 1967 (Fig. 1.4) (Anderson *et al.*, 1989; Anderson *et al.*, 1996a). In 1989, the natural *G. gracilis* population again experienced one of these die-offs and as a result no commercial harvesting was possible for the next three years (Anderson *et al.*, 1996a; Jaffray *et al.*, 1997; Rothman *et al.*, 2009). *G. gracilis* yields became more stable in 1992, with up to 400 tonnes dry weight being harvested annually (Fig. 1.4) (Anderson *et al.*, 1996a).



**Figure 1.3** Beach-cast *G. gracilis* collection in Saldanha Bay (Rob Anderson, <http://www.algaebase.org>).

In the summer of 1993-1994, much of the beach cast *Gracilaria* was contaminated with a bloom of *Ulva* which resulted from localized eutrophication by fish-processing waste released into the bay (Anderson *et al.*, 1996b). As a result, much of the beach-cast collection

during this time had to be discarded (Anderson *et al.*, 1996b). The population never fully recovered and became unreliable for export and local agar production (Anderson *et al.*, 1996a). In an effort to develop a stable *Gracilaria* industry, research on suspended cultivation in Saldanha Bay began in 1990 (Anderson *et al.*, 2001). The cultivation was adapted from successful methods employed at Lüderitz Bay in Namibia and initially, relatively high growth rates of approximately  $5\% \text{ day}^{-1}$  were obtained (Dawes, 1995; De Oliveira *et al.*, 2000). However, two subsequent attempts at commercial farming had to be abandoned due to poor growth and, fouling by mussels and tunicates in the summer months (Anderson *et al.*, 2001). Over the last decade, the yields of beach-cast *Gracilaria* have never been above 300 tonnes fresh weight per year and the most recent survey predicted the standing stock in Small Bay in 2006 at 538 tonnes fresh weight (Rothman *et al.*, 2009). The most recent documented collapse in the Saldanha Bay resource was in 2005 (Rothman *et al.*, 2009).



**Figure 1.4** Commercial yields of *Gracilaria* from Saldanha Bay showing the large population collapses after construction of the ore jetty in 1974 (Rothman *et al.*, 2009).

There are three possible reasons for the collapses in the *Gracilaria* population in Saldanha Bay, all of which may occur concurrently:

- The construction of the breakwater and ore-jetty generated changes in the water flow characteristics within the bay. This led to the development of strong thermal stratification of the water column, with oligotrophic surface waters prevailing in the

summer months (Anderson *et al.*, 1996b). The absence of utilizable carbon sources in the water column during these summer months can cause some of the bacterial epiphytes to become pathogenic to their host in response to this environmental stress. These bacteria then metabolise the agar in the *Gracilaria* cell wall by producing agarase enzymes. This results in thallus bleaching and eventual death of the algae (Jaffray and Coyne, 1998; Schroeder *et al.*, 2003). There are other instances of disease caused by agarolytic bacteria in *Gracilaria* species. These include 'rotten thallus' syndrome and 'white-tip disease' of *Gracilaria conferta* (Friendlander and Gunkel, 1992; Lavilla-Pitogo, 1992).

- The prolonged low nutrient levels during summer may also starve the *G. gracilis* plants of nitrogen and lead to poor growth and eventual death (Anderson *et al.*, 1996b). This hypothesis is supported by the results of growth trials carried at two different sites in Saldanha Bay by Anderson *et al.* (1999). The *Gracilaria* growing at the site where additional nitrogen was available from the discharge of fish-processing waste during the summer months, showed consistently high growth rates, while the *Gracilaria* that was grown at the control site 1.5 km away, showed decreased growth rates during the summer months and at one stage all of the planted *Gracilaria* in this control area died (Anderson *et al.*, 1999).
- A third possible reason for the collapses in the population is overgrazing by invertebrates and fish species (Anderson *et al.*, 1993).

St. Helena Bay, which is about 25 km north of Saldanha Bay, has been investigated as an alternative site for culturing gracilarioids in South Africa. The dominant species of gracilarioid occurring here is *Gracilariopsis longissima* (Govender, 2001). While this site is not subject to the problems experienced in Saldanha Bay during the summer months, 'black tides' occur in St. Helena Bay. During these black tides, low oxygen levels, which occur as a consequence of plankton blooms, result in toxic levels of hydrogen sulphide due to anaerobic decomposition of organic matter (Wakibia *et al.*, 2001; Rothman *et al.*, 2009). Black tides have proved fatal to the gracilarioids being cultivated in St. Helena Bay during these events (Wakibia *et al.*, 2001) and as a result, this site was also considered an unsuitable alternative for the culture of gracilarioids (Rothman *et al.*, 2009).

Although most of the gracilarioids collected in South Africa were exported for their agar, there has been increasing local use of these seaweeds for abalone feed (Rothman *et al.*, 2009). When used in a mixed diet with kelp and *Ulva*, they improve the growth rates of farmed abalone (Naidoo *et al.*, 2006; Troell *et al.*, 2006). Rothman *et al.* (2009) suggest that this might be a more suitable use for the South African *Gracilaria* resource.

In order to ensure a regular and healthy supply of the seaweed, the matter of maintaining a disease-free *G. gracilis* resource needs to be addressed. This would mean ensuring that cultured seaweed is not subjected to stresses causing disease, or selecting for and/or engineering macroalgal strains that are either more tolerant or resistant to these stresses. The success in higher plants and the progress in molecular transformation tools suggest this will soon be possible for macroalgae.

### **1.5 Uses of genetically engineered algae**

Besides the potential for producing strains with increased stress tolerance, genetic manipulation can lead to the use of macroalgae as marine bioreactors with potential applications in bioremediation, production of improved mariculture feeds and production of pharmaceuticals and oral vaccines (Qin *et al.*, 1999; Qin *et al.*, 2005). Substantial advances in these fields are already apparent in microalgae. Transgenic microalgae for monitoring and bioremediation of heavy metal-contaminated wastewaters and sediments have already been produced (Cai *et al.*, 1999; Siripornadulsil *et al.*, 2002; León-Bañares *et al.*, 2004; Rajamani *et al.*, 2007). Similarly, strains for bio-diesel, vaccine and therapeutic protein production have also been developed (León-Bañares *et al.*, 2004; Mayfield and Franklin, 2005; Walker *et al.*, 2005; Siripornadulsil *et al.*, 2007; Rasala *et al.*, 2010). Although none of these strains have been used in commercial applications to date, they do hold potential.

### **1.6 Current status of macroalgal genetics**

Seaweed biotechnology only began in the early 1990s and lags behind the great deal of progress made in our understanding and ability to exploit the genetics of higher plants and microalgae (Qin *et al.*, 2005). There are relatively few reports of transformation of a limited number of macroalgal species and, research has centred on the commercially important red and brown algae (Reddy *et al.*, 2008a). Initially, investigations focused on transient

expression of reporter genes under the control of promoters that had been used for this purpose in higher plants and unicellular algae (Qin *et al.*, 2004). Transient gene expression has been reported in: *Kappaphycus alvarezii* explants (Kurtzman and Cheney, 1991; Wang *et al.*, 2010a), *Porphyra miniata* protoplasts (Kübler *et al.*, 1994), *Porphyra yezoensis* protoplasts (Mizukami *et al.*, 2004) and explants (Kuang *et al.*, 1998; Fukuda *et al.*, 2008; Mikami *et al.*, 2009; Uji *et al.*, 2010; Takahashi *et al.*, 2010; Hirata *et al.*, 2011; Mikami *et al.*, 2011; Son *et al.*, 2011), *Porphyra haitanensis* protoplasts (Wang *et al.*, 1994 referenced in Qin *et al.*, 2005) and conchospores (Wang *et al.*, 2010b), in explants of *Porphyra tenera* (Hirata *et al.*, 2011; Mikami *et al.*, 2011; Son *et al.*, 2011), *Porphyra okamurae* (Hirata *et al.*, 2011; Mikami *et al.*, 2011), *Porphyra onoi* (Hirata *et al.*, 2011; Mikami *et al.*, 2011), *Porphyra variegata* (Hirata *et al.*, 2011; Mikami *et al.*, 2011), *Porphyra pseudolinearis* (Hirata *et al.*, 2011; Mikami *et al.*, 2011), *Bangia fuscopurpurea* (Hirata *et al.*, 2011; Mikami *et al.*, 2011), in *Ulva lactuca* protoplasts (Huang *et al.*, 1996), *Ulva pertusa* explants (Kakinuma *et al.*, 2009), and *Gracilaria changii* explants (Gan *et al.*, 2003). In many of these cases, transient gene expression was achieved with the cauliflower mosaic virus (CaMV) 35S promoter. Besides the CaMV35S promoter, the ubiquitin promoter from maize, the *FCP* promoter from the diatom fucoxanthin chlorophyll a/c-binding protein gene and the *AMT* promoter from the adenine methyltransferase gene of the *Chlorella* virus have been employed in transient  $\beta$ -glucuronidase (GUS) expression in kelp (Wu, 2001 referenced in Qin *et al.*, 2004).

However, CaMV 35S-mediated expression of the *uidA* (GUS) reporter gene in *Laminaria* and *Undaria* indicated that this promoter might be tissue specific (Qin *et al.*, 1994 referenced in Qin *et al.*, 2005). Subsequently, there have been various reports of transient and stable expression of various reporter genes in *Laminaria* using the Simian virus 40 (SV40) promoter (Qin *et al.*, 2005). Transient expression of the *lacZ* gene with the SV40 promoter has also been observed in *G. changii* (Gan *et al.*, 2003) and *Haematococcus pluvialis* (Teng *et al.*, 2002). More recently, the ability of the *FCP* promoter to drive stable expression of the *uidA* reporter gene in parthenogenetic sporophytes of *L. japonica* was shown by Li *et al.* (2009).

In the last few years, the use of endogenous promoters to drive the expression of reporter genes in *Porphyra* has received much attention. The promoter sequence of the ribulose-bisphosphate-carboxylase / oxygenase (Rubisco) gene was used for the transient expression of three reporter genes in *P. yezoensis* protoplasts: *uidA* (GUS), a mutant of green fluorescent

protein (S65T-GFP) and firefly luciferase (*luc*) genes (Mizukami *et al.*, 2004). The endogenous *U. pertusa* Rubisco small subunit gene (*UprbcS1*) promoter has also been used to express enhanced green fluorescent protein (EGFP) (Kakinuma *et al.*, 2009). In a separate study, endogenous beta-tubulin flanking sequences were used to transiently express GUS in *P. yezoensis* protoplasts (Gong *et al.*, 2007). Furthermore, the *P. yezoensis actin 1* gene (*PyAct1*) promoter and the *P. yezoensis* glyceraldehyde-3-phosphate dehydrogenase gene (*PyGAPDH*) promoter have been effectively employed to drive the expression of a number of reporter genes in *P. yezoensis* (Mikami *et al.*, 2009; Takahashi *et al.*, 2010; Uji *et al.*, 2010; Hirata *et al.*, 2011; Mikami *et al.*, 2011). The *P. tenera* heat shock protein 70 gene (*PtHSP70*) promoter has also been shown to be functional in driving efficient expression reporter gene expression in both *P. tenera* and *P. yezoensis* (Son *et al.*, 2011). In addition, the *PyAct1* promoter has been utilised for the establishment of a fluorescent protein reporter system in *P. yezoensis* (Mikami *et al.*, 2011). Successful expression of a number of humanised and plant-adapted fluorescent proteins, including humanised *Zoanthus* sp. green (ZsGFP) and yellow (ZsYFP) fluorescent proteins, plant-adapted sGFP (S65T) and humanised *Anemonia majano* cyan fluorescent protein (AmCFP), has been achieved in *P. yezoensis* (Mikami *et al.*, 2011). This system has allowed the visualisation and study of phosphoinositides (PIs) and their derivatives (Mikami *et al.*, 2009), and the visualisation of subcellular localization of *P. yezoensis* transcription factors, elongation factor 1 (PyElf1) and multiprotein bridging factor 1 (PyMBF1), in *P. yezoensis* cells (Uji *et al.*, 2010). These are the first studies of this nature to be performed in macroalgae and they demonstrate the value of a robust transient expression system.

The *P. yezoensis* reporter expression system has also been strengthened through the development of a codon-adapted *uidA* reporter gene (PyGUS). PyGUS was shown to be functionally superior to native *uidA* when expressed under the influence of the *PyGAPDH* promoter (Fukuda *et al.*, 2008), indicating the value of reporter gene codon optimization. In a recent study by Hirata *et al.* (2011), the applicability of the *P. yezoensis* system, employing PyGUS and sGFP as reporters and the *PyAct1* promoter as a heterologous promoter, in other Bangiophycean algae was shown. However, while the *PyAct1* promoter was functional in other Bangiophycean algae, promoter activity was significantly lower than in *P. yezoensis* (Hirata *et al.*, 2011). Furthermore, Hirata *et al.* (2011) were unable to successfully express either PyGUS or sGFP under the influence of the *PyAct1* promoter in a number of Florideophycean algae (*Chondrus ocellatus*, *Gloiopeltis furcate*, *Gracilaria vermiculophylla*

and *Mazzaella japonica*). These results indicate that systems established in one species may not be optimal, or even functional in another.

Targeted homologous recombination (HR) and matrix attachment regions (MARs) have also been studied in *Porphyra* for their ability to enhance transgene expression. The effect of 18S rDNA-targeted HR on the expression of GUS in *P. yezoensis* protoplasts was assessed (Liu *et al.*, 2003). Preliminary results showed that targeted HR was in fact possible in this species of macroalgae and, that the presence of 18S rRNA gene sequences flanking the promoter and reporter gene construct resulted in increased transformation efficiency of *P. yezoensis* protoplasts and therefore increased GUS expression (Liu *et al.*, 2003). However, the study of Liu *et al.* (2003) is limited in that GUS expression was only assessed two days post-transformation and thus the prolonged effect of 18S rDNA-targeted integration on foreign gene expression was not investigated. In a further study, the effect of 18S rDNA-targeted HR in conjunction with MARs from silkworms on chloramphenicol acetyltransferase (*cat*) gene expression was investigated in *P. haitanensis* protoplasts (Zuo *et al.*, 2007). Zuo *et al.* (2007) showed that both the presence of targeted HR regions and MARs had a positive effect on CAT expression. However, since no protocol for the selection of transformed *P. haitanensis* was available the study only assessed transient expression of CAT over the first six days following transformation (Zuo *et al.*, 2007).

Few research groups, however, have reported stable transformation in macroalgae since this requires clonal seaweed culture and techniques for plant regeneration from single cells. These systems have not yet been developed in many species. *Laminaria* transformation has been the most successful to date, with reports of stable expression of the hepatitis B surface antigen (HBsAg) (Jiang *et al.*, 2002), *lacZ* (Jiang *et al.*, 2003), CAT (Jiang *et al.*, 2002), a recombinant tissue-type plasminogen activator (rtPA) (Gao *et al.*, 2005) and recently, GUS (Li *et al.*, 2009). There are reports of stable expression of *lacZ* in *Undaria pinnatifida* (Qin *et al.*, 2003 referenced in Walker *et al.*, 2005) and although full publications are not available, many have reported stable transformation of *Porphyra* in conference abstracts (Cheney *et al.*, 2001; He *et al.*, 2001; Lin *et al.*, 2001; Bernasconi *et al.*, 2004). Reporter genes that have been stably expressed in *Porphyra yezoensis* include *gluc* (glucose oxidase), *cat*, *uidA*, GFP (Cheney *et al.*, 2001; He *et al.*, 2001) and the bacterial nitroreductase gene *nsfI* (Bernasconi *et al.*, 2004). Recently, the prolonged expression of PyGUS has also been reported in regenerating *P. yezoensis* protoplasts (Takahashi *et al.*, 2010).

## 1.7 Transformation techniques

The transformation techniques applicable to macroalgae are largely dependent on the tissue-type to be transformed. Microparticle bombardment has proved successful in macroalgae, particularly when thallus explants have been used (Kurtzman and Cheney, 1991; Qin *et al.*, 1994; Gan *et al.*, 2003; Jiang *et al.*, 2003; Qin *et al.*, 2003). Microparticle bombardment is a technique whereby DNA is coated onto “micron-sized” metal particles that are then accelerated, under high velocities, into the cell’s interior. This technique was developed in the 1980s as an alternative method for transforming plants which were initially thought to be recalcitrant to *Agrobacterium*-mediated transformation (Taylor and Fauquet, 2002). It has also been successfully employed in transformation of animal cells (Williams *et al.*, 1991), bacteria (Smith *et al.*, 1992), unicellular algae (Tan *et al.*, 2005) and subcellular organelles (Boynton and Gillham, 1993 and 1996). The major advantage of using this method is that it is applicable to the transformation of whole cells and tough cell walls do not present a significant barrier to DNA entry (Taylor and Fauquet, 2002).

Protoplasts are living cells which are devoid of their cell walls (Reddy *et al.*, 2006). The advantages of using protoplasts in transformation studies are that a large amount of cells can be handled with relative ease and the absence of the cell wall means that there is one less obstacle to successful transformation. Polyethylene glycol (PEG) and/or electroporation have been successfully employed in the transformation of macroalgal protoplasts (Kübler *et al.*, 1994; Huang *et al.*, 1996; Kuang *et al.*, 1998; He *et al.*, 2001; Lin *et al.*, 2001; Liu *et al.*, 2003; Mizukami *et al.*, 2004; Gong *et al.*, 2007; Zuo *et al.*, 2007). PEG is thought to mediate transformation by interacting with the negatively charged DNA to form a positively charged complex that then interacts with the anionic protoplast membrane. DNA uptake into the cell is then thought to occur through active uptake or endocytosis (Veilleux *et al.*, 2004). During electroporation, the electric pulse applied is thought to create temporary pores in the cell membrane through which the DNA can enter the cell (Veilleux *et al.*, 2004). These are both direct methods of transformation. *Agrobacterium*-mediated transformation, although not a commonly employed technique for transformation of algae, has been reported to be successful in *P. yezoensis*, though fully published methods are not available (Cheney *et al.*, 2001; Bernasconi *et al.*, 2004).

## 1.8 Algal tissue culture

Seaweed tissue culture, as with transformation studies, is a fairly recent field of research and lags behind that of the higher plants. Much of the anticipated success of transformation of macroalgae is however dependent on the development of this field (Stevens and Purton, 1997). There are three main areas of focus in this field: *in vitro* cultivation, callus induction and culture, and protoplast isolation and culture (Kaczyna and Megnet, 1993).

### 1.8.1 Algal callus culture

In higher plants, callus is described as disorganized cell growth in differentiated tissue resulting from wounding (Yeoman, 1987). The idea of callus from seaweeds is consequently somewhat of a controversial topic, since many seaweeds lack a high degree of organization and differentiation (Aguirre-Lipperheide *et al.*, 1995). Instead, the term “callus-like” has been applied in many cases to the uniseriate, pigmented and branched filamentous outgrowths seen in both the red (pseudo-parenchymatous type tissue) and brown (parenchymatous type tissue) seaweeds (Garcia-Reina *et al.*, 1991; Yokoya *et al.*, 1993; Reddy *et al.*, 2008b).

Many of the studies pertaining to seaweed callus culture have concentrated on basic induction of callus in various species of macroalgae and the effects of plant growth regulators and carbon sources on this process (Gusev *et al.*, 1987; Polne-Fuller and Gibor, 1987; Bradley and Cheney, 1990; Robaina *et al.*, 1990; De Nys *et al.*, 1991; Kaczyna and Magnet, 1993; Kawashima and Tokuda, 1993; Aguirre-Lipperheide *et al.*, 1995; Yokoya and Handro, 1996; Huang and Fujita, 1997a and 1997b; Yokoya, 2000; Yokoya *et al.*, 2004; Rajahrishna Kumar *et al.*, 2007; Reddy *et al.*, 2008b). Although induction of callus has been achieved in many species of macroalgae, the problem remains that seaweed callus is often slow growing and small in size (Reddy *et al.*, 2008b). Often rates of callus induction are very low, and appear to be sporadic and more dependent on internal factors of the explants rather than on the external culture conditions (Aguirre-Lipperheide *et al.*, 1995). True callus culture (excised from the explant) is also not a common occurrence and to date there are very few species for which this is possible. Protoplasts, however, offer an alternative to seaweed callus culture. The success in this field, when compared with callus induction and culture, has led some to suggest that protoplasts may be the method of choice for seaweed tissue culture (Aguirre-Lipperheide *et al.*, 1995; Baweja *et al.*, 2009).

### 1.8.2 Protoplasts

Protoplasts offer a unique opportunity in that they are potentially totipotent and therefore, one or more plants could theoretically be regenerated from a single cell (Davey *et al.*, 2005a). For this particular reason, protoplasts have become an increasingly attractive cell system for use in transformation and somatic hybridization (Davey *et al.*, 2005a). Although protoplasts seem to offer much potential in the field of macroalgal tissue culture, they are difficult to work with due to their fragile nature. Unfavourable changes in osmotic pressure of the bathing medium which needs to balance or slightly exceed that of the cytosol, or excess mechanical stresses such as excessive centrifugation or pipetting through a narrow bore, can result in damage to the protoplasts (Warren, 1991).

Isolation of protoplasts from macrophytic benthic marine algae by mechanical methods was reported as early as 1970 (Tatewaki and Nagata, 1970; Enomoto and Hirose, 1972; Kobayashi, 1975), but it was not until the development of an enzymatic method by Millner *et al.* (1979) for *Enteromorpha intestinalis* that it became possible to isolate large numbers of viable protoplasts. Currently there are many reports of protoplast isolation and culture for marine multicellular macrophytic algae. However, regeneration from protoplasts to complete thalli, especially with regard to the anatomically complex seaweeds, is a fairly recent development. Protoplasts have been isolated from 13 genera belonging to the Rhodophyta or red seaweeds (Reddy *et al.*, 2008a). Table 1 summarises the progress of Rhodophyta protoplast research to date. Within the *Gracilaria* species, plant regeneration has only been reported for four species: *G. asiatica* (Yan and Wang, 1993), *G. chilensis* (Cheney, 1990), *G. tikvahiae* (Cheney, 1990) and *G. changii* (Yeong *et al.*, 2008).

**Table 1** Status of protoplast isolation, regeneration and studies within the Rhodophyta (adapted from Reddy *et al.*, 2008a).

Species	Status	Reference
<i>Acrosorium polyneurum</i>	PI	Yamaguchi <i>et al.</i> (1989)
<i>Bangia atropurpurea</i>	PI	Araki <i>et al.</i> (1994)
<i>Chondrus crispus</i>	BS	Smith and Bidwell (1989)
	PI	Le Gall <i>et al.</i> (1990)
<i>Gelidium robustum</i>	PI	Coury <i>et al.</i> (1993)
<i>Gracilaria asiatica</i>	PR	Yan and Wang (1993)
<i>G. changii</i>	PR	Yeong <i>et al.</i> (2008)
<i>G. chilensis</i>	PR	Cheney (1990)
<i>G. chorda</i>	PI	Chou and Lu (1989) in Reddy <i>et al.</i> (2008a)
<i>G. dura</i>	PI	Gupta <i>et al.</i> (2011)
<i>G. filicina</i>	PI	Yamaguchi <i>et al.</i> (1989)
<i>G. gigas</i>	PI	Chou and Lu (1989) in Reddy <i>et al.</i> (2008a)
<i>G. lamaneiformis</i>	CW	Cheney <i>et al.</i> (1986)
	PI	Bjork <i>et al.</i> (1990)
<i>G. salicornia</i>	PI	Chou and Lu (1989) in Reddy <i>et al.</i> (2008a)
<i>G. sordida</i>	PI	Bjork <i>et al.</i> (1990)
<i>G. tenuestipitata</i>	PI	Chou and Lu (1989) in Reddy <i>et al.</i> (2008a); Bjork <i>et al.</i> (1990)
<i>G. tikvahiae</i>	PR	Cheney <i>et al.</i> (1986); Cheney (1990)
<i>G. verrucosa</i>	PI	Bjork <i>et al.</i> (1990); Araki <i>et al.</i> (1998); Gupta <i>et al.</i> (2011)
	PI & CW	Mollet <i>et al.</i> (1995)
<i>Grateloupia sparsa</i>	PI	Chen and Chiang (1994a and 1994b)
	CW	Chen and Chiang (1995)
<i>G. filicina</i>	PI	Yamaguchi <i>et al.</i> (1989); Chen and Chiang (1994a and 1994b)
<i>G. turuturu</i>	PI	Yamaguchi <i>et al.</i> (1989)
	PR	Lafontaine <i>et al.</i> (2011)
<i>Halymenia formosa</i>	PI	Chou and Lu (1989) in Reddy <i>et al.</i> (2008a)
<i>Kappaphycus alvarezii</i>	PI & BS	Zablackis <i>et al.</i> (1993)
	PR	Salvador and Serrano (2005)
<i>Laurencia obtuse</i>	PI	Balestri <i>et al.</i> (1989) in Reddy <i>et al.</i> (2008a)
<i>Palmaria palmata</i>	PI	Liu <i>et al.</i> (1992); Nikolaeva <i>et al.</i> (1999)
	BS	Le Gall <i>et al.</i> (2004)
<i>Plocamium cartilagineum</i>	PI	Balestri <i>et al.</i> (1989) in Reddy <i>et al.</i> (2008a)
<i>Porphyra sp.</i> (wild)	PI	Packer (1994)
<i>P. angusta</i>	PI	Chou and Lou (1989) in Reddy <i>et al.</i> (2008a)
<i>P. crispata</i>	PI	Chou and Lou (1989) in Reddy <i>et al.</i> (2008a); Ar Gall <i>et al.</i> (1993)

Table 1 (continued)

Species	Status	Reference
<i>P. dentata</i>	PI	Ar Gall <i>et al.</i> (1993)
<i>P. lanceolata</i>	PR	Polne-Fuller <i>et al.</i> (1984)
<i>P. leucosticta</i>	PR	Chen (1987)
<i>P. linearis</i>	PR	Chen <i>et al.</i> (1988) in Reddy <i>et al.</i> (2008a)
<i>P. nereocystis</i>	PR	Waaland <i>et al.</i> (1990)
<i>P. okamurae</i>	PI	Fujita and Saito (1990)
<i>P. okhaensis</i>	PI	Dipakkore <i>et al.</i> (2005)
<i>P. perforata</i>	PR	Polne-Fuller <i>et al.</i> (1984 and 1990); Saga <i>et al.</i> (1986) in Reddy <i>et al.</i> (2008a)
<i>P. pseudolinearis</i>	PR	Fujita and Saito (1990)
<i>P. seriata</i>	PI	Fujita and Saito (1990)
<i>P. suborbiculata</i>	CW	Tang (1982) in Reddy <i>et al.</i> (2008a)
	PI	Fujita and Saito (1990)
	PR	Araki <i>et al.</i> (1987)
<i>P. tenera</i>	PI	Song and Chung (1988); Fujita and Saito (1990)
<i>P. tenuipedalis</i>	PR	Saga and Sakai (1984); Fujita and Migita (1985) in Reddy <i>et al.</i> (2008a); Araki <i>et al.</i> (1987); Yamaguchi <i>et al.</i> (1989)
	PI	Fujita and Saito (1990)
<i>P. yezoensis</i>	PR	Takahashi <i>et al.</i> (2010)
	PI	Gomez-Pinchetti and Garcia Reina (1993)

BS: Biochemical study; CW: Cell wall regeneration; PI: Protoplast isolation; PR: Protoplast regeneration

### 1.8.2.1 The uses of protoplasts

Protoplasts as seed stocks for cultivation of *Ulva* and *Monostroma* have been developed and well tested (Chen, 1998; Chen and Shih, 2000; Dipakkore *et al.*, 2005; Reddy *et al.*, 2006). These seed stocks offer an alternative to the use of spores for seeding nets for field cultivation (Reddy *et al.*, 2006).

Protoplasts have also been used for a number of physiological studies. These include: the mechanism of inorganic carbon uptake in various species (Smith and Bidwell, 1989; Bjork *et al.*, 1992; Haglund *et al.*, 1992); the oxygen evolution rate in comparison to intact plant tissue (Millner *et al.*, 1979; Davison and Polne-Fuller, 1990; Bjork *et al.*, 1992; Benet *et al.*, 1994; Beer and Bjork, 1994); and the production of various compounds (Fujimura and Kajiwara, 1990; Zabacklis *et al.*, 1993; Hagen Rodde and Larsen, 1997; Kim *et al.*, 2005).

Somatic hybridization as a method for genetic improvement of crops requires isolation and fusion of intact protoplasts, sustained division of the fusion product and regeneration of plants (Pelletier, 1993). This method allows the creation of new strains which are impossible to produce by conventional breeding methods due to sexual incompatibility between species. Genetic manipulation through this method is also generally more widely accepted than genetic engineering (the introduction of recombinant DNA) and thus offers an attractive method for production of superior species of algae that produce new and valuable products or exhibit improved and predictable growth (Cheney, 1990). Plant regeneration of a limited number of fusion species have been reported in some algae.

### 1.9 Concluding remarks

*G. gracilis* belongs to a commercially important genus of red macroalgae that is used extensively in the production of two commercially important grades of agar (Schroeder *et al.*, 2003). *G. gracilis* (Stackhouse) M. Steentoft, L. M. Irvine & W. F. Farnham was previously known as *G. verrucosa*, prior to 1995. It is however evident through literature that not all species referred to as *G. verrucosa* prior to 1995 were in fact *G. verrucosa*, and there was considerable confusion between *Gracilaria* and *Gracilariopsis* species in many areas; there in fact still is. In addition there has been recent literature published (post 1998) on the species *G. verrucosa*. This is confusing since taxonomically this species no longer is relevant and now is used as a synonym for *Gracilariopsis longissima*. This brings into question the real identity of this species and highlights the need for using the correct taxonomic name for seaweed species.

The South African *Gracilaria* industry depended solely on the natural *G. gracilis* resource growing in Saldanha Bay. However, the industry experienced a number of setbacks over the past few years due to major collapses in the *G. gracilis* population which has proven to be unreliable for commercial collections (Schroeder *et al.*, 2003). Rothman *et al.* (2009) suggest that the only means of establishing a sustainable *Gracilaria* industry in South Africa is through suspended (open-water) cultivation.

A better understanding of how this commercially important seaweed responds at a genetic level to stresses faced in the aquaculture environment will be of great advantage to the South African *Gracilaria* industry. However, for a full understanding of the function of a particular gene, gene knockout is often necessary and for this to be possible *in vivo*, a transformation

system is required. The understanding of gene function in stress response and the development of a suitable transformation and tissue culture system for *G. gracilis* would also make it possible to select, breed and/or engineer strains that are either more tolerant or resistant to environmental stresses. Much progress has been made in this field with higher plants and although macroalgal crop improvement is not currently possible, the growing body of knowledge of transformation systems in macroalgal species can be used to build a platform for future biotechnological innovations.

### 1.10 Aims and objectives of this study

The broader objective of this investigation was to develop a transformation and tissue culture system for *G. gracilis* with a view to laying the necessary groundwork for future genetic manipulation studies in this alga. In order to achieve this, the specific aims of this study were three-fold:

- i) Transformation systems require an effective method to deliver foreign DNA into target cells. Microparticle bombardment was investigated and optimised as a method for achieving this in *G. gracilis*. The successful implementation of this technique could then allow critical and rapid assessment of suitable viral promoters for use in future *G. gracilis* transformation studies.
- ii) Stable transformation in macroalgae requires techniques for plant regeneration from single cells. Due to the problems associated with seaweed callus culture, as discussed in 1.8.1, protoplasts were investigated as a possible cell culture system for *G. gracilis*. Protocols for the isolation and culture of *G. gracilis* protoplasts were optimized to ensure maximal yield and survival of these cells. Furthermore, whole plant regeneration from *G. gracilis* protoplasts and the factors that affect this were investigated.
- iii) A *G. gracilis* cell culture system based on protoplasts requires an optimized method for PEG-mediated transformation of these cells. Using this system, the effect of expression enhancing strategies such as targeted homologous recombination (HR) and matrix attachment regions (MARs) on foreign gene expression was investigated as a possible means for increasing transgene expression. In addition, the sensitivity of *G. gracilis* protoplasts to possible selective agents was assessed in order to identify agents which could be used for selection of transformed cells. Selection of transformed *G. gracilis* protoplasts was then assessed using the chosen selective agent.

## **CHAPTER 2**

### **MICROPARTICLE BOMBARDMENT OF *GRACILARIA GRACILIS* THALLI**

## Contents

<b>2.1</b>	<b>Summary</b> .....	26
<b>2.2</b>	<b>Introduction</b> .....	27
2.2.1	Aims of this chapter.....	31
<b>2.3</b>	<b>Materials and methods</b> .....	32
2.3.1	Algal strains and culture .....	32
2.3.2	General molecular techniques .....	32
2.3.3	Thallus sterilization for microparticle bombardment .....	33
2.3.4	Optimization of rupture disc pressure for microparticle bombardment .....	34
2.3.5	Histological <i>lacZ</i> assay.....	34
2.3.6	Cloning and construction of vectors .....	35
2.3.6.1	Bacterial strains and plasmids.....	35
2.3.6.2	Media and culture conditions .....	36
2.3.6.3	Construction of vectors for microparticle bombardment .....	36
2.3.7	Testing of promoter function and strength .....	40
2.3.7.1	Microparticle bombardment.....	40
2.3.7.2	Histological <i>lacZ</i> staining .....	40
2.3.7.3	PCR detection of <i>lacZ</i> in microparticle bombarded thalli .....	41
2.3.7.4	$\beta$ -Galactosidase enzyme assay .....	42
<b>2.4</b>	<b>Results</b> .....	43
2.4.1	Testing sterilization process .....	43
2.4.2	Optimization of rupture disc pressure .....	44
2.4.3	Testing of viral promoter function and strength.....	45
<b>2.5</b>	<b>Discussion</b> .....	51

## 2.1 Summary

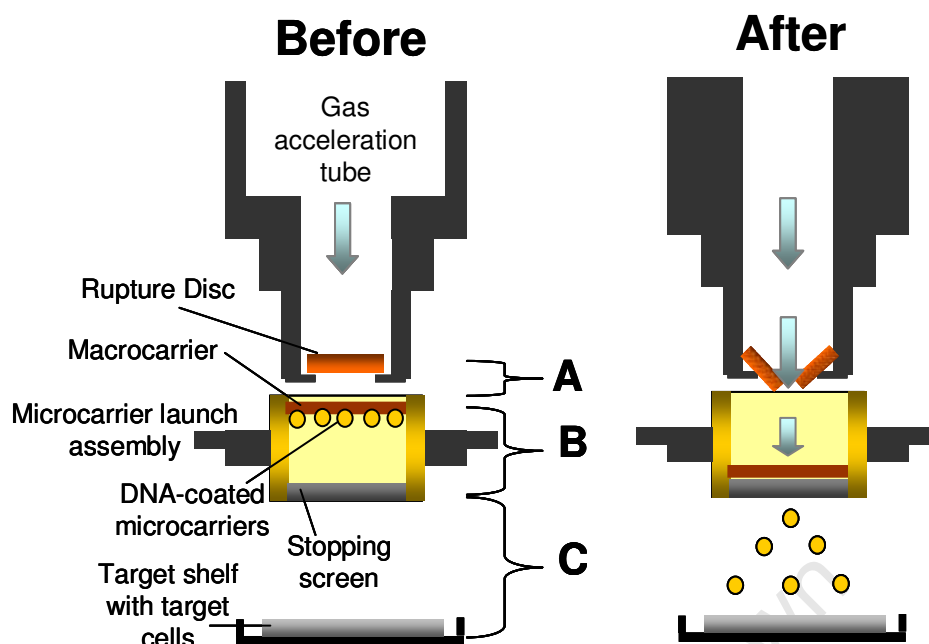
Microparticle bombardment was investigated and optimized as a method for the identification of a suitable promoter for use in future transformation studies in *G. gracilis*. This technique proved successful for transformation of thalli when a construct containing the *lacZ* reporter gene under the influence of the Simian virus 40 (SV40) promoter was employed. Transgene expression could be seen in thalli following *in situ* histochemical staining two days post-bombardment. Transformation efficiencies of 1.7-12.5% were obtained when thalli were bombarded under different helium pressures. A pressure of 650 psi was determined to be optimal for transformation of *G. gracilis* thalli and therefore was employed in all further procedures. Sectioning and histological staining of bombarded thalli showed that bombarded recombinant DNA penetrated into cells below the epidermal layer of the thallus and allowed expression within the cortical cells. Transient expression of the *lacZ* reporter gene was further compared under the control of three different viral promoters including the SV40 promoter, the Cytomegalovirus (CMV) promoter and the cauliflower mosaic virus (CaMV) 35S promoter. In thalli transformed with vectors containing either the SV40 or CMV promoter, *lacZ* presence was detected by histological staining 2 and 3 days post-bombardment. In thalli transformed with the vector containing the CaMV 35S promoter, *lacZ* presence was detected by histological staining up to 5 days post-bombardment. PCR analysis verified the presence of the *lacZ* gene in plasmid-bombarded *G. gracilis* thalli from the first day post-bombardment onwards.  $\beta$ -galactosidase activity in bombarded thalli varied in relation to the promoter used in the plasmid and allowed the SV40 promoter to be identified as a suitable promoter for further transformation studies.

## 2.2 Introduction

Microparticle bombardment is a technique that delivers DNA and other substances into a cell's interior through the use of "micron-sized" metal particles that are accelerated under high velocities. This technique was developed in the 1980s as an alternative method for transforming plants which were initially thought to be recalcitrant to *Agrobacterium*-mediated transformation (Taylor and Fauquet, 2002). The first particle delivery device was developed by Sanford and co-workers (Sanford *et al.*, 1987; Sanford, 1988). It used a blast of air to accelerate tungsten particles into large onion epidermal cells, but failed when smaller particles and smaller cells were used (Southgate *et al.*, 1995). This device was later modified to employ gunpowder to accelerate DNA-coated microparticles, under a vacuum, into target tissue (Southgate *et al.*, 1995). Further developments eventually gave rise to the PDS-1000/He Biolistic<sup>®</sup> particle delivery system which removed the need for gunpowder and allowed more control over the bombardment process (Taylor and Fauquet, 2002).

The PDS-1000/He Biolistic<sup>®</sup> particle delivery system effectively uses helium (He) pressure and vacuum circuits to accelerate microcarriers into the target tissue. Once all the materials are prepared and in place a vacuum is drawn in the system. The fire switch can then be triggered which allows the flow of He gas into the acceleration chamber. The He gas is held back until the burst pressure of the rupture disc is reached. At this point the plastic rupture disc breaks and releases a helium shock wave into the chamber. The shock wave impacts the microcarrier launch assembly which is placed inside the chamber below the rupture disc, and this shock wave propels a plastic macrocarrier holding DNA-coated microcarriers toward the target cells. The macrocarrier travels a short distance before being arrested by a metal stopping screen. This causes the microcarriers to be launched through the stopping screen and into the target tissue below (Kikkert, 1993; Taylor and Fauquet, 2002) (Fig. 2.1).

There are a number of physical parameters which can be optimized within the bombardment process. These parameters include the microparticle size, type and density, vacuum extent, target distance, gap distance, macrocarrier travel distance and He pressure (Taylor and Fauquet, 2002).



**Figure 2.1** Schematic representation of the PDS-1000/He Biolistic<sup>®</sup> particle delivery system (Bio-Rad) before and after activation. A: gap distance or distance between the rupture disc and the macrocarrier; B: macrocarrier travel distance or distance between the macrocarrier and the stopping screen; C: target distance or distance between the stopping screen and the target tissue. A, B and C represent mechanically adjustable distances which have an influence on microparticle velocity. The arrows indicate the direction of helium flow in the system. Figure is not drawn to scale. (Figure adapted from Dunder *et al.*, 1995 and Heiser, 1992)

The microparticle size and type is important since this has a direct effect on the depth of penetration of the microparticle. Traditionally, tungsten and gold have been used (Randolph-Anderson *et al.*, 1995). Gold, however, is most commonly used since its surface is more regular than tungsten, it does not agglomerate during the DNA coating procedure as tungsten does, and tungsten has had toxic effects in some cases, whereas gold has proven to be completely inert (Russell *et al.*, 1992; Southgate *et al.*, 1995). The concentration of the microparticles affects the coverage of the target tissue (Southgate *et al.*, 1995). If the concentration is too low, it is likely that the coverage area would be low, but at high concentrations, microparticles may also agglomerate and result in tissue damage when delivered (Southgate *et al.*, 1995).

The vacuum extent, target distance, gap distance, macrocarrier travel distance and He pressure all affect the momentum of the microparticles and thus the depth to which they

penetrate the target tissue. The chamber vacuum prevents deceleration of microparticles due to air friction. The higher the vacuum, the less the particles will be decelerated (Randolph-Anderson *et al.*, 1995). A longer target distance or distance between the stopping screen and the target tissue, the greater the spread of the particles across the target tissue and the less pronounced the helium shock wave will be. Conversely, longer travel distances result in decreased microparticle speed and hence decreased likelihood of target tissue penetration (Randolph-Anderson *et al.*, 1995). The gap distance or distance between the rupture disc and the macrocarrier is set during assembly and the smaller the distance, the faster the microparticles will travel. The macrocarrier travel distance or distance between the macrocarrier and the stopping screen influences the velocity of the microparticles and longer travel distances result in increased velocities (Kikkert, 1993). The He pressure is ultimately determined by the rupture disc rating. The higher the rupture disc rating, the more pronounced the shock wave and the faster the microparticles will travel (Kikkert, 1993).

Although microparticle bombardment was originally developed for use in plants, its application for use in many other species is well reported (Armaleo *et al.*, 1990; Williams *et al.*, 1991; Smith *et al.*, 1992; Qin *et al.*, 2005; Tan *et al.*, 2005). Microparticle bombardment has been successfully employed to transform mainly commercially important macroalgal species (Table 2.1). These efforts were largely focused on driving transient expression of reporter genes using promoters which had proved successful for similar applications in higher plants and microalgae (Qin *et al.*, 2004). The cauliflower mosaic virus (CaMV) 35S promoter and *uidA*, encoding  $\beta$ -glucuronidase (GUS) as a reporter gene, proved successful for transient expression in *Kappaphycus* (Kurtzman and Cheney, 1991) and *Porphyra* (Kuang *et al.*, 1998). However, reports of CaMV 35S promoter use in *Laminaria* and *Undaria* were not as successful. While this promoter was able to drive expression of the *uidA* reporter gene in regenerated plants, expression appeared to be tissue specific and was only observed in the rhizoids of *Laminaria* and blades of *Undaria* (Qin *et al.*, 1994 referenced in Qin *et al.*, 1999). The Simian virus 40 (SV40) promoter was consequently employed and shown to drive stable, uniform expression of *lacZ*, encoding  $\beta$ -galactosidase, in both *Laminaria* (Jiang *et al.*, 2003) and *Undaria* (Qin *et al.*, 2003 referenced in Walker *et al.*, 2005). This construct has also been successfully employed to drive transient expression of *lacZ* in *K. alvarezii* (Wang *et al.*, 2010a) and *G. changii* (Gan *et al.*, 2003).

**Table 2.1** Macroalgal species for which microparticle bombardment has been employed.

Algae/Species	Reporter gene <sup>b</sup>	Promoter <sup>c</sup>	Type of expression	Reference
<i>Gracilaria changii</i>	<i>lacZ</i>	SV40	transient	Gan <i>et al.</i> (2003)
<i>Kappaphycus alvarezii</i>	<i>uidA</i>	CaMV 35S	transient	Kurtzman and Cheney (1991)
	<i>lacZ</i>	SV40	transient	Wang <i>et al.</i> (2010a)
<i>Laminaria japonica</i>	<i>uidA</i>	CaMV 35S	transient	Qin <i>et al.</i> (1994) referenced in Qin <i>et al.</i> (1999)
	<i>lacZ</i>	SV40	stable	Jiang <i>et al.</i> (2003)
	<i>HBsAg / cat</i>	SV40	stable	Jiang <i>et al.</i> (2002)
	<i>rtPA</i>	SV40	stable	Zhang <i>et al.</i> (2008)
	<i>bar</i>	CaMV 35S	stable	Zhang <i>et al.</i> (2006); Zhang <i>et al.</i> (2008)
	<i>uidA</i>	CaMV 35S / UBI / AMT	transient	Li <i>et al.</i> (2009)
	<i>uidA</i>	FCP	stable	Li <i>et al.</i> (2009)
<i>Undaria pinnatifida</i>	<i>uidA</i>	CaMV 35S	transient	Qin <i>et al.</i> (1994) referenced in Qin <i>et al.</i> (1999)
	<i>lacZ</i>	SV40	stable	Qin <i>et al.</i> (2003) referenced in Walker <i>et al.</i> (2005)
<i>Ulva pertusa</i>	<i>egfp</i>	<i>UprbcS1</i>	transient	Kakimura <i>et al.</i> (2009)
<i>Porphyra yezoensis</i>	<i>uidA</i>	CaMV 35S	transient	Kuang <i>et al.</i> (1998)
	<i>uidA / PyGUS</i>	CaMV 35S / <i>PyGAPDH</i>	transient	Fukuda <i>et al.</i> (2008)
	<i>ZsGFP / ZsYFP / sGFP / AmCFP</i>	<i>PyAct1</i>	transient	Mikami <i>et al.</i> (2009); Uji <i>et al.</i> (2010); Mikami <i>et al.</i> (2011)
	PyGUS	<i>PyAct1</i>	transient (prolonged)	Takahashi <i>et al.</i> (2010)
	PyGUS	<i>PtHSP70</i>	transient	Son <i>et al.</i> (2011)
<i>Porphyra tenera</i>	PyGUS	<i>PtHSP70</i>	transient	Son <i>et al.</i> (2011)
Bangiophycean sp. <sup>a</sup>	PyGUS / sGFP	<i>PyAct1</i>	transient	Hirata <i>et al.</i> (2011)

<sup>a</sup> *P. yezoensis*, *P. tenera*, *Porphyra okamurae*, *Porphyra onoi*, *Porphyra variegata*, *Porphyra pseudolinearis*, *Bangia fuscopurpurea*

<sup>b</sup> *HBsAg*, hepatitis B surface antigen; *cat*, chloramphenicol acetyl transferase; *rtPA*, Reteplase; *bar*, phosphinothricin acetyl transferase; PyGUS,  $\beta$ -glucuronidase (GUS) coding region adapted to the codon usage of *P. yezoensis*; *ZsGFP* and *ZsYFP*, humanised *Zoanthus* sp. green and yellow fluorescent proteins, respectively; *sGFP* (S65T), plant adapted green fluorescent protein; *AmCFP*, *Anemonia majano* cyan fluorescent protein

<sup>c</sup> UBI, ubiquitin promoter from maize; AMT, adenine methyltransferase promoter from the *Chorellavirus*; *PyGAPDH*, *P. yezoensis* glyceraldehyde-3-phosphate dehydrogenase promoter; *PyAct1*, *P. yezoensis actin 1* promoter; *PtHSP70*, *P. tenera* heat shock protein 70 promoter

Success has been achieved mainly in *Laminaria japonica*, where stable expression of a number of genes under the influence of the SV40 promoter, and more recently the fucoxanthin chlorophyll a/c-binding protein gene (FCP) promoter, has been reported (Table 2.1; Jiang *et al.*, 2002; Jiang *et al.*, 2003; Zhang *et al.*, 2008; Li *et al.*, 2009). Significant progress has also been achieved in *P. yezoensis*, where the use of strong endogenous promoters and codon optimized reporter genes have enabled the establishment of a robust transient expression system (Table 2.1; Fukuda *et al.*, 2008; Mikami *et al.*, 2009; Takahashi *et al.*, 2010; Uji *et al.*, 2010; Hirata *et al.*, 2011; Mikami *et al.*, 2011).

### **2.2.1 Aims of this chapter**

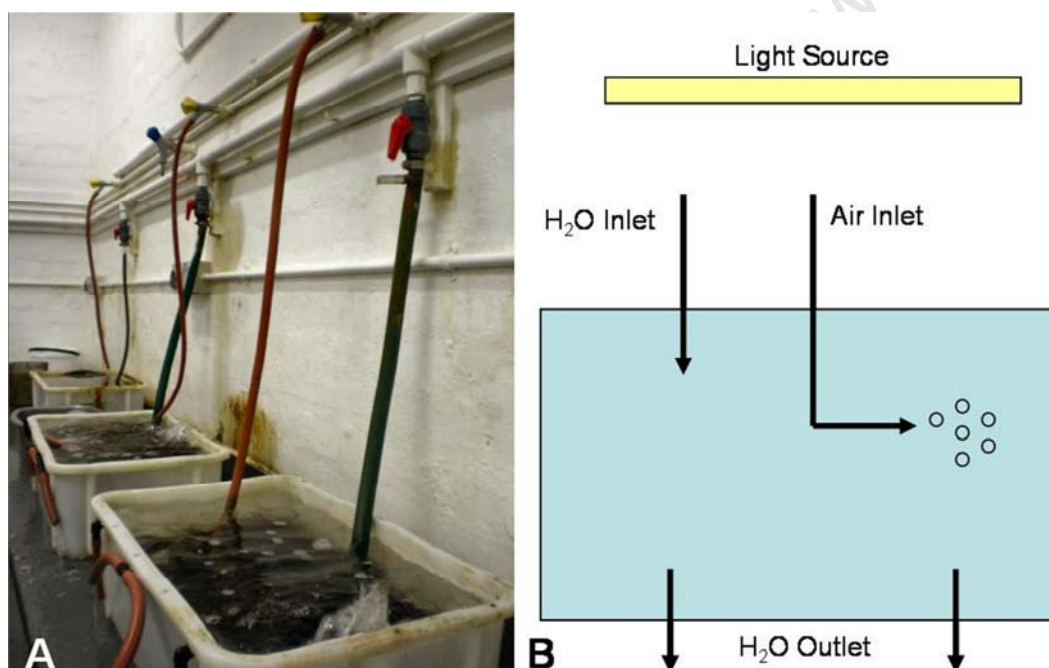
The aim of this chapter was to identify a suitable promoter for use in future transformation studies in the economically important species of macroalgae, *G. gracilis*. This was done by first establishing whether microparticle bombardment can be successfully applied to transiently transform *G. gracilis*, which has not been previously reported. The success of this technique was then employed to test the functionality of three viral promoters in *G. gracilis* using the *lacZ* reporter gene, and in so doing, identify a promoter which could be applied in future transformation studies. This is important since the establishment of transient gene expression is the first step towards the development of a stable transformation system.

## 2.3 Materials and methods

All media and solutions used in this study are listed in Appendix A.

### 2.3.1 Algal strains and culture

*G. gracilis* thalli were obtained from Irvine and Johnson Abalone Culture Division, Danger Point, Gansbaai, South Africa. Thalli were maintained in tanks with a flow-through system of aerated seawater at approximately 240 litres hr<sup>-1</sup> under a 16/8 hours (hrs) (day/night) photoperiod with a light intensity of 45  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  at 14–15 °C (Fig. 2.2).



**Figure 2.2** Aquarium tank setup in which *G. gracilis* was maintained in an aerated, flow-through seawater system (A). A schematic representation of an individual tank in which *G. gracilis* was maintained (B).

### 2.3.2 General molecular techniques

Agarose gel electrophoresis was performed in 1x TAE running buffer (Appendix A.2.3), as described by Ausubel *et al.* (1989) (Appendix B.5).

Small-scale preparation of plasmid DNA was achieved using the alkaline lysis mini-prep method of Ish-Horowitz and Burke (1981) (Appendix B.3), unless otherwise stated. Large-scale plasmid isolations were performed using the Qiagen Midi-prep kit according to manufacturer's instructions.

Restriction endonuclease digestions were performed as described by Ausubel *et al.* (1989) (Appendix B.4) and according to manufacturer's instructions. All restriction enzymes were purchased from Fermentas, unless otherwise stated.

Excised gel fragments were purified using the Qiagen gel extraction kit according to manufacturer's instructions.

Ligation of DNA fragments was carried out as described by Coyne *et al.* (2002) (Appendix B.8).

Competent *Escherichia coli* (*E. coli*) DH5 $\alpha$  (Table 2.2) were transformed as described in Appendix B.2 and plated on Luria-Bertoni agar (LA) (Appendix A.1.6) containing 100  $\mu\text{g ml}^{-1}$  ampicillin or 100  $\mu\text{g ml}^{-1}$  kanamycin (Appendix A.2.2), and incubated overnight at 37 °C, unless otherwise stated. *E. coli* DH5 $\alpha$  capable of growth on ampicillin or kanamycin, were then inoculated into 5 ml Luria-Bertoni broth (LB) (Appendix A.1.5) supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin or kanamycin and grown overnight with shaking at 37 °C. Plasmid DNA was isolated from these overnight cultures for screening purposes. All overnight cultures were prepared in this way, unless otherwise stated.

### **2.3.3 Thallus sterilization for microparticle bombardment**

In preparation for microparticle bombardment, thalli were rinsed and visible epiphytes removed before sterilising by placing them in sterile distilled water for 3 hrs, followed by a 5 minute (min) incubation in 0.1% (v/v) sodium hypochlorite and a final incubation in 1% (w/v) KI for 1 min. Between each treatment, thalli were rinsed three times in sterile artificial seawater (ASW) (Appendix A.1.1). Following sterilization, thalli were cultured overnight in ASW enriched with PES (Provasoli, 1968) (1/3 strength) (Appendix A.1.4) and supplemented with 0.5  $\text{mg ml}^{-1}$  each of penicillin G and kanamycin (Appendix A.2.2) at 15 °C. Sterility of thalli was confirmed by placing thallus sections on Marine Agar (MA; Appendix A.1.10) and

incubating at 22 °C. Thalli were monitored for the presence of bacterial and/or fungal contamination over a period of a week by monitoring microbial growth on MA. Plates containing thalli that were subjected to the sterilization protocol were compared to unsterilized thalli.

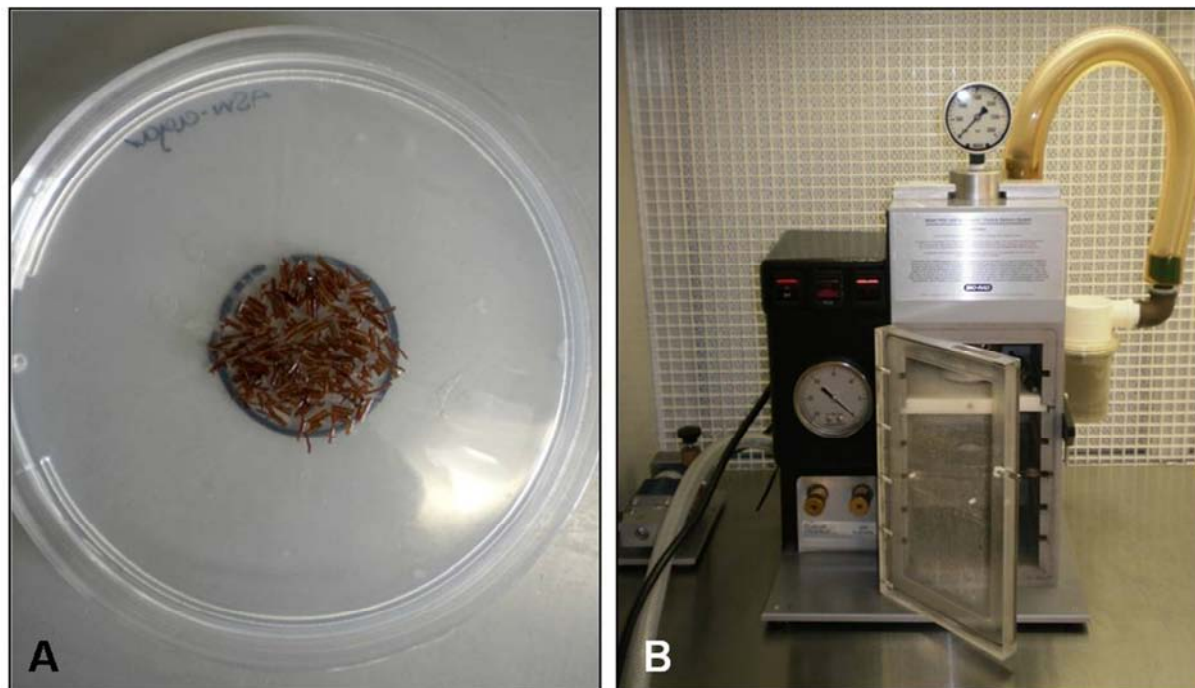
### 2.3.4 Optimization of rupture disc pressure for microparticle bombardment

Thalli, sterilized as described in 2.3.3, were cut into lengths of approximately 0.5 cm using a sterile scalpel blade. Approximately 70 of these thalli pieces were placed in the centre of a solid ASW agar (0.8%; w/v) (Appendix A.1.11) surface (Fig. 2.3A) prior to particle bombardment. The plasmid, pSV- $\beta$ -Galactosidase (Promega) (Table 2.3; Fig. 2.4), was precipitated onto gold particles (1  $\mu$ m in diameter, Bio-Rad) as described by Dunder *et al.* (1995) (Appendix B.10.2). A Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) (Fig. 2.3B) was used for particle delivery. Each bombardment delivered 0.48 mg of gold particles and 1.0  $\mu$ g plasmid DNA in accordance with Teng *et al.* (2002), Gan *et al.* (2003) and Jiang *et al.* (2003). The He pressure was varied by employing different rupture discs (450, 650, 900 and 1100 psi, Bio-Rad). A particle travel distance of 6 cm and a vacuum extent of 27 inches of mercury (in Hg) were employed for all the bombardments. Negative controls were bombarded with non-DNA-attached gold particles at each of the He pressures investigated. A total of three plates were bombarded for each of the four He pressures tested. Each plate was bombarded twice. Following bombardment, thalli were thoroughly rinsed with ASW and thalli from each bombarded plate were maintained separately in ASW enriched with PES (1/3 strength) at 15 °C in the dark for 2 days.

### 2.3.5 Histological *lacZ* assay

The presence of the  $\beta$ -galactosidase enzyme was assayed 2 days post-bombardment, in accordance with Teng *et al.* (2002), Gan *et al.* (2003) and Jiang *et al.* (2003), by employing an *in situ* histochemical stain. The histochemical stain uses 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal), a chromogenic substrate that turns blue following cleavage by the  $\beta$ -galactosidase enzyme. A total of 40 thalli pieces were randomly selected from each of the bombarded plates and processed separately. Thalli were rinsed twice in ASW, followed by a rinse in 1x phosphate buffered saline (PBS, Appendix A.2.1), fixed for 0.5 hrs in 1x PBS (pH 7.0) containing 1 mM MgCl<sub>2</sub> and 1.25% glutaraldehyde (v/v), placed in stain

solution (Appendix A.2.8) and incubated at 37 °C for 4 hrs. Following the incubation, thalli were thoroughly rinsed in 1x PBS (pH 7.0), viewed and photographed under a Nikon Stereoscopic Zoom Microscope SMZ1500.



**Figure 2.3** Thalli, sterilized and cut into lengths of approximately 0.5 cm were placed in the centre of a solid ASW agar (0.8%; w/v) surface (A) prior to microparticle bombardment with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) (B).

In addition, a thallus showing blue staining was transversely sectioned through the blue stained region using a sterile scalpel blade. This was done in order to determine where the blue stained cells were localized within the seaweed thallus. The section was viewed and photographed on a slide under a light microscope.

### 2.3.6 Cloning and construction of vectors

Diagrams of the cloning strategies used to construct the vectors for microparticle bombardment can be found in Appendix C.

#### 2.3.6.1 Bacterial strains and plasmids

*E. coli* strains and plasmids used in this chapter are listed in Table 2.2 and 2.3, respectively.

**2.3.6.2 Media and culture conditions**

*E. coli* strains were either grown in LB or on LA at 37 °C. *E. coli* strains harbouring vectors listed in Table 2.3 were cultured in LB or on LA containing 100 µg ml<sup>-1</sup> ampicillin or 100 µg ml<sup>-1</sup> kanamycin at 37 °C.

**Table 2.2** Bacterial strains used in this study

Strain	Genotype/ relevant feature(s) <sup>a</sup>	Reference
<i>Escherichia coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F'(traD36 proAB' lacI<sup>q</sup> lacZΔM15)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>Escherichia coli</i> DH5α	(φ80 <i>lacZΔM15</i> ) <i>recA1 endA1 gyrA96 thi-1 hsdR17(r<sub>K</sub><sup>-</sup>m<sub>K</sub><sup>+</sup>) supE44 (Nal<sup>r</sup>) relA1 Δ(lacZYA-argF)<sub>U169</sub>phoA</i>	Hanahan (1983)

<sup>a</sup> Nal<sup>r</sup>, naladixic acid resistant

**2.3.6.3 Construction of vectors for microparticle bombardment**Construction of pCMV-β-Galactosidase

Large-scale plasmid isolation was performed in order to isolate the plasmids pSV-β-Galactosidase (Promega) and pEGFP (BD Biosciences) (Table 2.3). The plasmid pEGFP was digested with the restriction enzymes *AgeI* and *XbaI*. The resulting fragments were resolved on a 1% (w/v) TAE agarose gel and the desired 745 bp fragment (Appendix C Fig. C1a) containing the *egfp* gene was gel purified.

The plasmid pSV-β-Galactosidase was subjected to restriction enzyme digestion with the restriction enzymes *AgeI*, *EcoRV* and *XbaI*. The resulting fragments were resolved on a 0.8% (w/v) TAE agarose gel and the desired 3285 bp fragment (Appendix C Fig. C1b), containing the β-lactamase (*bla*) gene encoding ampicillin resistance, the origin of replication and the SV40 promoter/enhancer region, was gel purified. The *egfp*-containing fragment was subsequently cloned into the 3285 bp fragment from pSV-β-Galactosidase and the resulting constructs were transformed into competent *E. coli* DH5α. Plasmid DNA was isolated from overnight cultures and screened for the presence of the 745 bp insert by *AgeI* and *XbaI* restriction enzyme analysis. A recombinant construct containing the 745 bp DNA fragment

was identified and designated pSV-egfp (Table 2.3; Appendix C Fig. C1c). This sub-cloning strategy resulted in substitution of the *lacZ* gene with the *egfp* gene.

**Table 2.3** Plasmids used in this study

Plasmid	Relevant feature(s) <sup>a</sup>	Reference
pSV-β-Galactosidase	Amp <sup>r</sup> , β-galactosidase ( <i>lacZ</i> ), SV40 promoter/enhancer	Promega
pEGFP	Amp <sup>r</sup> , EGFP ( <i>egfp</i> )	BD Biosciences
pSV-egfp	Derivative of pSV-β-Galactosidase with the <i>egfp</i> gene substituted for the <i>lacZ</i> gene; Amp <sup>r</sup>	This study
pcDNA3.1/Zeo/CAT	Amp <sup>r</sup> , Cm <sup>r</sup> , CMV promoter	Invitrogen
pCMV-egfp	Derivative of pSV-egfp with the CMV promoter substituted for the SV40 promoter/enhancer; Amp <sup>r</sup>	This study
pCMV-β-Galactosidase	Derivative of pCMV-egfp with the <i>lacZ</i> gene substituted for the <i>egfp</i> gene; Amp <sup>r</sup>	This study
pEarleyGate201Δ <i>ccdB</i>	Km <sup>r</sup> , <i>bar</i> , CaMV 35S promoter, Gateway	Smart (2011); Earley <i>et al.</i> (2006)
pCaMV-egfp	Derivative of pSV-egfp with the CaMV 35S promoter substituted for the SV40 promoter/enhancer; Amp <sup>r</sup>	This study
pCaMV-β-Galactosidase	Derivative of pCaMV-egfp with the <i>lacZ</i> gene substituted for the <i>egfp</i> gene; Amp <sup>r</sup>	This study

<sup>a</sup> Amp<sup>r</sup>, ampicillin resistant; Cm<sup>r</sup>, chloramphenicol resistant; Km<sup>r</sup>, kanamycin resistant

The plasmid pSV-egfp (Table 2.3) was isolated and digested with the restriction enzymes *EcoRI* and *HindIII*. Resulting fragments were resolved on a 1% (w/v) TAE agarose gel and the desired 3610 bp fragment (Appendix C Fig. C2a) was purified.

Plasmid pcDNA3.1/Zeo/CAT (Invitrogen) (Table 2.3), containing the Cytomegalovirus (CMV) promoter, was isolated from an overnight culture. A 749 bp fragment (Appendix C Fig. C2b), containing the CMV promoter, was PCR amplified (Appendix B.15.1) from pcDNA3.1/Zeo/CAT (Invitrogen) (Table 2.3) using the high fidelity *Pfu* Polymerase (Fermentas) and the oligonucleotide primers CMVpro-F and CMVpro-R (Table 2.4). The PCR primers CMVpro-F and CMVpro-R were designed to include an *EcoRI* and a *HindIII*

restriction site, respectively, allowing simplified cloning into the destination vector pSV-egfp. The amplified PCR product was subjected to restriction digest with *EcoRI* and *HindIII* and gel purified. The restricted CMV promoter-containing fragment was subsequently sub-cloned into the 3610 bp fragment from pSV-egfp. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures and screened for the presence of the 728 bp CMV promoter-containing insert by *EcoRI* and *HindIII* restriction enzyme analysis. A recombinant construct containing the 728 bp DNA fragment was identified and designated pCMV-egfp (Appendix C Fig. C2c; Table 2.3). This sub-cloning strategy resulted in the substitution of the CMV promoter for the SV40 promoter/enhancer.

**Table 2.4** Oligonucleotide primers used in this study

Primer	Sequence (5' – 3') <sup>a</sup>	Reference
18S-F	GAGTGAATTGTACAACGAAACTGC	This study
18S-R	GATCTGAATAATCAGTTCATCTAGC	This study
CMV <sub>pro</sub> -F	TTGACCGAGAATTCCATGAAG	This study
CMV <sub>pro</sub> -R	CGCTAGCA <u>AAGCTT</u> GGGTCT	This study
CaMV <sub>pro</sub> -F	ATCTCAGAATTCCAATCCC	This study
CaMV <sub>pro</sub> -R	GTAAAAATA <u>AAGCTT</u> TTATACTCG	This study
lacZ-F	GGTTGAACTGCACACCGCCG	This study
lacZ-R	GATGGACCATTTCGGCACAG	This study

<sup>a</sup> Single underlined sequences (GAATTC) indicate the inclusion of an *EcoRI* restriction site in the oligonucleotide primer, and those doubly underlined (AAGCTT) indicate the inclusion of a *HindIII* restriction site within the oligonucleotide primer.

The vectors pCMV-egfp and pSV- $\beta$ -Galactosidase were digested (Appendix C Fig. C3a-b) with *AgeI* and *PstI*. The resulting fragments were resolved on 0.8% (w/v) TAE agarose gel before the 3551 bp fragment, containing the *lacZ* gene, from pSV- $\beta$ -Galactosidase and the 3577 bp fragment, lacking the *egfp* gene, from pCMV-egfp were gel purified. The 3551 bp *lacZ* fragment was ligated (Appendix B.8) to the 3577 bp fragment from pCMV-egfp. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ , plated on LA containing 100  $\mu\text{g ml}^{-1}$  ampicillin and 40  $\mu\text{g ml}^{-1}$  X-gal (Appendix A.2.1), and incubated overnight at 37 °C. *E. coli* DH5 $\alpha$  capable of growth on ampicillin and showing a blue colour, were

inoculated into 5 ml LB supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin and grown overnight with shaking at 37 °C. Plasmid DNA was isolated from the overnight cultures and screened for the expected restriction pattern by *PvuII* restriction enzyme analysis. A recombinant construct showing the desired restriction digest pattern was identified and designated pCMV- $\beta$ -Galactosidase (Fig. 2.4; Table 2.3).

#### Construction of pCaMV- $\beta$ -Galactosidase

Plasmid pEarleyGate201 $\Delta$ *ccdB* (Earley *et al.*, 2006; Smart, 2011) (Table 2.3), containing the CaMV 35S promoter, was isolated from an overnight culture. A 1378 bp fragment, containing the CaMV 35S promoter, was PCR amplified (Appendix B.15.2) from pEarleyGate201 $\Delta$ *ccdB* using high fidelity *Pfu* Polymerase (Fermentas) and the oligonucleotide primers CaMVpro-F and CaMVpro-R (Table 2.4). The PCR primers CaMVpro-F and CaMVpro-R were designed to include an *EcoRI* and a *HindIII* restriction site, respectively, allowing simplified cloning into the destination vector pSV-egfp. The amplified PCR product (Appendix C Fig. C4b) was subjected to restriction enzyme digestion with *EcoRI* and *HindIII* and gel purified. The CaMV promoter-containing fragment was subsequently sub-cloned into the 3610 bp fragment from pSV-egfp (Appendix C Fig. C4a) (prepared as described above). The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures and screened for the presence of the 1348 bp CaMV promoter-containing insert by *EcoRI* and *HindIII* restriction enzyme analysis. A recombinant construct containing the 1348 bp DNA fragment was identified and designated pCaMV-egfp (Appendix C Fig. C4c; Table 2.3). This sub-cloning strategy resulted in substitution of the SV40 promoter/enhancer with the CaMV 35S promoter.

The vectors pCaMV-egfp and pSV- $\beta$ -Galactosidase were subjected to restriction enzyme digestion with *HindIII* and *PstI* (Appendix C Fig. C5a-b). The resulting fragments were resolved on 0.8% (w/v) TAE agarose gel before the 3759 bp fragment, containing the *lacZ* gene, from the pSV- $\beta$ -Galactosidase restriction and the 3989 bp fragment, lacking the *egfp* gene, from the pCaMV-egfp restriction were gel purified. The 3759bp *lacZ* fragment was ligated (Appendix B.8) to the 3989 bp fragment from pCaMV-egfp. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ , plated on LA containing 100  $\mu\text{g ml}^{-1}$  ampicillin and 40  $\mu\text{g ml}^{-1}$  X-gal, and incubated overnight at 37 °C. *E. coli* DH5 $\alpha$  colonies

capable of growth on ampicillin and showing a blue colour, were inoculated into 5 ml LB supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin and grown overnight with shaking at 37 °C. Plasmid DNA was isolated from the overnight cultures and screened by *EcoRI* and *HindIII* restriction enzyme analysis. A recombinant construct having the expected restriction digest pattern was identified and designated pCaMV- $\beta$ -Galactosidase (Fig. 2.4; Table 2.3).

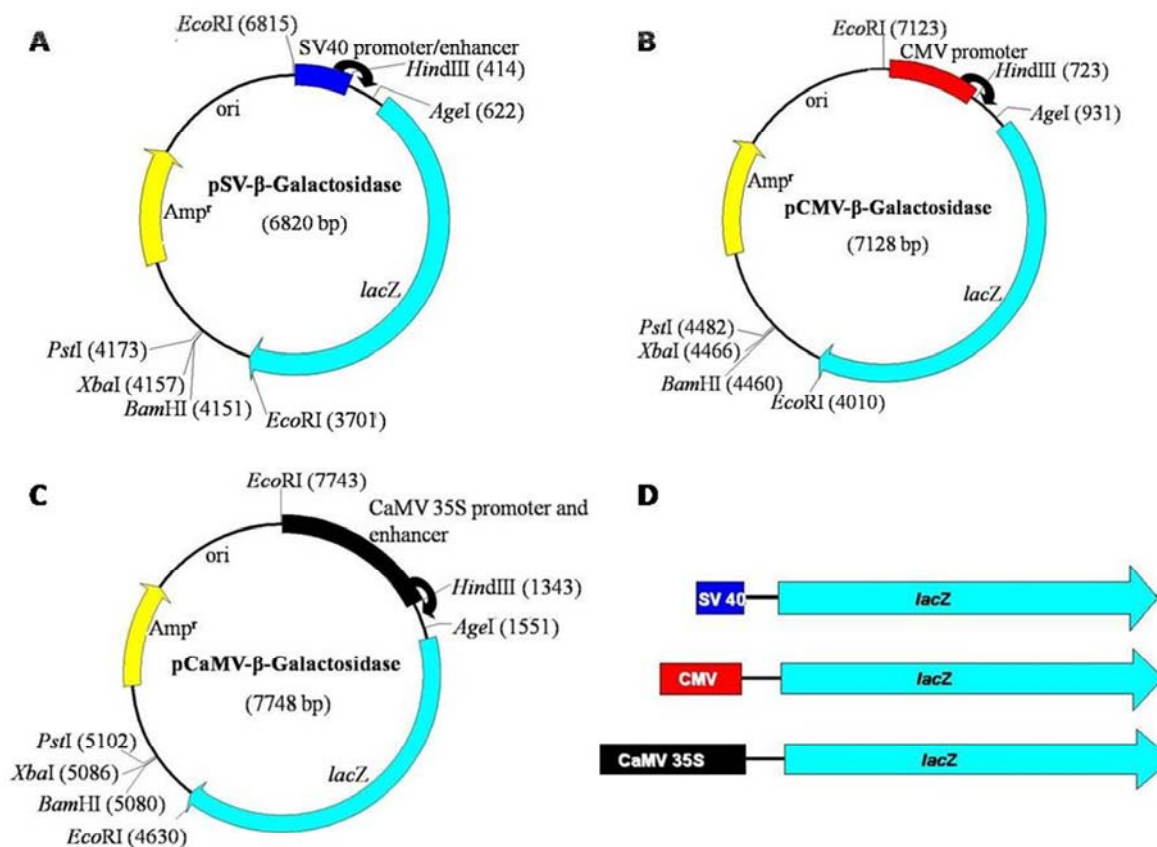
### **2.3.7 Testing of promoter function and strength**

#### **2.3.7.1 Microparticle bombardment**

*G. gracilis* samples for microparticle bombardment were sterilized and cultured as described in 2.3.3, one day prior to bombardment. Thallus preparation, microparticle bombardment and gold preparation was carried out as described in 2.3.4 with the following amendments. Gold particles were coated with one of three vectors, pSV- $\beta$ -Galactosidase, pCaMV- $\beta$ -Galactosidase or pCMV- $\beta$ -Galactosidase (Fig. 2.4; Table 2.3) or without any vector (negative control). Four plates were bombarded per treatment. A rupture disc pressure of 650 psi, a particle travel distance of 6 cm and a vacuum extent of 27 in Hg were employed for all bombardments. Following microparticle bombardment the plates were treated as described in 2.3.4 and each plate was considered a biological repeat.

#### **2.3.7.2 Histological *lacZ* staining**

Histological *lacZ* staining was carried out as described in 2.3.5. A total of forty randomly selected thalli sections were stained per plate at each of the following time points: 1, 2, 3, 5 and 7 days post-bombardment. Thalli sections were viewed and photographed under a Nikon Stereoscopic Zoom Microscope SMZ1500.



**Figure 2.4** Vectors employed in the microparticle bombardment of *G. gracilis* thalli (A-C). The recombinant vectors pCMV-β-Galactosidase (B) and pCaMV-β-Galactosidase (C) are derivatives of the control vector pSV-β-Galactosidase (Promega) (A). All vectors contain the *lacZ* reporter gene under the influence of a different viral promoter (D): SV40 promoter/enhancer (■) (A, D), CMV promoter (■) (B, D) and the CaMV 35S promoter (■) (C, D). Relevant restriction sites are shown. Elements of vectors are as follows: ►, Amp<sup>r</sup>; ►, *lacZ*; the black arrow (►), indicates direction of transcription from the promoter.

### 2.3.7.3 PCR detection of *lacZ* in microparticle bombarded thalli

Total DNA was isolated from randomly selected *G. gracilis* thalli from both the plasmid-bombarded samples and the negative controls at the time points mentioned in 2.3.7.2 using the Qiagen DNeasy Plant Mini Kit with minor modifications (Appendix B.9.3). Primers lacZ-F and lacZ-R (Table 2.4) were used to PCR amplify (Appendix B.15.3) a 624 bp fragment of the *lacZ* gene, while primers 18S-F and 18S-R (Table 2.4) were used to PCR amplify (Appendix B.15.4) a 1615 bp fragment of the *G. gracilis* 18S rRNA gene from

both the negative and plasmid-bombarded samples at each time point. The latter control confirmed the presence of intact genomic DNA in each of the samples. Amplified products for both sets of primers were analysed by electrophoresis through a 1% (w/v) TAE agarose gel.

#### **2.3.7.4 $\beta$ -Galactosidase enzyme assay**

*G. gracilis* samples for microparticle bombardment were sterilized and cultured as described in 2.3.3 one day prior to bombardment. Thallus preparation, gold preparation, microparticle bombardment and thallus culture following microparticle bombardment was carried out as described in 2.3.7.1. Three plates were bombarded per treatment. Algal tissue was homogenized two days post-bombardment in Z-buffer (Miller, 1972) supplemented with  $\beta$ -mercaptoethanol (Appendix A.2.9). The extract was clarified by microcentrifugation and the protein content was determined using the standard Bradford protein assay (Bradford, 1976; Appendix B.11).  $\beta$ -Galactosidase activity was assayed in the extract by measuring hydrolysis of o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (Appendix A.2.9) at 37 °C (Appendix B.12). One unit of  $\beta$ -galactosidase is defined as the amount of enzyme that will hydrolyze 1  $\mu$ mol of ONPG to o-nitrophenol and D-galactose per minute at pH 7.5 at 37 °C. In order to control for the coloured compounds released from the algal tissue itself, a blank reaction with no substrate was performed for each sample. Activity data is expressed as specific activity, i.e. total activity in mU per mg of total soluble protein (mU mg<sup>-1</sup> protein). The  $\beta$ -galactosidase activity data was analyzed by one-way ANOVA. When the results of the ANOVA were significant, the Tukey Test was used in order to determine the significant differences in  $\beta$ -galactosidase activity due to the presence of the various promoters, using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at  $P < 0.05$ .

## 2.4 Results

### 2.4.1 Testing sterilization process

The surface of macroalgae serves as an attachment site for a wide variety of epiphytes, including bacterial and fungal species (Jaffray *et al.*, 1997). It is therefore necessary to sterilize the seaweed surface in order to be certain that any heterologous gene expression detected is the result of macroalgal expression of introduced genes and not due to bacterial contamination. These contaminants can result in false-positives, particularly when *lacZ* or *uidA* are employed as reporter genes (Qin *et al.*, 1999; Qin *et al.*, 2005).

The sterilization process developed in this study was effective in establishing axenic thalli as no bacterial or fungal growth was observed when sterilized thalli were cultured on MA at 22 °C for 1 week (Fig. 2.5A). Sterilised thalli continued to show no bacterial or fungal contamination when cultured for up to 2 weeks. In contrast, thalli that were not sterilized showed the presence of bacterial contamination after just one day on MA. The bacterial contamination continued to increase over the 7 day period until thalli were completely overgrown with bacteria (Fig. 2.5B), including agarolytic bacterial isolates capable of pitting the surface of the agar medium (Fig. 2.5C). Fungal growth also became apparent after 4 days of cultivation.



**Figure 2.5** *G. gracilis* thalli that had been sterilized (A) and cultured on MA for 1 week showing no bacterial or fungal contamination. Control thalli (B & C) which had not been subjected to sterilization showing bacterial and fungal contamination after 1 week cultivation on MA. Arrow indicates the presence of agarolytic bacteria causing pitting of the agar surface (C).

The sodium hypochlorite treatment employed in this study had previously been determined to be effective in eliminating microorganisms without causing bleaching of thalli (data not shown).

#### 2.4.2 Optimization of rupture disc pressure

As outlined in the introduction to this chapter, successful transformation by microparticle bombardment has been reported for a limited number of macroalgal species. In all reported cases, the bombardment parameters employed are fairly similar and there is little variation in the physical parameters within the process. However, Gan *et al.* (2003), Fukuda *et al.* (2008) and Wang *et al.* (2010a) report variation in transformation efficiencies in relation to different He pressures. Since microparticle bombardment of *G. gracilis* had never previously been reported, it was necessary to test whether this was a suitable method of transformation, and which He pressure would result in optimal transformation of *G. gracilis* thalli. The other physical parameters used in this study were the same as those employed to transform other macroalgal species by microparticle bombardment.

Microparticle bombardment proved a successful method for transforming *G. gracilis* thalli with a construct containing the *lacZ* reporter gene under the influence of the SV40 promoter. The presence of blue-stained areas within the thalli indicates areas of  $\beta$ -galactosidase activity and therefore, *lacZ* expression (Fig. 2.6A). Blue staining was seen for thalli bombarded under all He pressures tested with an efficiency ranging from 1.7-12.5% (Table 2.5). A He pressure of 650 psi proved optimal, resulting in the highest transformation efficiency of 12.5% (Table 2.5). He pressures of 450, 900 and 1100 psi resulted in transformation efficiencies of 2.5, 4.2 and 1.7%, respectively. However, not every bombardment experiment performed at 450, 900 and 1100 psi resulted in blue stained thalli (Table 2.5). Significantly, none of the *G. gracilis* thalli bombarded with gold lacking vector DNA showed any blue staining at any of the He pressures employed.

**Table 2.5** Effect of helium pressure on transformation efficiency of *G. gracilis* thalli during microparticle bombardment.

Helium pressure (psi) <sup>a</sup>	Number of thalli showing blue staining (n=40) <sup>b</sup>				Transformation efficiency (%) <sup>c</sup>
	Exp.1	Exp.2	Exp.3	Mean ± SD	
450	2	0	1	1.0 ± 1.0	2.5
650	4	5	6	5.0 ± 1.0	12.5
900	3	2	0	1.7 ± 1.5	4.2
1100	0	1	1	0.7 ± 0.6	1.7

<sup>a</sup> Helium (He) pressure was varied by employing different rupture discs.

<sup>b</sup> Number of thalli showing staining out of a total of 40 stained thalli for each plate.

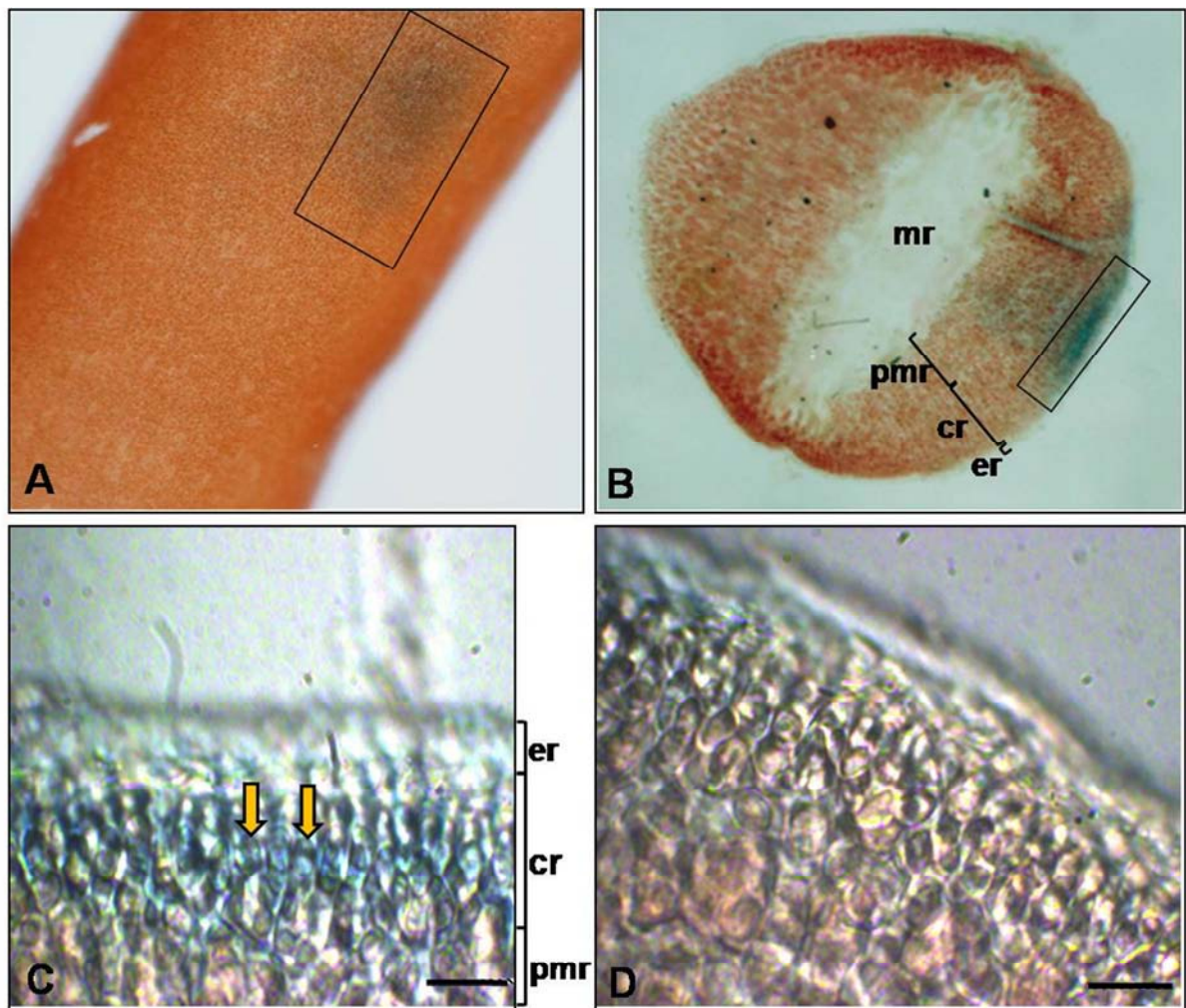
<sup>c</sup> Transformation efficiency = number of thalli showing blue staining/total number of thalli stained.

Observation of blue stained areas on *G. gracilis* thalli does not indicate the extent of penetration of bombarded DNA into the thalli, and therefore, which cells contain the transiently expressed *lacZ*. In order to determine this, areas of thalli exhibiting blue staining were transversely sectioned to examine which particular cells showed  $\beta$ -galactosidase staining. Blue-stained cells were observed within the inner cortical cells bordering the medullary region and not in the surface epidermal cells of the thallus (Fig. 2.6B, C & D).

### 2.4.3 Testing of viral promoter function and strength

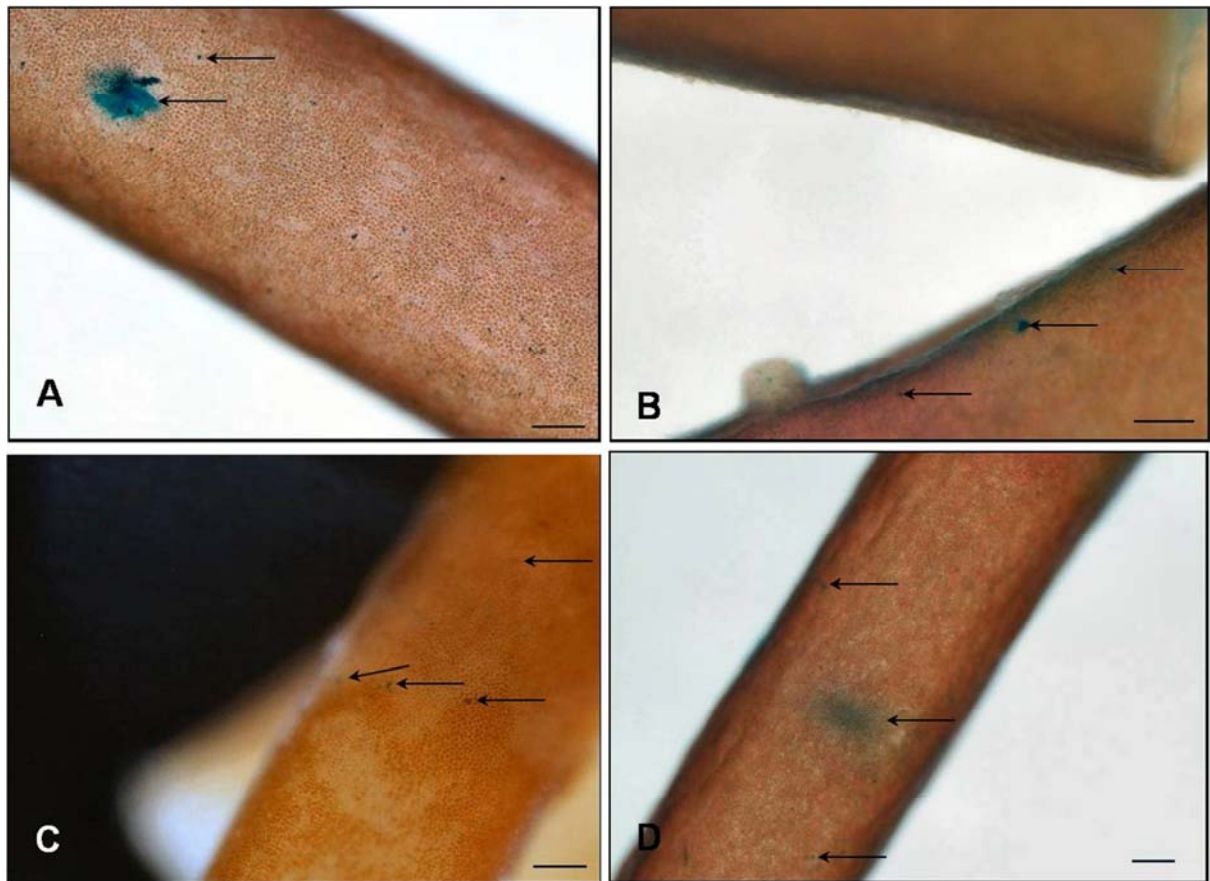
In order to test successful expression of *lacZ* under the influence of three viral promoters, *G. gracilis* thalli were bombarded with the recombinant vectors pSV- $\beta$ -Galactosidase, pCMV- $\beta$ -Galactosidase and pCaMV- $\beta$ -Galactosidase (Fig. 2.4; Table 2.3). Thalli were sampled at five post-bombardment time points (1, 2, 3, 5 and 7 days) and stained for the presence of *lacZ*. A negative control employing gold particles lacking vector DNA was included. *LacZ* expression was visible on days 1, 2, 3 and 5 post-bombardment in the *G. gracilis* samples bombarded with pCaMV- $\beta$ -Galactosidase (Fig. 2.7). No *lacZ* expression was detected on day 7 in the pCaMV- $\beta$ -Galactosidase-bombarded samples.

*LacZ* expression was visible on 2 and 3 days post-bombardment in the samples bombarded with pCMV- $\beta$ -Galactosidase (Fig. 2.8) and pSV- $\beta$ -Galactosidase (Fig. 2.9). No *lacZ* expression was seen on days 1, 5 and 7 in these plasmid-bombarded samples.

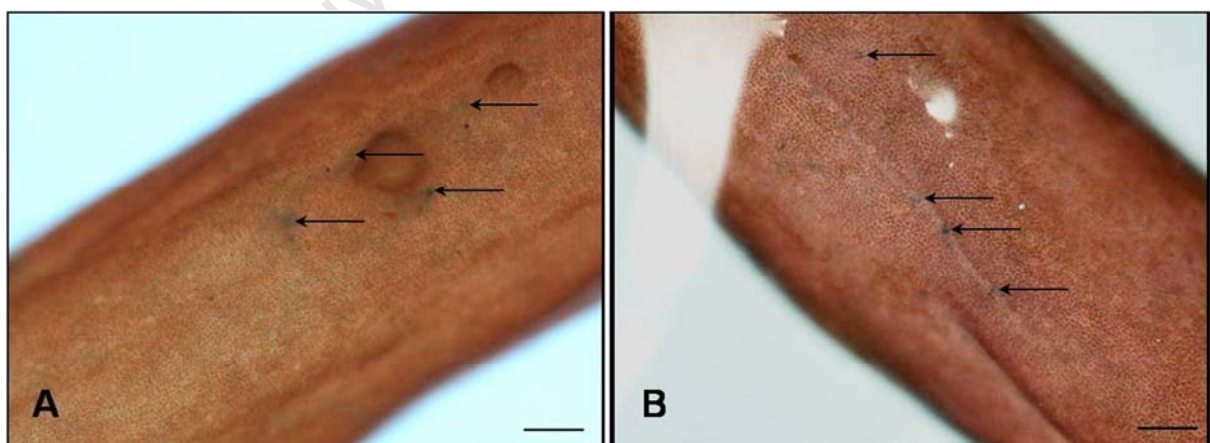


**Figure 2.6** *G. gracilis* thalli two days post-bombardment (A) with the stained blue area (block) indicating  $\beta$ -galactosidase activity after *in situ* histochemical staining. A transverse section (B) was made through the stained section of the thallus shown in A. The stained area is indicated by the block (B) which shows that staining occurs within the inner cell layers of the thallus. C is a magnified view of the blocked area in the section shown in B. Whole cells can be seen to be blue in colour (arrows) and these cells are within the cortical region (cr) which lie beneath the epidermal cells (er) (C). View of a similar region of section of a thallus which did not show blue staining following X-gal *in situ* histochemical staining (D). mr, medullary region; pmr, perimedullary region; cr, cortical region; er, epidermal region. Scale bar = 100  $\mu$ m.

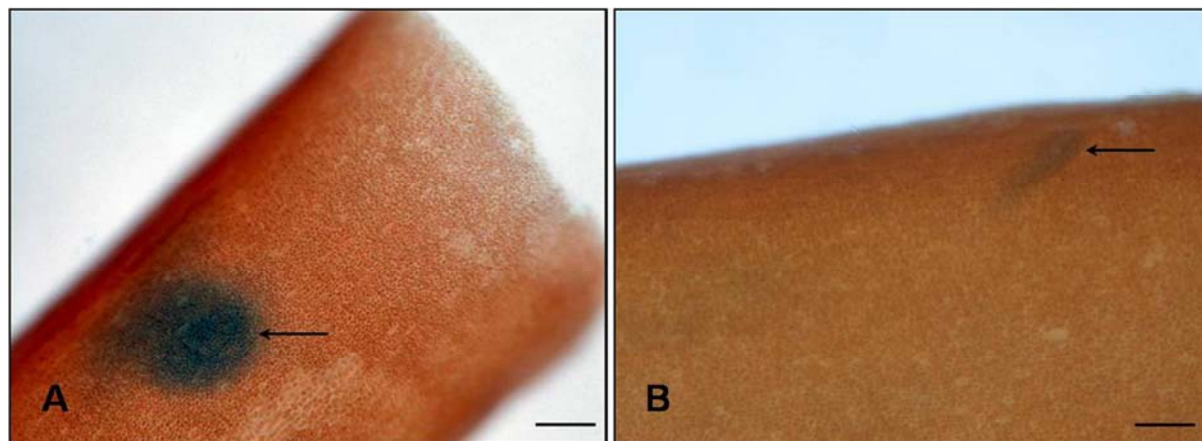
Macroalgal thalli bombarded with gold particles lacking vector DNA showed no blue-stained areas at any of the sampling time points when compared with the plasmid-bombarded samples expressing *lacZ* (Fig. 2.10).



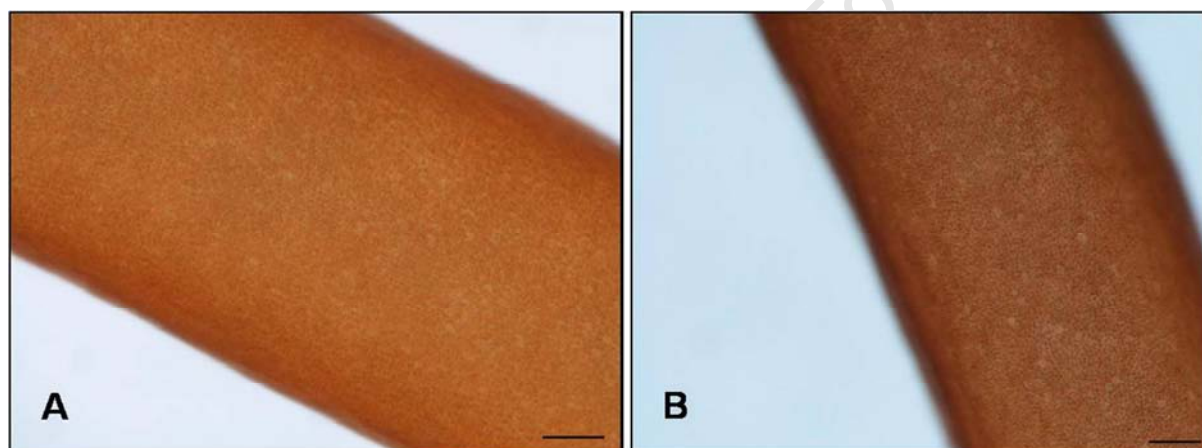
**Figure 2.7** pCaMV- $\beta$ -Galactosidase bombarded *G. gracilis* thalli 1 (A), 2 (B), 3 (C) and 5 days (D) post-bombardment with the stained blue areas (indicated by the black arrows) showing  $\beta$ -galactosidase activity following the addition of X-gal. Scale bar = 100  $\mu$ m.



**Figure 2.8** pCMV- $\beta$ -Galactosidase bombarded *G. gracilis* thalli 2 (A) and 3 (B) days post-bombardment with the stained blue areas (arrows) indicating  $\beta$ -galactosidase activity after the addition of X-gal. Scale bar = 100  $\mu$ m.

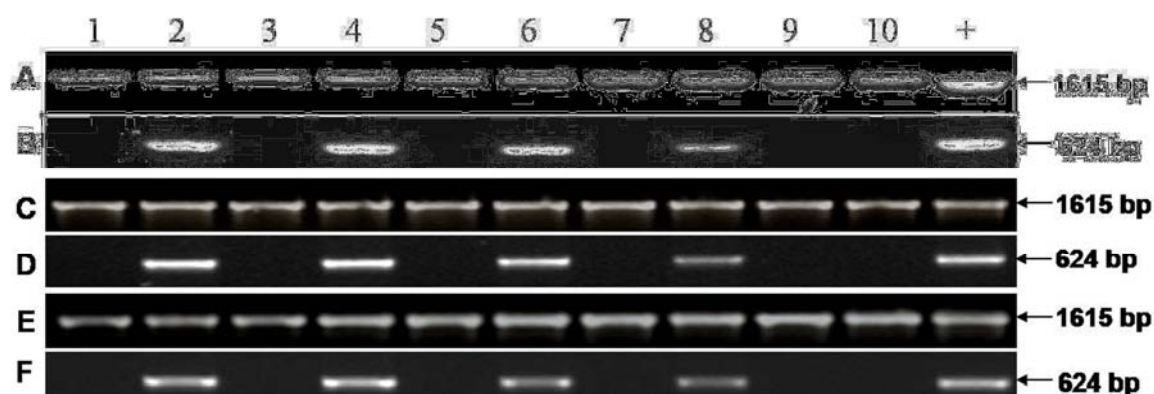


**Figure 2.9** pSV- $\beta$ -Galactosidase bombarded *G. gracilis* thalli 2 (A) and 3 (B) days post-bombardment with the stained blue areas (arrows) indicating  $\beta$ -galactosidase activity after the addition of X-gal. Scale bar = 100  $\mu$ m.



**Figure 2.10** *G. gracilis* thalli 2 (A) and 3 (B) days post-bombardment with no visible areas of blue staining. Samples were bombarded with gold microparticles lacking vector DNA. Scale bar = 100  $\mu$ m.

PCR analysis of genomic DNA extracted from thalli sampled at different time points post-bombardment (days 1, 2, 3, 5 and 7) was carried out to confirm the presence of the *lacZ* gene in the host cells. A 624 bp *lacZ* PCR product was amplified from all the DNA samples from *G. gracilis* tissue bombarded with all three vectors, pSV- $\beta$ -Galactosidase, pCMV- $\beta$ -Galactosidase and pCaMV- $\beta$ -Galactosidase, 1-5 days post-bombardment (Fig. 2.11), but not amplified from any of the algal samples bombarded with gold particles lacking vector DNA. No *lacZ* PCR product was detected in tissue bombarded with any of the vectors at 7 days post-bombardment. The PCR amplification of a *G. gracilis* 18S rDNA PCR product confirmed the presence of intact *G. gracilis* DNA in all the samples tested (Fig. 2.11).



**Figure 2.11** PCR analysis of total DNA isolated from bombarded thalli. Lanes 1, 3, 5, 7 and 9, seaweed bombarded with non-DNA-attached gold particles (negative control) sampled 1, 2, 3, 5 and 7 days post-bombardment respectively; lanes 2, 4, 6, 8 and 10, plasmid-bombarded seaweed (test sample) sampled 1, 2, 3, 5 and 7 days post-bombardment respectively; +, positive control. Panels A, C and E: DNA fragments amplified with *G. gracilis* 18S rRNA gene specific primers in samples bombarded with pSV- $\beta$ -Galactosidase, pCaMV- $\beta$ -Galactosidase and pCMV- $\beta$ -Galactosidase, respectively. Panels B, D and F: DNA fragments amplified with *lacZ* specific primers in samples bombarded with pSV- $\beta$ -Galactosidase, pCaMV- $\beta$ -Galactosidase and pCMV- $\beta$ -Galactosidase, respectively.

In order to compare the strength of the three promoters, *in vivo*  $\beta$ -galactosidase activity assays were carried out on *G. gracilis* sampled two days post-bombardment with the three recombinant vectors (Fig 2.4; Table 2.3). This time point was selected as it was the first day on which thalli bombarded with the vectors showed  $\beta$ -galactosidase activity by *in situ* histological staining.  $\beta$ -galactosidase activity in *G. gracilis* thalli samples bombarded with gold particles lacking vector DNA showed that *G. gracilis* tissue exhibited background  $\beta$ -galactosidase activity levels of approximately  $7.738 \pm 0.154$  mU mg<sup>-1</sup> protein (Table 2.6). However, *G. gracilis* thalli bombarded with both pSV- $\beta$ -Galactosidase and pCMV- $\beta$ -Galactosidase vectors showed significantly ( $P < 0.05$ ) higher levels of  $\beta$ -galactosidase activity of  $9.367 \pm 0.161$  mU mg<sup>-1</sup> protein and  $9.031 \pm 0.316$  mU mg<sup>-1</sup> protein, respectively (Table 2.6). Thalli bombarded with pCaMV- $\beta$ -Galactosidase did not show significantly ( $P > 0.05$ ) higher activity ( $7.828 \pm 0.244$  mU mg<sup>-1</sup> protein) when compared to negative control *G. gracilis* thalli (Table 2.6). Overall, *G. gracilis* thalli samples bombarded with pSV- $\beta$ -Galactosidase, pCMV- $\beta$ -Galactosidase and pCaMV- $\beta$ -Galactosidase vectors resulted in a 21.1, 16.7 and 1.2% increase in thallus  $\beta$ -galactosidase activity, respectively (Table 2.6).

**Table 2.6** Effect of viral promoters on transient  $\beta$ -galactosidase activity in microparticle bombarded *G. gracilis* thalli.

Promoter	$\beta$ -Galactosidase activity $\pm$ SEM (mU mg <sup>-1</sup> protein) <sup>†</sup>	Relative $\beta$ -Galactosidase activity (%)
Negative	7.738 $\pm$ 0.154 <sup>b</sup>	-
CMV	9.031 $\pm$ 0.316 <sup>c</sup>	16.7
CaMV 35S	7.828 $\pm$ 0.244 <sup>b</sup>	1.2
SV 40	9.367 $\pm$ 0.161 <sup>c</sup>	21.1

<sup>†</sup> Values are means  $\pm$  SEM of three independent samples.  
Activities with different superscripts are significantly different ( $P < 0.05$ ; one-way ANOVA).

University of Cape Town

## 2.5 Discussion

Microparticle bombardment allows the delivery of DNA into a cell's interior through the introduction of "micron-sized" metal particles under high velocities. This technique is able to overcome the significant barrier that the plant cell wall represents. Besides the potential for producing genetically transformed plants and tissues, researchers also use microparticle bombardment in transient expression studies for qualitative and quantitative assessment of transgene expression levels as an indicator of promoter efficacy (Taylor and Fauquet, 2002). We tested whether this technique was applicable for use in the establishment of a transformation system for *G. gracilis*.

Jaffray *et al.* (1997) reported that healthy *G. gracilis* thalli had between  $3.78 \times 10^5$  and  $1.51 \times 10^6$  CFU g<sup>-1</sup> (wet weight) of culturable bacteria associated with them. This number was as high as  $10^9$  cells g<sup>-1</sup> wet weight when non-culturable bacteria were included (Jaffray *et al.*, 1997). This is not an uncommon occurrence and bacterial populations of  $10^1$  to  $10^7$  bacteria g<sup>-1</sup> dry weight (Conover and Sieburth, 1964; Chan and McManus, 1969) have been found colonising surfaces of other species of macroalgae. However, these natural bacteria prove problematic in transformation studies, particularly when *lacZ* or *uidA* are employed as reporter genes (Qin *et al.*, 1999; Qin *et al.*, 2005). Gan *et al.* (2003) reported that decaying thalli or thalli infected with bacteria could result in false-positives, since blue spots could be associated with the bacteria themselves. They suggested that it was essential to select healthy thalli for bombardment purposes in order to further reduce the possibility of false-positives. Therefore, it was necessary in this study to ensure that *G. gracilis* thalli employed in bombardment experiments were axenic.

The sterilization process developed proved successful in eliminating culturable bacterial and fungal contaminants, and thalli continued to show no bacterial or fungal growth on MA for a period of up to 2 weeks and could thus be considered axenic. MA was employed for the sterility test since this media is known to support the growth of a wide variety of marine bacterial and fungal species. The sterilization process made use of a combination of osmotic shock and treatment with oxidizing agents (sodium hypochlorite and KI) as primary sterilizers, which are common strategies employed for surface sterilization of seaweeds (Baweja *et al.*, 2009). Unfortunately, obtaining axenic seaweed is more difficult than for higher plants since seaweeds lack a thick protective outer surface and many of the chemical

agents employed in the sterilization process, such as sodium hypochlorite, can potentially damage the explants (Baweja *et al.*, 2009). It is for this reason the incubation time and concentration of sodium hypochlorite used for sterilization of *G. gracilis* thalli was optimized to prevent damage of *G. gracilis* thalli. The use of sodium hypochlorite for surface sterilization of many macroalgal species is well reported in the literature (Fries, 1983; Kaczyna and Megnet, 1993; Yokoya and Handro, 1996; Yokoya *et al.*, 1999; Yokoya, 2000; Yokoya *et al.*, 2004; Hayashi *et al.*, 2008). The use of antibiotics in the sterilization of seaweed species is also common and often a cocktail of antibiotics is used (Polne-Fuller and Gibor, 1984). A combination of two bacteriocidal antibiotics proved effective for *G. gracilis* thalli sterilization. However, it should be kept in mind that sample material collected at different periods of the year may have differing loads of epiphytic bacteria and in some cases establishing axenic explants may prove extremely difficult and potentially impossible (Baweja *et al.*, 2009). The health of the thalli during the selection process should be a serious consideration for this reason.

*LacZ* as a reporter gene is exceptionally valuable due to its amenability to histochemical detection. Its gene product is stable and can be immobilised in tissue by using a cross-linking fixative without negatively affecting enzyme activity. This fixation has the added advantage of inactivating endogenous enzymes which is particularly important for organisms which may have endogenously high levels of  $\beta$ -galactosidase activity, as is the case with many plant species (Teeri *et al.*, 1989). *LacZ* use in macroalgae, although limited, has proved successful for a number of species (Gan *et al.*, 2003; Jiang *et al.*, 2003; Qin *et al.*, 2003 referenced in Walker *et al.*, 2005; Wang *et al.*, 2010a). The *lacZ* reporter gene was therefore employed in this study to determine whether *G. gracilis* thalli could be successfully transformed through microparticle bombardment. Furthermore, success has been reported in a variety of macroalgal species using the SV40 promoter/enhancer to drive expression of reporter genes (Qin *et al.*, 1999; Gan *et al.*, 2003; Qin *et al.*, 2003 referenced in Walker *et al.*, 2005; Wang *et al.*, 2010a) and for this reason it was included for microparticle bombardment of *G. gracilis*.

Microparticle bombardment of *G. gracilis* thalli showed that the SV40 promoter was functional in *G. gracilis* and that the *lacZ* reporter gene could also be transcribed and expressed by the transformed thalli. This was not surprising as the *lacZ* reporter gene, under the influence of the SV40 promoter, had been shown to be functional in other species of

macroalgae, as previously mentioned. The optimization indicated that all helium pressures employed in this study resulted in some thalli showing *lacZ* expression. Varying helium pressures did however affect the transformation efficiency. A helium pressure of 650 psi resulted in the highest transformation efficiency, 12.5%. Although Randolph-Anderson *et al.* (1995) state that helium pressure employed in bombardment is of less importance than the size and density of microparticles used, the target distance and the vacuum extent, it has been demonstrated that helium pressure for microparticle bombardment of seaweeds seriously affects transformation efficiencies (Gan *et al.*, 2003; Fukuda *et al.*, 2008; Wang *et al.*, 2010a). Similarly, a helium pressure of 650 psi was shown to be optimal for bombardment of *K. alvarezii* when other physical bombardment parameters were kept constant (Wang *et al.*, 2010a). Fukuda *et al.* (2008) also showed that intermediate helium pressures were effective for the transformation of *P. yezoensis*, although a different system was used for delivery. Conversely, Gan *et al.* (2003) reported that helium pressures of 1100 and 1300 psi resulted in optimal transformation efficiencies for bombardment of *G. changii*. However, only 5 to 10 thalli were used in each treatment, which is much fewer than used in this study and the authors also state that thallus thickness should dictate the choice of rupture disc. This may explain the difference between optimal rupture disc ratings for microparticle bombardment of the two species of *Gracilaria*. Since the penetration power of the microparticles is dependent on the helium pressure (Kikkert, 1993), it is likely that using lower pressures results in insufficient penetration of *G. gracilis*, while the higher pressures might be damaging to the tissue. The gas blast and acoustic shock generated during microparticle bombardment have been shown to be the major causes of cell damage (Russell *et al.*, 1992; Tadesse *et al.*, 2003) and these are more pronounced at higher pressures.

The sectioning of *G. gracilis* thalli through the blue-stained region indicated that the plasmid coated gold particles penetrated the outer thallus surface during the bombardment process and became lodged within the underlying cortical cells, resulting in *lacZ* expression within these cells. This is not the first reported case of gold particles penetrating the thallus exterior cells to allow transgene expression in the underlying cells. Wang *et al.* (2010a) reported that when a rupture disc pressure of 650 psi was used in the bombardment of *K. alvarezii*, transgene expression could be identified in the epidermal cells as well as in the medullary cells. It is likely that the depth of particle penetration is related to the thallus thickness at any particular helium pressure.

Transformation efforts in macroalgae have largely focused on using promoters which have proved successful in higher plants and microalgae (Qin *et al.*, 2004). The SV40 and CaMV 35S promoter, although seemingly controversial in some cases (Qin *et al.*, 1994 referenced in Qin *et al.*, 1999), being the most widely employed. There has been relatively little research conducted on the functionality of various promoters 'endogenous or viral' and comparisons of their relative strengths. Therefore, in order to establish a suitable promoter for use in future transformation studies in *G. gracilis*, the relative strengths of three viral promoters, SV40, CaMV 35S and CMV promoters, were assessed. Table 2.7 summarises these results. All three promoters were shown to be functional in expressing *lacZ* as seen by *in situ* histochemical staining 2 and 3 days post-bombardment. This time frame is generally employed when assessing transient expression (Taylor and Fauquet, 2002). However, only the CaMV 35S promoter was shown to drive expression for as long as 5 days post-bombardment (Table 2.7). No expression was detected 7 days post-bombardment for any of the promoters assessed in this study. The negative control samples showed no evidence of  $\beta$ -galactosidase activity at any of the time points, and this indicates that  $\beta$ -galactosidase activity detected in the plasmid bombarded samples was not the result of possible bacterial contamination. Instead the  $\beta$ -galactosidase activity could be directly correlated with the presence of the vector.

Furthermore, PCR analysis supported the histochemical staining data that showed  $\beta$ -galactosidase activity in thalli bombarded with pCaMV- $\beta$ -Galactosidase after 1, 2, 3 and 5 days (Table 2.7). Li *et al.* (2009) reported high transient expression of the *uidA* reporter gene under the influence of the CaMV 35S promoter, but this promoter was unable to drive stable expression of GUS in kelp. The fact that *lacZ* expression was only observed in thalli 2 and 3 days post-bombardment with pSV- $\beta$ -Galactosidase or pCMV- $\beta$ -Galactosidase, despite the detection of *lacZ* DNA in thallus samples 1, 2, 3, 5 and 7 days post-bombardment (Table 2.7), could be explained by the possible origins of the *lacZ* PCR template. During the bombardment process, the gold particles coated with plasmid DNA enter many cells within a thallus. Gene expression is however ultimately related to location of the microprojectiles within the cells (Southgate *et al.*, 1995). The *lacZ* DNA product detected by PCR in the samples could therefore not only have originated from cells in which transient *lacZ* expression occurred, but also from cells into which recombinant DNA was successfully delivered but not necessarily expressed. It is also possible that the *lacZ* gene may have been silenced by the time five days had elapsed, which could explain why  $\beta$ -galactosidase activity

was not observed in thalli when stained 5 days after bombardment, but evidence to support this is inconclusive.

**Table 2.7** Summary of  $\beta$ -galactosidase staining and *lacZ* PCR results observed over the sampling time period following microparticle bombardment of *G. gracilis* thalli with three different vectors.

Sample time point <sup>a</sup> (Day)	Vector	$\beta$ -galactosidase activity <sup>b</sup>	PCR <sup>c</sup>
1	pCaMV- $\beta$ -Galactosidase	+	+
	pCMV- $\beta$ -Galactosidase	-	+
	pSV- $\beta$ -Galactosidase	-	+
2	pCaMV- $\beta$ -Galactosidase	+	+
	pCMV- $\beta$ -Galactosidase	+	+
	pSV- $\beta$ -Galactosidase	+	+
3	pCaMV- $\beta$ -Galactosidase	+	+
	pCMV- $\beta$ -Galactosidase	+	+
	pSV- $\beta$ -Galactosidase	+	+
5	pCaMV- $\beta$ -Galactosidase	+	+
	pCMV- $\beta$ -Galactosidase	-	+
	pSV- $\beta$ -Galactosidase	-	+
7	pCaMV- $\beta$ -Galactosidase	-	-
	pCMV- $\beta$ -Galactosidase	-	-
	pSV- $\beta$ -Galactosidase	-	-

<sup>a</sup> Sampling time point given as days post-bombardment.

<sup>b</sup>  $\beta$ -galactosidase activity is given as present (+) if thalli showed blue staining following the application of X-gal and absent (-) if no staining was seen.

<sup>c</sup> Presence (+) or absence (-) of a *lacZ* PCR product following amplification using specific primers.

When comparing the PCR results with results from the  $\beta$ -galactosidase histological staining, it was seen that the presence of *lacZ* DNA did not necessarily mean that  $\beta$ -galactosidase activity was observed in the host cells at comparable time points (Table 2.7). It is possible that this variation in appearance of  $\beta$ -galactosidase activity is influenced by differences in promoter activity, and it was for this reason that the  $\beta$ -galactosidase activity was assessed directly through a quantitative enzyme assay.

Enzyme activity levels in thalli samples bombarded with gold particles lacking any vector DNA showed that the algal tissue did exhibit  $\beta$ -galactosidase activity. This is not surprising as marine macroalgal tissue has previously been shown to exhibit  $\beta$ -galactosidase activity

(Davies *et al.*, 1994). However, the levels of  $\beta$ -galactosidase activity detected in this study are very low when compared to levels detected for some higher plant species. The use of *lacZ* as a reporter gene in plants is limited by the fact that many plants have endogenous  $\beta$ -galactosidase activity at neutral pH values which complicates the direct measurement of heterologous  $\beta$ -galactosidase activity using enzymatic assays (Teeri *et al.*, 1989). The levels of  $\beta$ -galactosidase in tobacco has been reported to be between 10 and 20 U mg<sup>-1</sup> protein, depending on the type of tissue sampled (Teeri *et al.*, 1989). The levels of endogenous activity measured for *G. gracilis* during the course of this study are approximately 1,000-fold less. The fact that *G. gracilis* shows such a low endogenous  $\beta$ -galactosidase activity in thalli at a neutral pH, means that measuring exogenous *lacZ* expression using enzymatic assays should not be skewed by high endogenous  $\beta$ -galactosidase levels. *LacZ* use as a reporter gene in macroalgae species may also then not be limited to the same extent as it is in higher plants, and direct  $\beta$ -galactosidase assays may well be possible as long as the endogenous activity of each species is low enough.

Thalli samples bombarded with pSV- $\beta$ -Galactosidase, pCMV- $\beta$ -Galactosidase and pCaMV- $\beta$ -Galactosidase vectors resulted in 21.1, 16.7 and 1.2% increases in thallus  $\beta$ -galactosidase activity, respectively. This means that the presence of the vector was positively correlated with an increase in thallus  $\beta$ -galactosidase activity above that of the negative control. However, only thalli bombarded with pSV- $\beta$ -Galactosidase and pCMV- $\beta$ -Galactosidase showed significant ( $P < 0.05$ ) increases over that of the control thalli. This indicated that either the CMV or SV40 promoter could be of particular use in short term transient expression studies in *G. gracilis*. The SV40 promoter has already been proven to be functional in a number of algal species (Qin *et al.*, 1999; Gan *et al.*, 2003; Qin *et al.*, 2003 referenced in Walker *et al.*, 2005; Wang *et al.*, 2010a). The CMV promoter has however never before been employed in macroalgal transformation, but it has been shown to be active in fungi (Lorang *et al.*, 2001), a marine diatom (Sakaue *et al.*, 2008), a moss (Horstmann *et al.*, 2004), and potato protoplasts (Vlasák *et al.*, 2003) and as such, it is not surprising that it is active in *G. gracilis*.

Transformation efficiencies obtained for *G. gracilis* are lower than what has been reported for *G. changii* (Gan *et al.*, 2003) and *K. alvarezii* (Wang *et al.*, 2010a) when using the pSV- $\beta$ -Galactosidase vector construct. Transformation efficiencies obtained for *G. gracilis* in this study were 1.7-12.5%, depending on the rupture disc employed, while Gan *et*

*al.* (2003) reported an efficiency of 94% for *G. changii*, and Wang *et al.* (2010a) reported transformation efficiencies of 87% for *K. alvarezii*. The differences may be due to experimental setup, but may also be attributable to possible variations in the strength of the SV40 promoter and/or the functionality of the *lacZ* reporter gene in these three macroalgal species. Discrepancies concerning the functionality of the commonly employed *uidA* reporter gene and CaMV 35S promoter have also been reported for microparticle bombardment of *P. yezoensis* (Fukuda *et al.*, 2008). Fukuda *et al.* (2008) reported that codon optimization of the *uidA* gene was required for successful expression employing the CaMV 35S promoter in *P. yezoensis*, while successful expression of this gene had been reported in other macroalgal species even without codon optimization (Kübler *et al.*, 1994; Qin *et al.*, 1994 referenced in Qin *et al.*, 1999; Huang *et al.*, 1996; Okauchi and Mizukami, 1999). The transformation efficiency attained for *P. yezoensis* when employing the CaMV 35S promoter was very low and increased GUS expression levels could be achieved when the endogenous glyceraldehyde-3-phosphate dehydrogenase (*PyGAPDH*) promoter was employed instead (Fukuda *et al.*, 2008). The CaMV 35S promoter has however been successfully employed for the expression of foreign genes in many other species of macroalgae (Kurtzman and Cheney, 1991; Kuang *et al.*, 1998; Zhang *et al.*, 2006; Zhang *et al.*, 2008; Li *et al.*, 2009). Furthermore, while the endogenous *P. yezoensis actin 1* (*PyAct1*) promoter has been shown to be functional for the expression of reporter genes in a number of other Bangiophycean algae (Table 2.1; Hirata *et al.*, 2011), the frequency of expression was significantly reduced for a number of the species tested (Mikami *et al.*, 2011). In addition, the *P. yezoensis* reporter expression system was also tested for its applicability for the transformation of Florideophycean species and found to be unsuitable (Hirata *et al.*, 2011). This evidence suggests that promoters employed for algal transformation do function with varying efficiency between species, and therefore, each should be tested for its applicability to a new system.

A lack of knowledge concerning native algal promoters or algal-associated viral promoters, has negatively affected the progress of macroalgal transformation (Qin *et al.*, 1999). However, until sequences of macroalgal genes along with their regulatory elements become readily available, transformation of these macroalgae will continue to rely on promoters that have been successfully implemented in microalgae and higher plants.

In conclusion, we established a protocol for microparticle bombardment of *G. gracilis* thalli. This optimized bombardment system was then used to test the extent of *lacZ* expression under the influence of three different viral promoters: SV40, CaMV 35S and CMV promoters. All three promoters were functional in expressing *lacZ*; however, the SV40 promoter was shown to be the most efficient and therefore chosen as the most suitable promoter for further *G. gracilis* transformation studies.

University of Cape Town

## **CHAPTER 3**

### **REGENERATION OF WHOLE PLANTS FROM *GRACILARIA GRACILIS* PROTOPLASTS**

University of Cape Town

## Contents

<b>3.1</b>	<b>Summary</b> .....	61
<b>3.2</b>	<b>Introduction</b> .....	62
3.2.1	Aims of this chapter.....	65
<b>3.3</b>	<b>Materials and Methods</b> .....	66
3.3.1	Algal material and culture conditions.....	66
3.3.2	Isolation of protoplasts from <i>G. gracilis</i> thalli .....	66
3.3.2.1	Cytological methods .....	67
3.3.3	Optimization of factors affecting protoplast isolation.....	67
3.3.3.1	Optimization of enzyme constituents.....	68
3.3.3.2	Optimization of pre-treatment of thalli .....	68
3.3.3.3	Optimization of incubation period, pH and temperature .....	69
3.3.3.4	Optimization of osmoticum in the enzymatic medium.....	69
3.3.4	Optimized protocol for <i>G. gracilis</i> protoplast isolation and purification .....	70
3.3.5	Optimization of factors affecting regeneration of whole plants .....	71
3.3.5.1	Optimization of culture media .....	71
3.3.5.2	Optimization of seeding density .....	72
3.3.6	Cell wall regeneration.....	73
3.3.6.1	Calcofluor white staining.....	73
3.3.6.2	Scanning electron microscopy .....	74
3.3.7	Effect of temperature and light intensity on protoplast development .....	74
<b>3.4</b>	<b>Results</b> .....	76
3.4.1	Isolation of protoplasts from <i>G. gracilis</i> thalli .....	76
3.4.2	Optimization of factors affecting protoplast isolation.....	77
3.4.2.1	Effect of enzyme constituents and concentrations on protoplast yields .....	78
3.4.2.2	Effect of pre-treatment on protoplast yields .....	80
3.4.2.3	Effect of incubation period, pH and temperature on protoplast yields .....	80
3.4.2.4	Effect of osmoticum in the enzymatic medium on protoplast yields.....	83
3.4.3	<i>G. gracilis</i> protoplast purification .....	84
3.4.4	Optimization of factors affecting regeneration of whole plants .....	87
3.4.5	Cell wall regeneration.....	88
3.4.6	Effect of temperature and light intensity on protoplast development .....	91
<b>3.5</b>	<b>Discussion</b> .....	105

### 3.1 Summary

The possibility of using *G. gracilis* protoplasts as a suitable cell culture system was investigated. Initially, an isolation protocol was optimized by investigation of the effects of the enzyme constituents and concentrations, the pre-treatment of thalli, the incubation period and temperature, the pH of the enzymatic medium and the osmoticum in the enzymatic medium on protoplast yields. Reliably high yields ( $20.0\text{-}30.0 \times 10^5$  protoplasts  $\text{g}^{-1}$  f.wt) of protoplasts could be obtained from *G. gracilis* thalli when this optimized protocol was used. Furthermore, an OptiPrep<sup>®</sup> discontinuous density gradient was designed and employed for purification of *G. gracilis* protoplasts free of contaminating cell wall debris. The effect of culture media and seeding density on the survivability of *G. gracilis* protoplasts over the period of 1 week was also investigated. Filter-sterilized natural seawater with no supplementation and a seeding density of  $7\text{-}8 \times 10^4$  cells  $\text{ml}^{-1}$  were found to be optimal for the initial culturing of *G. gracilis* protoplasts. Cell wall re-synthesis by *G. gracilis* protoplasts was followed using calcoflour staining and scanning electron microscopy. Protoplasts were shown to complete the initial stages of cell wall re-synthesis within the first 24 hrs of culturing. The effect of light intensity and incubation temperature on whole plant regeneration from *G. gracilis* protoplasts was then investigated. Under conditions of low light intensity ( $5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and high incubation temperature ( $18\text{-}19 \text{ }^\circ\text{C}$ ), *G. gracilis* protoplasts underwent cell division that resulted in the formation of cell clumps, similar to those produced in cell suspension culture of *Porphyra*. The cells produced under these conditions could not be induced to regenerate whole plants. However, whole plants could be regenerated under conditions of higher light intensity ( $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and either high ( $18\text{-}19 \text{ }^\circ\text{C}$ ) or low ( $14\text{-}15 \text{ }^\circ\text{C}$ ) culture temperatures. Under these conditions, protoplasts divided to produce callus-like cell masses which showed the presence of uniseriate, filamentous outgrowths. Buds were produced from these callus-like cell masses and ultimately whole plants were regenerated. The protoplasts either regenerated slowly to produce plants which resembled the parental plants, exhibiting slender, branched thalli, or they regenerated rapidly to produce plants which remained small with thalli that were thick and unbranched.

### 3.2 Introduction

Algal cells and protoplasts, unlike plant cells, are all potentially totipotent and can regenerate into a complete thallus even without the addition of external growth substances. Despite this, regeneration of anatomically complex seaweeds has only been achieved fairly recently. So while regeneration in seaweeds, with their simple morphological and anatomical structure, may seem a simple feat, the factors that affect regeneration are fairly complex in practice.

Many factors affect the yield, viability and regeneration rate of protoplasts. These factors include: pre-treatment of the tissue, enzyme constituents and concentrations, pH, osmotic strength and ionic strength of the isolation medium, incubation temperature, the physiological state of the donor plant including the age of the plant, the growth rate, the duration of laboratory culture and the season in which the plants were harvested, as well as the culture medium and culture conditions in which the protoplasts are kept after isolation (Butler *et al.*, 1989; Bjork *et al.*, 1990; Zablackis *et al.*, 1993; Araki *et al.*, 1994 and 1998; Chen and Chiang, 1994a; Mollet *et al.*, 1995; Benet *et al.*, 1997; Chen, 1998; Chen and Shih, 2000; Dipakkore *et al.*, 2005; Reddy *et al.*, 2008a). Thus, when preparing protoplasts it is critical that each of these conditions should be optimized for each plant or seaweed species.

One method for improving protoplast yields, is through the pre-treatment of the tissue prior to cell wall removal with either plasmolytic solutions or proteases (Butler *et al.*, 1989; Fujita and Saito, 1990; Polne-Fuller and Gibor, 1990; Bjork *et al.*, 1992; Araki *et al.*, 1994 and 1998; Chen and Chiang, 1994a and 1995; Mollet *et al.*, 1995; Dipakkore *et al.*, 2005; Yeong *et al.*, 2008; Gupta *et al.*, 2011). Tribe (1955) was the first to find that plasmolysis of plant tissue inhibited the toxic effects of cell separation. This is because pre-plasmolysis ensures that all the cells have reduced turgor pressure prior to digestion of the cell walls (Butler *et al.*, 1989). Protoplasts often burst when plasmolysis is carried out during cell wall digestion since they have not reached equilibrium with the osmoticum. Plasmolysing protoplasts prior to digestion can also help inhibit the uptake of toxic substances such as proteases, lipases and ribonucleases from crude enzyme preparations by infolding of the cell membrane (Butler *et al.*, 1989). Pre-treatment of thalli with proteolytic enzymes is thought to improve protoplast yields by digesting the surface proteins of the thallus, and thereby,

improving penetration of the polysaccharide-degrading enzymes into the cell wall matrix (Waaland *et al.*, 1990; Dipakkore *et al.*, 2005).

Seaweeds, unlike higher plants, exhibit a highly variable cell wall in both complexity and chemical composition, particularly in the abundance and type of amorphous matrix compounds. The matrix polysaccharides of the Rhodophyta are generally linear sulphated galactans composed of repeated galactose units with an alternating sequence of  $\beta(1-4)$  and  $\alpha(1-3)$  linkages (Murano, 1995), while the cellulose content of the cell walls ranges from 1-8% of the dry weight of the thallus (Reddy *et al.*, 2008a). The cellulose content of the Phaeophyta is similar to that of the Rhodophyta, but the major matrix component of these brown seaweeds, accounting for 10 to 45% of the thallus dry weight, is alginic acid which is composed of  $\beta$ -1,4-D-mannuronic acid residues with varying amounts of 1,4-L-guluronic acid (Lee, 1980; Reddy *et al.*, 2008a). The Chlorophyta have either xylogalactoarabinans or glucuronoxylorhamnans with varying sulphate content as matrix polysaccharides, while the cellulose content of these green seaweeds may contribute up to 70% of the thallus dry weight (Reddy *et al.*, 2008a). These fundamental differences in cell wall composition mean that a unique set of specific enzymes is required to efficiently digest the cell wall and yield protoplasts for each different phylum of seaweeds. For example, protoplasts of green seaweeds can be prepared by using cellulases singly or in combination with macerozyme. In addition to cellulase, the red and brown seaweeds require either agarase/carragenase or alginase, respectively (Reddy *et al.*, 2008a).

The optimum pH for protoplast isolation has been said to be largely dependent on the enzymes used in the digestion of the cell walls (Butler *et al.*, 1989). This, along with the optimal temperature and incubation time, should be assessed for each particular species of seaweed under investigation.

In most cases, either mannitol or sorbitol is used as an osmotic stabilizer (Cheney *et al.*, 1986; Chen, 1987 and 1989; Bjork *et al.*, 1990; Polne-Fuller and Gibor, 1990; Coury *et al.*, 1993; Yan and Wang, 1993; Zablackis *et al.*, 1993; Araki *et al.*, 1994 and 1998; Beer and Bjork, 1994; Chen and Chiang, 1994a and 1995; Mollet *et al.*, 1995). Uppalapati and Fujita (2002), Dipakkore *et al.* (2005) and Reddy *et al.* (2006) have reported increased protoplast yields for a number of species of seaweeds when a low strength ionic

medium (1% NaCl in deionised water) was used. The increased yields in these cases were attributed to higher activities of the cell wall degrading enzymes in the low ionic strength media (Reddy *et al.*, 2006).

The first step in protoplast regeneration is cell wall development. The course of cell wall regeneration in protoplasts can be monitored through the use of scanning and transmission electron microscopy or by staining the protoplasts with a fluorescent brightener agent, such as calcofluor white (Nagata and Takebe, 1970). The detailed regeneration of cell walls in two species of seaweed, *Palmaria palmate* (Liu *et al.*, 1992) and *Grateloupia sparsa* (Chen and Chiang, 1995), have been followed through the use of transmission and scanning electron microscopy. Liu *et al.* (1992) suggest that the process of cell wall regeneration in protoplasts differs to the process of existing cell wall secretion in intact cells and spores, and that it is rather a response to the removal of the cell wall than a continuation of existing cell wall secretion.

The second step in protoplast regeneration is cell division. The prolonged presence of osmoticum in the culture medium has been shown to negatively affect cell division and further development of protoplasts in a number of species (Reddy *et al.*, 1989; Polne-Fuller and Gibor, 1990; Reddy and Fujita, 1991; Benet *et al.*, 1997). As a result, in most cases, the osmoticum is diluted out of the culture medium either prior to the onset of culturing, or as soon as the cell wall has been regenerated.

Protoplasts are generally cultured in the same medium that was used to cultivate the intact thallus (Reddy *et al.*, 2008a). However, there are particular instances where media constituents have been found to be toxic to the developing protoplasts. Yeong *et al.* (2008) stated that *G. changii* protoplasts were sensitive to the presence of ammonium and nitrate in the PES culture medium, and as a result they did not observe any further development of protoplasts. Similarly, it has been reported that although ammonium nitrate enhances cell division in higher plants, concentrations higher than 20 mM are toxic to plant protoplasts (Collin and Edwards, 1998; Compton *et al.*, 2000 referenced in Yeong *et al.*, 2008). Benet *et al.* (1997) noted that natural seawater without enrichment was the optimal culture medium for the long-term cultivation of *Laminaria* protoplasts.

The seeding density of protoplasts is an important factor in the successful cultivation and regeneration of protoplasts, since cells have the potential to provide undefined chemicals that can stimulate both cell wall regeneration and cell division in neighbouring cells. This phenomenon has been exploited in higher plants through the use of ‘feeder’ or ‘nurse’ cells which are fast-growing protoplasts or cells from either the same or a different plant that can enhance protoplast division (Davey *et al.*, 2005b).

Detailed algal regeneration has been reported for four species of *Gracilaria*: *G. changii*, (Yeong *et al.*, 2008), *G. asiatica*, (Yan and Wang, 1993), *G. tikvahiae* (Cheney, 1990) and *G. chilensis* (Cheney, 1990). However, the growing list of seaweed species for which protoplast regeneration has been achieved indicates that this may soon become an economically viable method of culturing seaweeds.

### **3.2.1 Aims of this chapter**

The aim of this chapter was to investigate the complex factors affecting protoplast yield from *G. gracilis* thalli and to establish an optimal protocol which would give reliably high yields of protoplasts. Furthermore, once a reliable method of protoplast isolation had been established, protoplast regeneration and the factors affecting this were investigated.

### 3.3 Materials and Methods

All media and solutions used in this study are listed in Appendix A.

#### 3.3.1 Algal material and culture conditions

As described in Chapter 2.3.1.

#### 3.3.2 Isolation of protoplasts from *G. gracilis* thalli

Initially, protoplasts were isolated by a method modified from Mollet *et al.* (1995), Araki *et al.* (1998) and Yeong *et al.* (2008). The method, together with the modifications, is described below.

Limited thallus sterilization was carried out prior to protoplast isolation since extensive sterilization processes negatively affected protoplast yield from thallus tissue. The sterilization process consisted of rinsing thalli three times in ASW (Appendix A.1.1), followed by washing the thalli with liquid detergent (Coss Ultra<sup>®</sup>) for 30 s and finally rinsing in ASW as before.

Following sterilization, *G. gracilis* thalli were chopped into 2-3 mm long pieces using a sterile scalpel blade and rinsed twice in sterile ASW prior to plasmolysis. For plasmolysis, thalli were immersed for 30 min in plasmolysis medium (Appendix A.2.10.13) at 22 °C in the dark on a rotary shaker (40 rpm). Following this, the plasmolysis medium was removed and replaced with enzymatic medium (Appendix A.2.10.8). During the enzymatic treatment, *G. gracilis* thalli fragments were incubated on a rotary shaker (40 rpm) in the dark at 22 °C for 6 hrs. Approximately 1 g of fresh weight (f.wt) tissue was added per 10 ml of enzymatic medium.

Following enzymatic cell wall digestion, the enzymatic medium containing protoplasts was collected and filtered through a 40 µm nylon mesh filter (Millipore) to remove any undigested tissue and cellular debris. The protoplasts were then harvested by centrifugation (160x g for 10 min at 22 °C) and the resulting pellet was subsequently resuspended in rinse medium (Appendix A.2.10.9).

### 3.3.2.1 Cytological methods

Protoplast numbers were determined using a haemocytometer (Neubauer improved bright-line Haemocytometer).

Viability of protoplasts was assessed by their ability to exclude Trypan Blue (Sigma). Trypan Blue was used at a final concentration of 0.02% (w/v) in sterile ASW.

In order to confirm that protoplasts were in fact true protoplasts lacking cell walls, they were stained with 0.01% (w/v) calcofluor white M2R (Sigma), a fluorescent dye that binds to the cellulose of cell walls (Nagata and Takebe, 1970), and examined under a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 400 nm emission filter. Bursting of protoplasts following the addition of sterile distilled water was also carried out to assess the absence of cell walls (Björk *et al.*, 1990).

### 3.3.3 Optimization of factors affecting protoplast isolation

Since the factors that affect protoplast yield from seaweeds are so numerous (Reddy *et al.*, 2008a), it was first necessary to optimize the factors that were immediately under our control. These included the enzyme constituents and concentrations, the pre-treatment of thalli, the incubation period and temperature, the pH of the enzymatic medium and the osmoticum in the enzymatic medium.

During the optimization experiments, thalli were pre-treated and protoplasts isolated and collected as described in 3.3.2, unless otherwise stated. Three replicate isolations, containing a known mass of chopped thalli (0.3–0.4 g f.wt), were carried out per treatment for each optimization experiment. During the optimization experiments, tissue digestions were carried out in 35 x 10 mm tissue culture dishes (Falcon). Optimization experiments were also never carried out on seaweed which had been cultured for longer than a week under laboratory conditions. One-way or two-way ANOVAs were used to analyze the optimization data. When the results of the ANOVA were significant, the Tukey Test was used to determine the significant differences due to various treatments using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at  $P < 0.05$ .

### 3.3.3.1 Optimization of enzyme constituents

The effect of various concentrations of cell wall degrading enzymes on protoplast yields was tested, either singly or in combination, in order to maximize the number of viable protoplasts which could be generated from *G. gracilis* thalli.

The effect of different concentrations of cellulase Onozuka R-10 (Yakult) (Appendix A.2.10.4), on protoplast yield from *G. gracilis* thalli, was tested by varying the final concentration (0, 1, 2 and 3% (w/v)) of cellulase in the enzymatic medium (Appendix A.2.10.7).

The effect of different concentrations (0, 1.5, 10 and 15 U ml<sup>-1</sup>) of agarase from *Pseudomonas atlantica* (Sigma) (Appendix A.2.10.6), in combination with either 2 or 3% (w/v) cellulase Onozuka R-10, on protoplast yield from *G. gracilis* thalli was tested. One unit (U) of agarase is defined as the amount of enzyme that will produce 1 µg of reducing sugar (measured as D-galactose) from agar per minute at pH 6.0 at 40 °C (Sigma). The various enzymatic concentrations were achieved by varying their final concentrations in the enzymatic medium (Appendix A.2.10.8).

The effect of different concentrations of Macerozyme R-10 (Yakult) (Appendix A.2.10.10), in combination with optimal levels of cellulase Onozuka R-10 and agarase, on protoplast yields from *G. gracilis* thalli was tested by varying the final concentration (0, 1 and 2% (w/v)) of macerozyme in the enzymatic medium (Appendix A.2.10.11).

All further experiments were conducted using a combination of 2% (w/v) cellulase Onozuka R-10, 1% (w/v) Macerozyme R-10 and 10 U ml<sup>-1</sup> agarase.

### 3.3.3.2 Optimization of pre-treatment of thalli

The effect of pre-treatment of thalli with various concentrations (0, 1, 2 and 5% (w/v)) of papain from papaya latex (Sigma) on *G. gracilis* protoplast yields was tested. The papain treatment was carried out at the same time as the pre-plasmolysis step, and the final concentration of papain was varied by altering the amount of papain in the plasmolysis medium (Appendix A.2.10.14). Following the 30 min papain and pre-plasmolysis treatment, the papain solution was removed and the thalli were gently rinsed three times with

plasmolysis medium before the addition of enzymatic medium (Appendix A.2.10.15). In order to counteract any toxic effects from any remaining papain, a protease inhibitor cocktail (EDTA-free protease inhibitor cocktail, Roche) (Appendix A.2.10.12) was included in the enzymatic solution.

A final concentration of 1% (w/v) papain was selected as optimal and employed for all further protoplast isolations.

### **3.3.3.3 Optimization of incubation period, pH and temperature**

In order to determine the optimal incubation period of enzymatic digestion for optimal protoplast production, *G. gracilis* thalli were incubated for periods of 1, 2, 3, 4, 5 and 6 hrs in enzymatic medium (Appendix A.2.10.15). The yield of protoplasts and protoplast viability (%) was determined for each time period.

The optimal pH of the enzymatic medium was determined by altering the pH (pH 6.0, 6.15, 6.3 and 6.5) of the Tris-MES buffer (Appendix A.2.10.2) in the enzymatic medium (Appendix A.2.10.15).

The optimal temperature for enzymatic treatment of the thalli was assessed by employing a range of incubation temperatures (18, 22 and 25 °C) during *G. gracilis* protoplast isolation. Protoplasts were collected after 3 hrs of enzymatic treatment and the temperature that resulted in the highest yields was determined. This incubation temperature was employed for all further protoplast isolations.

### **3.3.3.4 Optimization of osmoticum in the enzymatic medium**

The optimal concentration of mannitol for protoplast isolation was determined by altering the mannitol concentration (0.6, 0.8 and 1 M) (Appendix A.2.10.1) in the enzymatic medium (Appendix A.2.10.15) and determining the effect this had on *G. gracilis* protoplast yields.

There have been reports of increased protoplast yields when a low ionic strength solution of 1% NaCl in deionised water replaced the traditional use of sterile seawater in the enzymatic medium. Therefore, the effect of this substitution on the yield of *G. gracilis* protoplasts was assessed. The yields from enzymatic solutions (Appendix A.2.10.16) modified from those of

Reddy *et al.* (2006) were compared to yields obtained with the enzymatic solution (Appendix A.2.10.15) optimized in this study. A summary of the various media compositions assessed in this study are shown in Table 3.1.

**Table 3.1** Composition of enzymatic media employed in optimization of protoplast isolation.

Composition <sup>a</sup>	Medium X <sup>b</sup>	Medium Y	Medium Z
Cellulase Onozuka R-10	2%	2%	2%
Macerozyme R-10	1%	1%	1%
Agarase	10 U ml <sup>-1</sup>	10 U ml <sup>-1</sup>	10 U ml <sup>-1</sup>
Dextran sulphate	-	0.5%	0.5%
CaCl <sub>2</sub>	10 mM	10 mM	1 mM
NaCl	-	1%	3%
ASW	50%	-	-
Mannitol	0.8 M	0.8 M	0.6 M
MES	10 mM	10 mM	10 mM
pH	6.15	6.15	6.15

<sup>a</sup> Dissolved in de-ionised water.

<sup>b</sup> Medium found to be optimal in this study.

### 3.3.4 Optimized protocol for *G. gracilis* protoplast isolation and purification

Axenic starting material is required for protoplast culture and plant regeneration (Reddy *et al.*, 1989). Therefore, *G. gracilis* thalli used for the production of protoplasts for culturing purposes were subjected to a more rigorous sterilization protocol than had been employed in the protoplast yield optimization experiments. The method developed for thallus sterilization in Chapter 2.3.3 severely affected protoplast yields and therefore, it was adapted by excluding the 3 hr incubation in sterile distilled water step and replacing it with a 30 s wash in liquid detergent (Coss Ultra<sup>®</sup>). The sterilization protocol was otherwise unchanged.

Following sterilization, *G. gracilis* thalli were processed as described in 3.3.2. For plasmolysis, thalli were immersed for 30 min in plasmolysis medium containing 1% papain (w/v) (Appendix A.2.10.14) at 22 °C in the dark on a rotary shaker (40 rpm). Following the 30 min papain and pre-plasmolysis treatment, the papain solution was removed and the thalli were gently rinsed three times with plasmolysis medium before the addition of the optimized enzymatic medium (Medium X, Table 3.1; Appendix A.2.10.15). During the enzymatic

treatment, *G. gracilis* thalli fragments were incubated on a rotary shaker (40 rpm) in the dark at 22 °C for 3 hrs. Approximately 1 g of fresh weight (f.wt) tissue was added per 10 ml of enzymatic medium.

In addition to more stringent sterilization of algal material, the collection and purification of protoplasts was also altered. Protoplasts were harvested as described in 3.3.2 and following resuspension in rinse medium, the protoplasts were once again harvested by centrifugation (160x g for 10 min at 22 °C). Following centrifugation, approximately a quarter (500 µl) of the supernatant was removed and replaced with an equal volume of ASW to dilute the osmoticum in the rinse medium. Protoplasts were then gently resuspended. The process of centrifugation, medium dilution and resuspension of protoplasts was repeated a further four times, in order to gradually dilute out the osmoticum. The *G. gracilis* protoplast pellet was finally resuspended in ASW.

Protoplasts were further purified through a discontinuous isotonic density gradient employing OptiPrep<sup>®</sup> (Sigma). OptiPrep<sup>™</sup> is a sterile endotoxin tested solution of 60% (w/v) iodixanol (5,5'-[(2-hydroxy-1-3 propanediyl)-bis(acetylamino)] bis [N,N'-bis (2,3 dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide)]) in water. It is non-toxic, non-ionic and metabolically inert. The OptiPrep<sup>®</sup> discontinuous density gradient was constructed as described in Appendix B.13. Density gradients were centrifuged at 160x g for 10 min and the purified protoplasts were collected from the interface between the top two layers of the OptiPrep<sup>®</sup> density gradient. Protoplast numbers were determined as described in 3.3.2.1.

### **3.3.5 Optimization of factors affecting regeneration of whole plants**

Since both protoplast culture media and protoplast seeding densities for cultivation have been shown to affect the regeneration potential of protoplasts (Reddy *et al.*, 2008a; Yeong *et al.*, 2008), it was first necessary to assess the effects that these two parameters had on protoplast survival.

#### **3.3.5.1 Optimization of culture media**

The effect of various culture media on the survival of freshly isolated *G. gracilis* protoplasts was assessed.

*G. gracilis* protoplasts, isolated and purified as described in 3.3.4, were dispensed into 2 ml of filter-sterilized natural seawater with or without PES supplementation (Provasoli, 1968) ( $\frac{1}{3}$ ,  $\frac{1}{2}$  or full strength) (Appendix A.1.4) in 35 x 10 mm tissue culture dishes (Falcon) at a density of  $7 \times 10^4$  cells  $\text{ml}^{-1}$ . All cultures were supplemented with penicillin G ( $100 \mu\text{g ml}^{-1}$ ) and amphotericin B ( $0.1 \mu\text{g ml}^{-1}$ ) (Appendix A.2.2). The protoplast density was based on what had previously been observed to be optimal for *G. changii* protoplast culture (Yeong *et al.*, 2008). Three biological repeats were conducted per treatment. Culture plates containing protoplasts were incubated at 14–15 °C in the dark for 2 days, before gradual exposure to a light intensity of  $10 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 16/8 hrs (day/night) for 1 week and thereafter, protoplasts were maintained at  $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 16/8 hrs (day/night). Half the culture volume was replaced with fresh culture medium every five days. Antibiotics were maintained in protoplast cultures for the first 2 weeks of culturing.

Protoplasts were counted by selecting 10 random fields of view under a microscope and counting the number of protoplasts visible within each particular field of view. These counts were done 2 days after seeding, thereby allowing the protoplasts sufficient time to settle, and again 7 days later. Thus, protoplast survival after 9 days was expressed as a percentage of the total number of protoplasts counted initially at 2 days. Statistical analysis was carried out as described in 3.3.3.

### 3.3.5.2 Optimization of seeding density

The effect of seeding density on the survival of freshly isolated *G. gracilis* protoplasts was tested.

Protoplasts isolated and purified as described in 3.3.4, were dispensed into 2 ml of filter-sterilized natural seawater in 35 x 10 mm tissue culture dishes (Falcon) at densities ranging from  $2 \times 10^4$ – $2 \times 10^5$  cells  $\text{ml}^{-1}$ . Protoplasts were cultured as described in 3.3.5.1.

The percentage of *G. gracilis* protoplast survival was calculated as described in 3.3.5.1. Statistical analysis was carried out as described in 3.3.3.

### 3.3.6 Cell wall regeneration

The first step to protoplast regeneration is cell wall formation. This process was followed through observation of protoplasts sampled and stained with calcofluor white (Nagata and Takebe, 1970) at different time points, as well as by observation of protoplasts sampled and fixed at different time points by scanning electron microscopy (SEM).

Protoplasts were isolated and purified as described in 3.3.4 and cell wall regeneration was observed immediately over a 24 hr period. Protoplasts that were defined as 0 hr samples were purified slightly differently in order to minimize the sample handling time prior to either fixing or staining of samples. If the original purification protocol had been followed, 0 hr samples would have already had approximately 1 hr (sample handling time) to undergo cell wall deposition. Thus, the 0 hr protoplast samples were resuspended in ASW containing 0.4 M sorbitol (Appendix A.2.10.18) and immediately purified using the OptiPrep<sup>®</sup> discontinuous density gradient method as outlined in section 3.3.4. Alterations of buffer constituents are outlined in Appendix B.13. Purified protoplasts were either immediately processed (0 hr samples) or dispensed into 2 ml of filter-sterilized natural seawater containing penicillin G (100  $\mu\text{g ml}^{-1}$ ) and amphotericin B (0.1  $\mu\text{g ml}^{-1}$ ) in 35 x 10 mm tissue culture dishes (Falcon) at a density of  $7.5 \times 10^4$  cells  $\text{ml}^{-1}$ . Culture plates containing protoplasts were incubated at 14-15 °C in the dark.

#### 3.3.6.1 Calcofluor white staining

Cell wall regeneration of *G. gracilis* protoplasts was followed over the initial 24 hr period. In order to track protoplast cell wall regeneration, 0.01% (w/v) calcofluor white was included in the culture medium. The percentage of protoplasts showing resynthesized cell walls was determined by counting the number of protoplasts showing blue fluorescence (indicating those with cell walls) and those appearing red (indicating no cell walls) in 5 randomly selected fields of view under a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 400 nm emission filter. Counts were carried out at 0, 3, 12 and 24 hrs after initiating the cultures.

### 3.3.6.2 Scanning electron microscopy

The 0 hr *G. gracilis* protoplast samples were immediately fixed, whereas protoplasts sampled at the later time points were cultured for 3, 12 or 24 hrs before collection and fixation. Protoplasts were initially fixed at 4 °C for 1 hr in culture medium (either 0.4 M sorbitol-ASW or natural seawater) containing 2% (v/v) glutaraldehyde (Merck). Thereafter, protoplasts were gently passed through a syringe and into a filter unit containing 1 µm filter paper (Millipore). All further treatments of the protoplasts were carried out in the filter unit. Following collection, protoplasts were re-fixed with 5% (v/v) glutaraldehyde in 1x base buffer (bb) (Appendix A.2.10.20) containing 10 mM MgCl<sub>2</sub> and 0.2 M sucrose for 1 hr at 4 °C. Protoplasts were then rinsed four times with 1x bb containing 10 mM MgCl<sub>2</sub>, with the sucrose concentration being successively reduced to 0.05 M, and then rinsed twice in sucrose-free 1x bb containing 10 mM MgCl<sub>2</sub>. Thereafter, the *G. gracilis* protoplasts were rinsed 3 times with aqueous ethanol (50% (v/v)) and sequentially dehydrated in a graduated ethanol series (70, 80, 85, 90, 95 and 100% (v/v)). Following dehydration, the filter paper was removed from the filter unit and glued onto an aluminium stub. The samples were then further dehydrated with hexamethyldisilazane (HMDS) and critical point dried. Stubs were sputter coated with gold palladium alloy and then viewed in the SEM.

### 3.3.7 Effect of temperature and light intensity on protoplast development

The effects of both cultivation temperature and light intensity on protoplast cultures were assessed, since these have been reported to be important factors that influence plant regeneration patterns in *Porphyra* (Chen, 1989; Polne-Fuller and Gibor, 1990). Plant regeneration has also never before been reported in *G. gracilis* and therefore it was important to investigate the effects that these parameters had on plant regeneration.

Protoplasts isolated and purified as described in 3.3.4 were dispensed into 2 ml of filter-sterilized natural seawater containing penicillin G (100 µg ml<sup>-1</sup>) and amphotericin B (0.1 µg ml<sup>-1</sup>) in 35 x 10 mm tissue culture dishes (Falcon) at a density of 7.5 x 10<sup>4</sup> cells ml<sup>-1</sup>. Culture plates containing protoplasts were incubated at 14–15 °C in the dark for 2 days to allow protoplasts to settle, before combinations of low (5 µmol photons m<sup>-2</sup>s<sup>-1</sup>) and high (10 µmol photons m<sup>-2</sup>s<sup>-1</sup> for 1 week and thereafter, 30 µmol photons m<sup>-2</sup>s<sup>-1</sup>) light intensities, in combination with either high (18-19 °C) or low (14-15 °C) culture temperatures, were applied. In all cases a photoperiod of 16/8 hrs (day/night) was applied to cultures. Half the

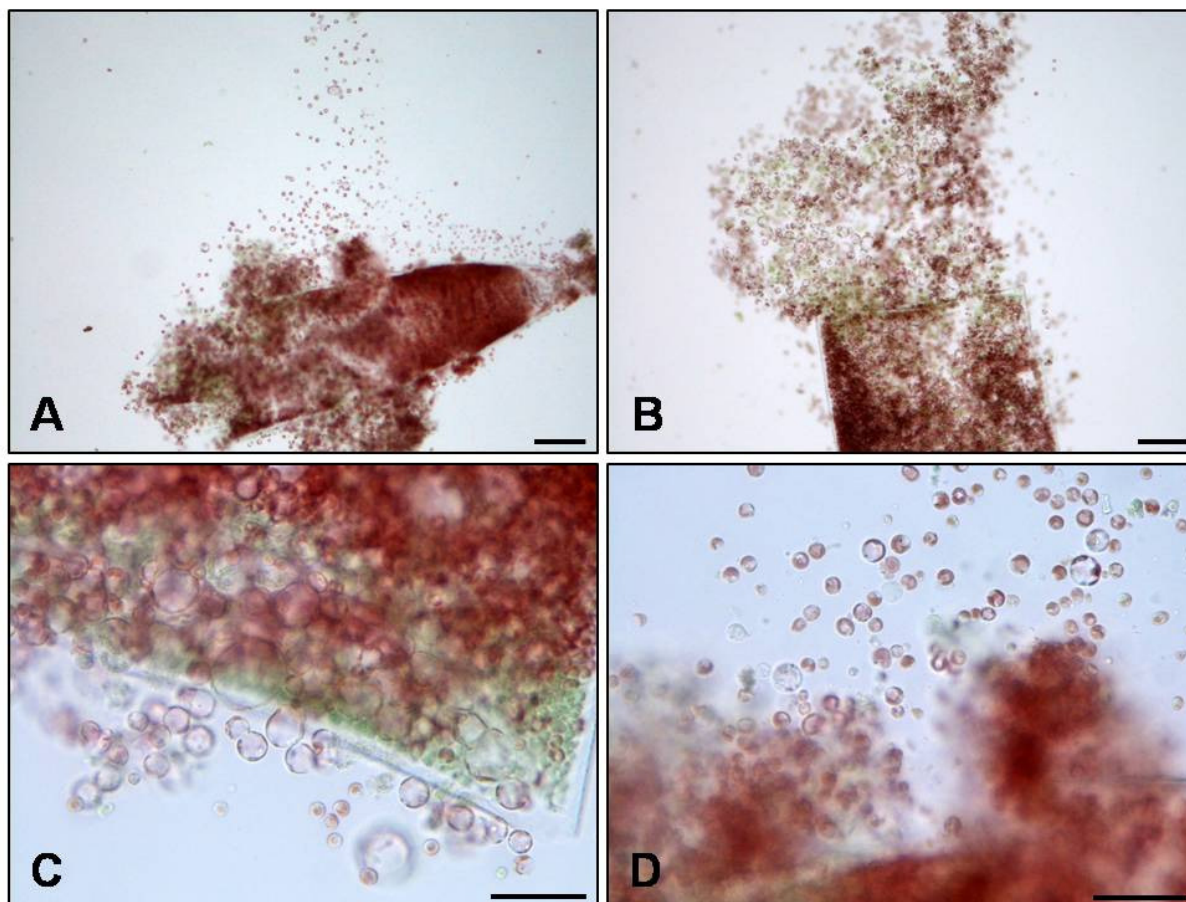
culture volume was replaced with fresh culture medium every five days. After 2 weeks of cultivation, the culture medium was changed from filter-sterilized natural seawater to filter-sterilized natural seawater supplemented with PES (full strength). Antibiotics were maintained in protoplast cultures for the first 2 weeks of culturing.

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### 3.4 Results

#### 3.4.1 Isolation of protoplasts from *G. gracilis* thalli

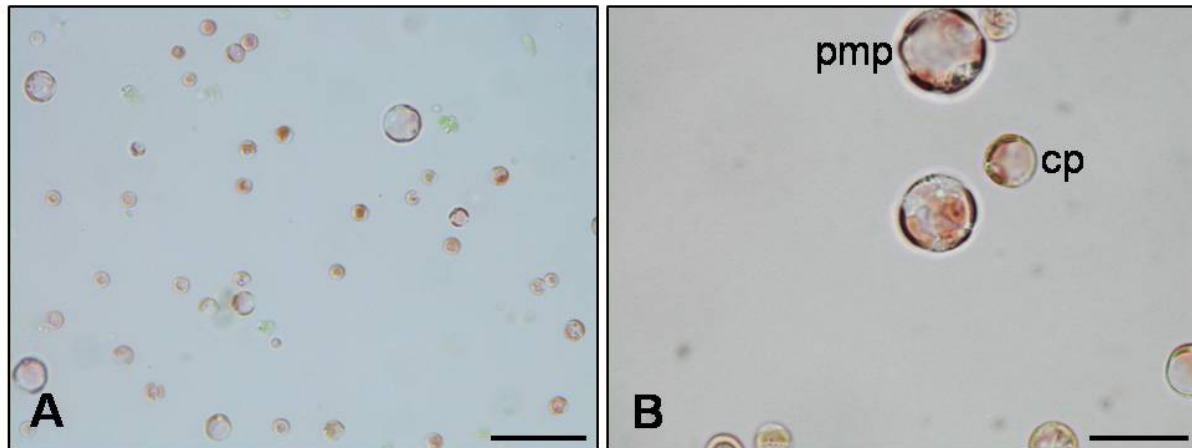
The protoplast isolation method modified from those of Mollet *et al.* (1995), Araki *et al.* (1998) and Yeong *et al.* (2008) proved successful for the liberation of protoplasts from *G. gracilis* thalli (Fig. 3.1A-D). Protoplasts were released mainly from the apical tip (Fig. 3.1A) and cut surfaces of the thalli (Fig. 3.1B & C).



**Figure 3.1** Protoplasts of *G. gracilis* being released from thalli during enzymatic treatment (A-D). Images were taken with a Nikon Eclipse 50i Compound Microscope. Scale bar = 100  $\mu\text{m}$  (A & B) and 50  $\mu\text{m}$  (C & D).

Purified protoplasts were typically spherical, pigmented red-pink and varied in size (Fig. 3.2A). Two main types of protoplasts were readily isolated, i) those which were smaller in size (5-15  $\mu\text{m}$  diameter), more highly pigmented and derived from the cortical region of the thallus where cells have larger and more numerous chloroplasts, and ii) those which were larger in size (20-40  $\mu\text{m}$  diameter), less pigmented and derived from the perimedullary region of the thallus where cells tend to have smaller chloroplasts and larger vacuoles (Fig. 3.2B).

The smaller cortical protoplasts were always more numerous than the larger perimedullary protoplasts and comprised approximately 80-90% of the protoplasts isolated (Fig. 3.2A). Large protoplasts (100-200  $\mu\text{m}$  in diameter), originating from the medullary region were rarely seen. All newly isolated *G. gracilis* protoplasts did not display blue fluorescence following staining with calcofluor white and burst when placed in fresh water, indicating that they were true protoplasts (data not shown).



**Figure 3.2** Freshly isolated protoplasts from *G. gracilis* thalli (A). Two size classes of protoplasts are obtained from *G. gracilis* thalli (B). Abbreviations: pmp, perimedullary protoplast; cp, cortical protoplast. Images were taken with a Nikon Eclipse 50i Compound Microscope. Scale bar = 50  $\mu\text{m}$  (A) and 20  $\mu\text{m}$  (B).

### 3.4.2 Optimization of factors affecting protoplast isolation

The modified method originally employed to isolate protoplasts from *G. gracilis* thalli was successful, routinely yielding  $5 \times 10^5$ – $1 \times 10^6$  protoplasts  $\text{g}^{-1}$  f.wt. However, previous studies pertaining to protoplast isolation from various red macroalgal species suggest that yields in excess of  $10^6$  protoplasts  $\text{g}^{-1}$  f.wt could be regularly obtained (Zablackis *et al.*, 1993; Araki *et al.*, 1998; Dipakkore *et al.*, 2005; Yeong *et al.*, 2008). Many factors affect the yield of protoplasts (Reddy *et al.*, 2008a), including tissue pre-treatment, cell wall degrading enzymes and concentrations, pH, osmotic and ionic strength of the isolation medium, and incubation temperature. Therefore, it was necessary that these factors be optimized for *G. gracilis* protoplast isolation, before developing protocols for whole plant regeneration.

### 3.4.2.1 Effect of enzyme constituents and concentrations on protoplast yields

Cellulase Onozuka R-10 at concentrations ranging from 1–3% (w/v), even in the absence of agarase, resulted in the release of protoplasts from *G. gracilis* thalli (Fig. 3.3A). A concentration of 3% (w/v) cellulase resulted in the highest yield of protoplasts ( $10.04 \pm 0.83 \times 10^5$  protoplasts  $g^{-1}$  f.wt) ( $P < 0.05$ ), while 1 and 2% cellulase (w/v) resulted in protoplast yields of  $3.68 \pm 0.97 \times 10^5$  and  $6.45 \pm 0.54 \times 10^5$  protoplasts  $g^{-1}$  f.wt, respectively (Fig. 3.3A). No protoplasts were released when cellulase was omitted from the enzymatic medium.

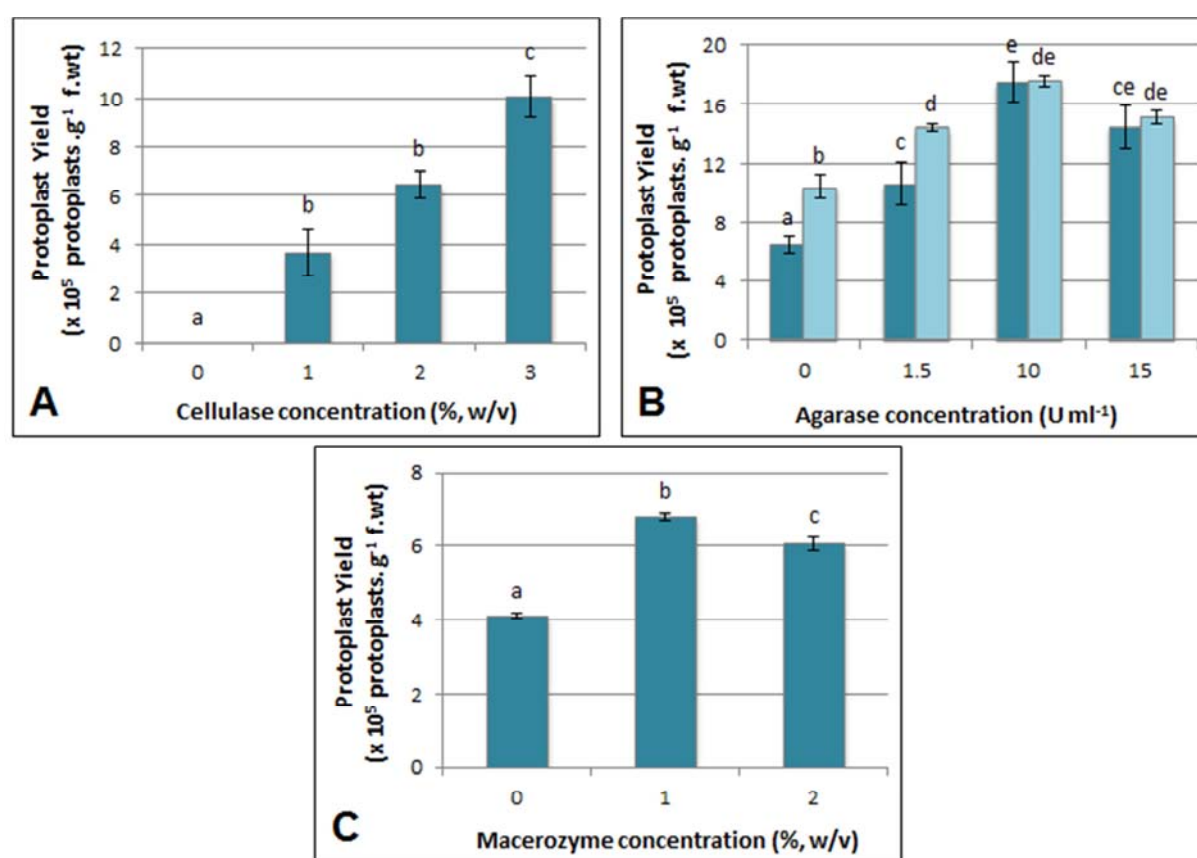
While the addition of 3% (w/v) cellulase resulted in the production of protoplasts from *G. gracilis* thalli on its own, it has been previously shown that a cocktail of cell wall degrading enzymes can produce higher yields of protoplasts than the respective enzymes individually (Yeong *et al.*, 2008). Therefore, the addition of various concentrations of agarase (0–15 U  $ml^{-1}$ ) in combination with either 2 or 3% cellulase (w/v), which resulted in the highest yields of protoplasts, was investigated as a means of obtaining increased protoplast yields from *G. gracilis* thalli.

The inclusion of agarase, at any concentration, in the enzymatic medium with either 2 or 3% cellulase resulted in significantly ( $P < 0.05$ ) higher yields of protoplasts when compared to protoplast yields obtained with cellulase on its own (Fig. 3.3B). A combination of either 2 or 3% cellulase and 10 U  $ml^{-1}$  of agarase resulted in the highest yields of protoplasts ( $17.5 \pm 1.3 \times 10^5$  and  $17.6 \pm 0.34 \times 10^5$  protoplasts  $g^{-1}$  f.wt, respectively) (Fig. 3.3B). Since the protoplast yields obtained with a higher agarase concentration (15 U  $ml^{-1}$ ) and either 2 or 3% cellulase were not significantly ( $P > 0.05$ ) different from the yields obtained with 10 U  $ml^{-1}$  of agarase, a combination of 2% cellulase and 10 U  $ml^{-1}$  of agarase was included in the enzymatic medium used for all further isolations.

Protoplast yields varied between batches of seaweed received from the supplier. Optimization of the agarase and cellulase concentration (Fig. 3.3B) and optimization of the macerozyme concentration (Fig. 3.3C) were done using two different batches of seaweed. The employment of enzymatic medium containing 2% (w/v) cellulase and 10 U  $ml^{-1}$  of agarase (Fig. 3.3B) resulted in protoplast yields of  $17.6 \pm 0.34 \times 10^5$  protoplasts  $g^{-1}$  f.wt for one batch of seaweed, while this same enzymatic medium (0% macerozyme) used for

protoplast isolation from a separate batch of seaweed resulted in protoplast yields of only  $4.11 \pm 0.20 \times 10^5$  protoplasts  $g^{-1}$  f.wt (Fig. 3.3C). Thus, comparisons of protoplast yields should only be made when the same batch of seaweed is being considered.

Macrozyme R-10 has been shown to enhance protoplast yields when used in combination with other cell wall degrading enzymes (Chen and Chiang, 1994; Araki *et al.*, 1998; Reddy *et al.*, 2005; Yeong *et al.*, 2008). Therefore, the effect of macrozyme R-10 on the yields of protoplasts from *G. gracilis* thalli was tested by including it in the enzymatic medium, together with 2% (w/v) cellulase and 10 U  $ml^{-1}$  of agarase.



**Figure 3.3** Effect of enzyme constituents and concentrations, either singly or in combination, on protoplast yield from *G. gracilis* thalli. The effect of cellulase Onozuka R-10 concentrations (% w/v) on protoplast yield was tested in isolation (A), while various concentrations of agarase (units  $ml^{-1}$ , U  $ml^{-1}$ ) were tested in combination with either 2% (■) or 3% (□) cellulase (B). The effect of macrozyme R-10 concentration (% w/v) on protoplast yield, in combination with 2% cellulase and 10 U  $ml^{-1}$  agarase, is shown in (C). In each case data represents the mean  $\pm$  standard error. Different postscripts indicate a significant difference ( $P < 0.05$ ; one-way or two-way ANOVA) between sample means (a, b, c, d or e).

The inclusion of either 1 or 2% (w/v) macerozyme in the enzymatic medium resulted in significantly higher ( $P < 0.05$ ) protoplast yields ( $6.82 \pm 0.10 \times 10^5$  and  $6.07 \pm 0.20 \times 10^5$  protoplasts  $g^{-1}$  f.wt, respectively) (Fig. 3.3C). Macerozyme at a concentration of 1%, in combination with 2% (w/v) cellulase and  $10 U ml^{-1}$  agarase, proved optimal for *G. gracilis* protoplast isolation (Fig. 3.3C).

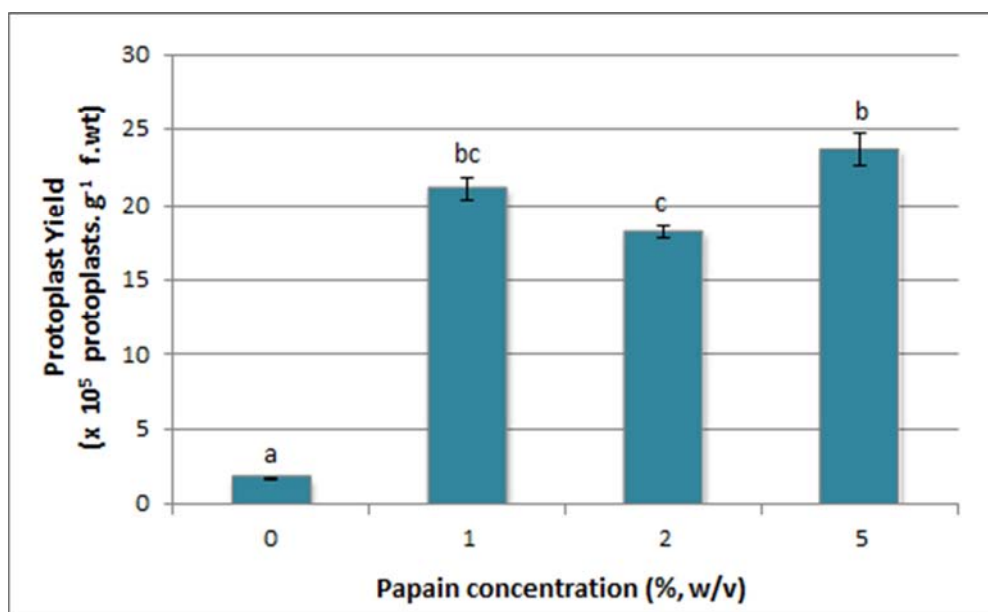
#### 3.4.2.2 Effect of pre-treatment on protoplast yields

Pre-treatment of thalli with proteolytic enzymes has previously been shown to improve the yield of protoplasts from many species of macroalgae (Araki *et al.*, 1994 and 1998; Chen and Chiang, 1994a and 1995; Yeong *et al.*, 2008). Therefore, the effect of pre-treatment of *G. gracilis* thalli with the proteolytic enzyme preparation, papain, on protoplast yield was investigated.

The pre-treatment of *G. gracilis* thalli with 1, 2 or 5% (w/v) papain resulted in significantly ( $P < 0.05$ ) increased yields of 12.5, 10.8 and 14.1 fold, respectively, when compared to the yield from thalli that were not pre-treated with papain (Fig. 3.4). A papain concentration of 1 or 5% (w/v) resulted in the highest protoplast yields ( $2.11 \pm 0.70 \times 10^5$  and  $23.7 \pm 1.02 \times 10^5$  protoplasts  $g^{-1}$  f.wt, respectively) (Fig. 3.4). However, there was no significant difference ( $P > 0.05$ ) between the yields of protoplasts obtained when either 1 or 5% (w/v) papain was used to pre-treat *G. gracilis* thalli (Fig. 3.4). Since papain and other protease treatments can have toxic effects on protoplast viability (Chen and Chiang, 1994a), and due to the fact that there was no significant difference in protoplast yields between the lower or higher concentrations of papain, it was decided that 1% (w/v) papain was to be used for all further protoplast isolations.

#### 3.4.2.3 Effect of incubation period, pH and temperature on protoplast yields

The time period of enzymatic digestion for the production of protoplasts varies amongst species of macroalgae. The optimal incubation period has even been reported to vary greatly between different studies conducted on the macroalga, *G. verrucosa* (Mollet *et al.*, 1995; Araki *et al.*, 1998; Gupta *et al.*, 2011). It was for this reason that the effect of incubation period on both the yield and viability of protoplasts was tested.



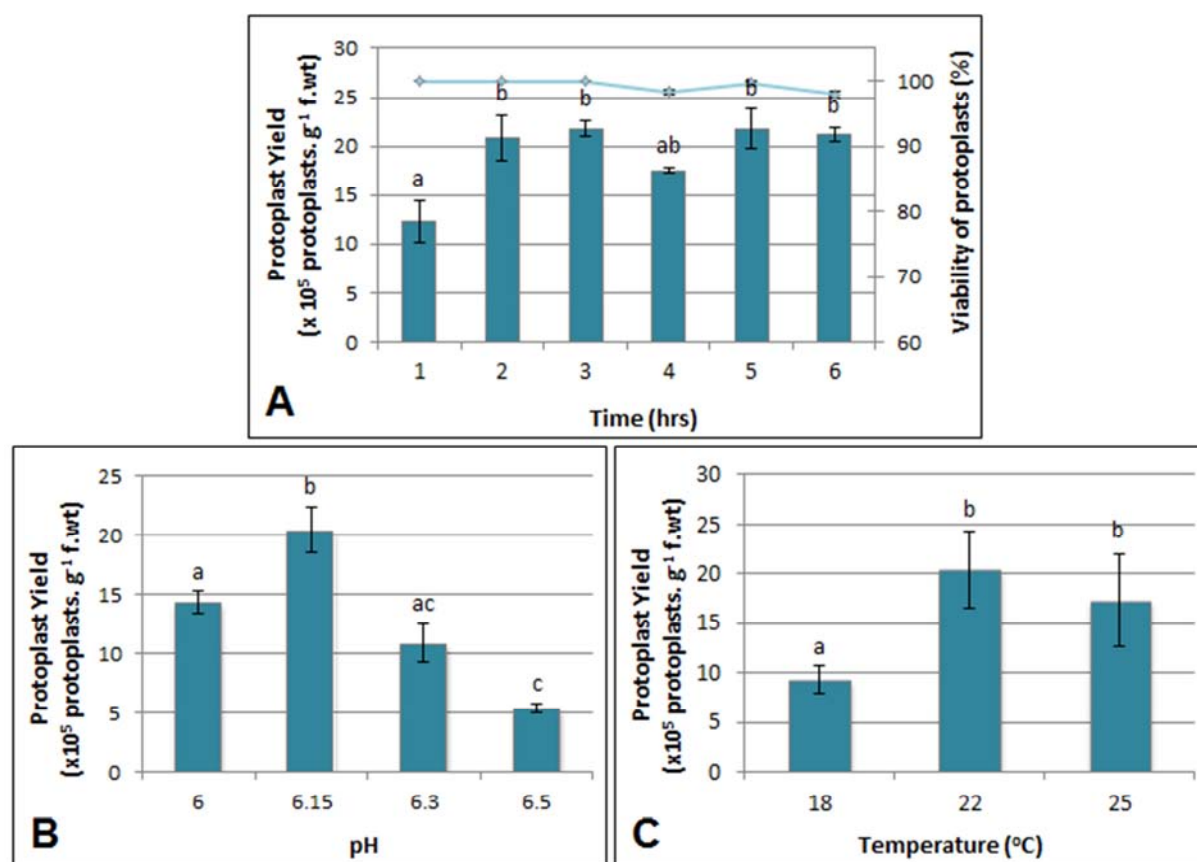
**Figure 3.4** Effect of papain (% w/v) pre-treatment on protoplast yield from *G. gracilis* thalli. Data represents the mean  $\pm$  standard error. Different postscripts indicate significant differences ( $P < 0.05$ ; one-way ANOVA) between sample means.

Protoplasts were released from *G. gracilis* thalli as early as 30 min after the addition of enzymatic medium, although protoplast yields were very low at this point (data not shown). Protoplast yields increased steadily over the first 3 hrs of digestion, reaching a maximum yield of  $21.8 \pm 0.86 \times 10^5$  protoplasts g<sup>-1</sup> f.wt (Fig. 3.5A). There was no significant ( $P > 0.05$ ) difference in protoplast yields obtained between 2 and 6 hrs of digestion, and furthermore, the viability of the *G. gracilis* protoplasts began to decrease after 3 hrs of incubation (Fig. 3.5A). An enzymatic digestion period of 3 hrs was thus chosen for all further protoplast isolations, since it was at this point that yields were maximized and protoplast viability was not compromised.

The optimal pH for enzymatic digestion of *G. gracilis* cell walls was tested by varying the pH of the digestion medium. The pH range chosen for the optimization experiments was based both on the pH optima for the cell wall degrading enzymes and on what has been previously used for other species of red macroalgae (Mollet *et al.*, 1995; Araki *et al.*, 1998; Yeong *et al.*, 2008).

A pH of 6.15 was found to be optimal for *G. gracilis* protoplast yields, resulting in significantly ( $P < 0.05$ ) increased yields of protoplasts ( $20.4 \pm 0.86 \times 10^5$  protoplasts g<sup>-1</sup> f.wt) when compared to enzymatic media at the other pH values assessed (Fig. 3.5B). Protoplast

yields decreased as the pH increased or decreased from the optimum of pH 6.15. Therefore, this pH was employed for all further protoplast isolation procedures.

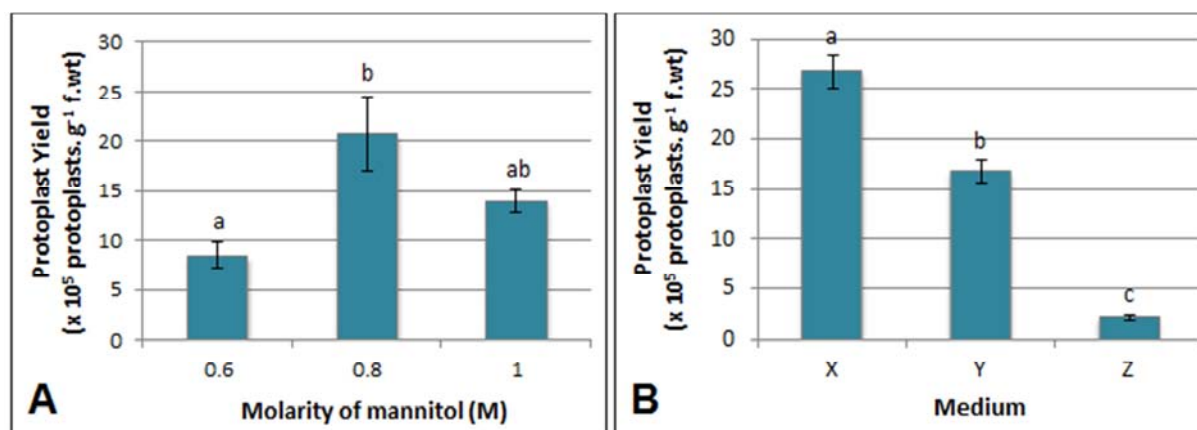


**Figure 3.5** Effect of enzymatic treatment period (A), pH of the enzymatic medium (B) and incubation temperature (C) on protoplast yield (■) and viability (—) from *G. gracilis* thalli. Data represents the mean  $\pm$  standard error. Different postscripts indicate significant differences ( $P < 0.05$ ; one-way ANOVA) between sample means.

The optimal temperature for *G. gracilis* enzymatic digestion was shown to be 22 °C with yields of  $20.4 \pm 3.80 \times 10^5$  protoplasts  $g^{-1}$  f.wt being obtained (Fig. 3.5C). Incubation temperatures of 22 and 25 °C produced significantly ( $P < 0.05$ ) higher yields of protoplasts when compared to the yields obtained at an incubation temperature of 18 °C (Fig. 3.5C). Although there was no significant difference ( $P > 0.05$ ) between protoplast yields obtained at incubation temperatures of 22 and 25 °C, the yields were higher and less varied at 22 °C (Fig. 3.5C). Therefore, an incubation of 22 °C was used for all further protoplast isolations.

### 3.4.2.4 Effect of osmoticum in the enzymatic medium on protoplast yields

The effect of different concentrations of mannitol (0.6-1.0 M) as osmotic stabilizer during protoplast isolation was investigated. Mannitol, at a concentration of 0.8 M, proved optimal with yields of  $20.4 \pm 3.80 \times 10^5$  protoplasts  $g^{-1}$  f.wt being obtained (Fig. 3.6A). Protoplast yields obtained when using 0.8 M mannitol were significantly higher ( $P < 0.05$ ) than those obtained using 0.6 M mannitol (Fig. 3.6A). However, there was no significant difference ( $P > 0.05$ ) between the yields obtained when protoplasts were isolated in the presence of 0.8 or 1.0 M mannitol ( $14.0 \pm 1.06 \times 10^5$  protoplasts  $g^{-1}$  f.wt) (Fig. 3.6A).



**Figure 3.6** Effect of mannitol concentration (M) in the enzymatic medium (A) and the composition of enzymatic media (B) on protoplast yield from *G. gracilis* thalli. Data represents the mean  $\pm$  standard error. Different postscripts indicate significant differences ( $P < 0.05$ ; one-way ANOVA) between sample means. Medium X, 0.8 M mannitol, 50% ASW; Medium Y, 0.8 M mannitol, 1% NaCl; Medium Z, 0.6 M mannitol, 3% NaCl.

There have been reports of increased protoplast yields when a low strength ionic medium (1% NaCl in deionised water) has been used instead of the more traditional ASW (Uppalapati and Fujita, 2002; Dipakkore *et al.*, 2005; Reddy *et al.*, 2006). Therefore, the effect of this substitution on protoplast yield from *G. gracilis* thalli was assessed. The substitution of ASW with 1 or 3% NaCl, however, resulted in significantly decreased ( $P < 0.05$ ) protoplast yields (Fig. 3.6B). Yields obtained when using the enzymatic medium optimized in this study (Medium X) were  $26.7 \pm 1.69 \times 10^5$  protoplasts  $g^{-1}$  f.wt compared to yields of  $16.8 \pm 1.09 \times 10^5$  and  $2.13 \pm 0.20 \times 10^5$  protoplasts  $g^{-1}$  f.wt when either 1 or 3% NaCl (Medium Y and Z) were employed, respectively (Fig. 3.6B).

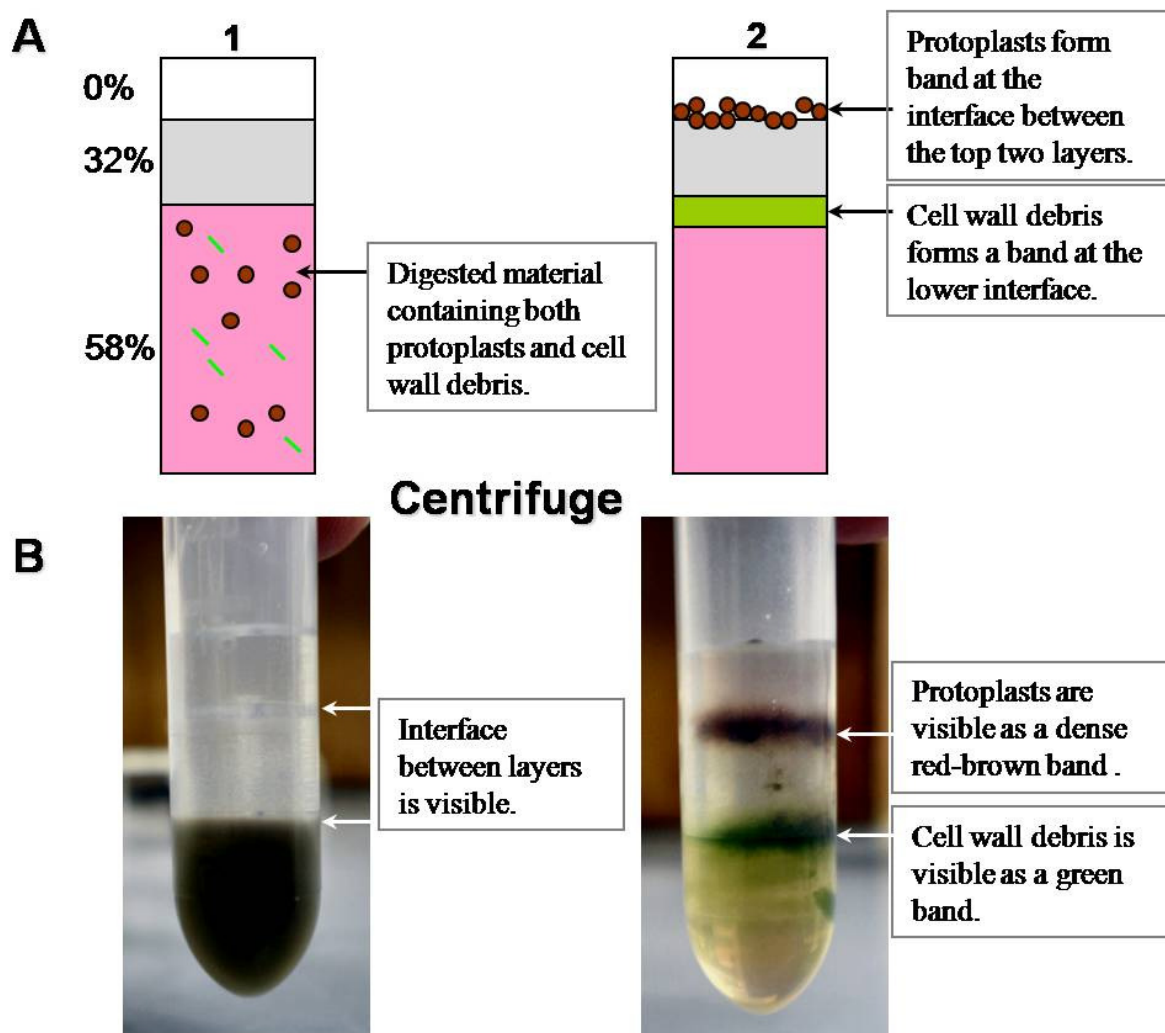
### 3.4.3 *G. gracilis* protoplast purification

Having developed a protocol for the generation of high yields of protoplasts from *G. gracilis*, it was then necessary to optimize a method for the collection and purification of protoplasts for culturing purposes. A discontinuous density gradient was tested using OptiPrep<sup>®</sup> density gradient medium. Initial density gradients based on those employed for plant protoplasts (0:10:25% (v/v) OptiPrep<sup>®</sup>) were not sufficient for the purification of *G. gracilis* protoplasts, since protoplasts never separated from contaminating matter. Instead, higher concentrations of Optiprep<sup>®</sup> had to be used for the density gradient, resulting in density gradient layers with higher densities sufficient for the purification of *G. gracilis* protoplasts. The ideal densities could easily be determined by observing where the position of the protoplast fraction would appear in the gradient following centrifugation. Protoplasts of *G. gracilis* are red in colour and therefore, the band of pure protoplasts within the density gradient would also be reddish in colour. By employing the optimal amount of OptiPrep<sup>®</sup> in the three layers of the gradient (0:32:58% (v/v) OptiPrep<sup>®</sup>), the protoplasts were easily separated from contaminating cell wall debris (Fig. 3.7).

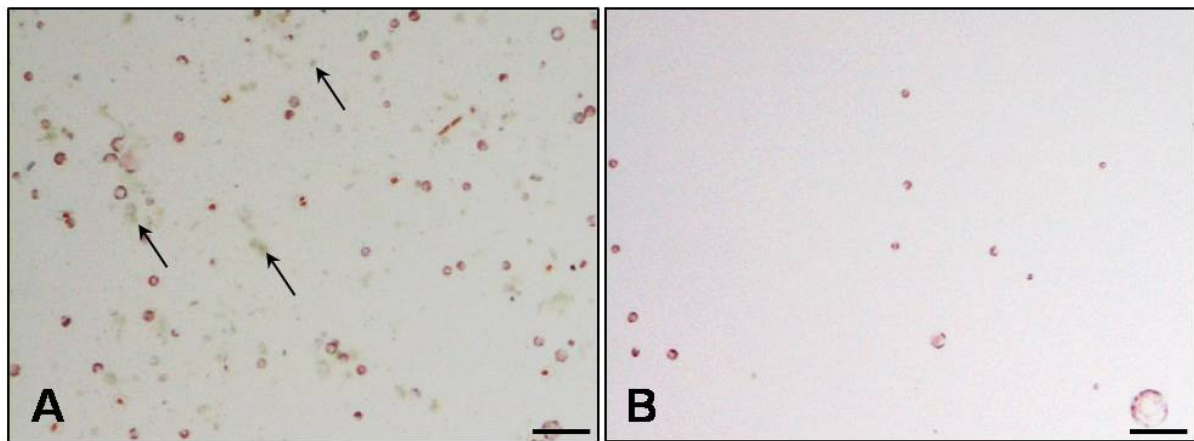
The difference in the purity of the protoplast fraction before and after density gradient purification is clearly evident (Fig. 3.8). Prior to density gradient purification, cell wall debris and plant material could be seen together with protoplasts (Fig. 3.8A). However, following the density gradient protoplast purification a pure fraction of *G. gracilis* protoplasts, with little to no contaminating materials, were clearly visible (Fig. 3.8B).

Although a rigorous sterilization protocol was applied to the *G. gracilis* thalli prior to protoplast isolation, in some cases bacterial contamination was evident from approximately the fourth day of protoplast culture (data not shown). Microbial contamination appeared to be batch dependent, i.e. related to the donor thalli received from the supplier, and was detrimental to protoplast survival, in that fewer protoplasts were visible in contaminated cultures (data not shown). Contamination appeared most prevalent during the summer months. The inclusion of penicillin G (100  $\mu\text{g ml}^{-1}$ ) and amphotericin B (0.1  $\mu\text{g ml}^{-1}$ ) made a considerable difference to lowering the levels of microbial contamination within the protoplast culture media (Fig. 3.9). Neither of these antibiotics had any apparent negative effects on *G. gracilis* protoplast cultivation and survivability. The only difference noted was that inclusion of the antibiotics, and in particular penicillin G, resulted in the protoplasts

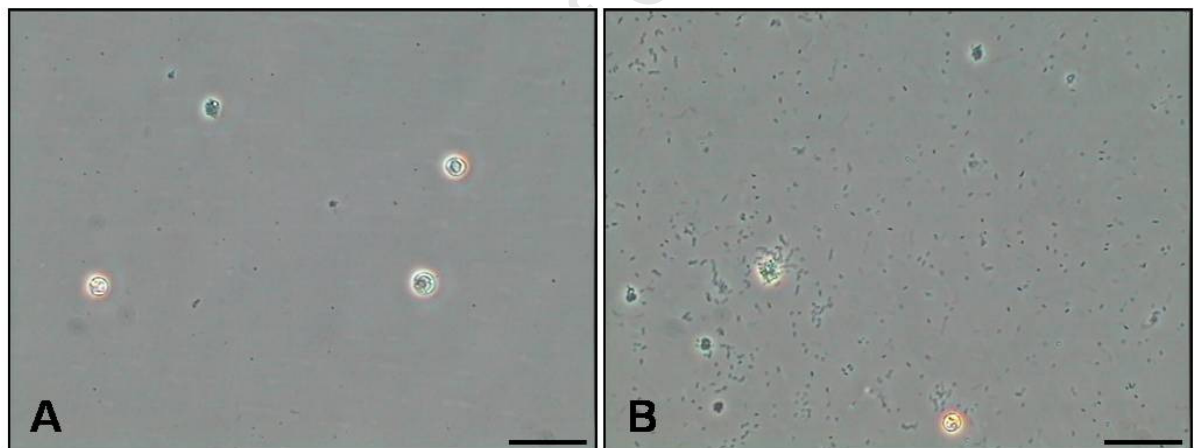
taking 2 days to settle, instead of only one day when no antibiotics were employed. Antibiotics were therefore included in the protoplast culture media for the first 2 weeks. After this period, the antibiotics were diluted out of the protoplast culture medium during subsequent media changes.



**Figure 3.7** OptiPrep<sup>®</sup> discontinuous density gradient showing the composition of the various layers before (1) and after (2) centrifugation during the purification of *G. gracilis* protoplasts. Percentage values indicate the amount of OptiPrep<sup>®</sup> (v/v) in the 3 layers of the gradient. When the gradient is constructed, the protoplasts along with any cell wall debris, are loaded as the bottom most layer of the gradient (A1) and as a consequence this layer shows a dark red-brown colouring (B1). During centrifugation, the protoplasts migrate to the interface between the top two layers of the gradient (A2) and are visible as a red-brown band. This allows the protoplasts to be separated from other contaminating matter which forms a band at the interface between the lower two layers (A2), visible as a green band (B2).



**Figure 3.8** *G. gracilis* protoplasts before (A) and after (B) OptiPrep<sup>®</sup> discontinuous density gradient purification. Protoplasts are contaminated with a large amount of cell wall debris (→) prior to purification (A). After density gradient purification the protoplast fraction appears free of contaminating cell wall material (B). Images were taken with a Nikon Eclipse 50i Compound Microscope. Scale bar = 50  $\mu$ m.

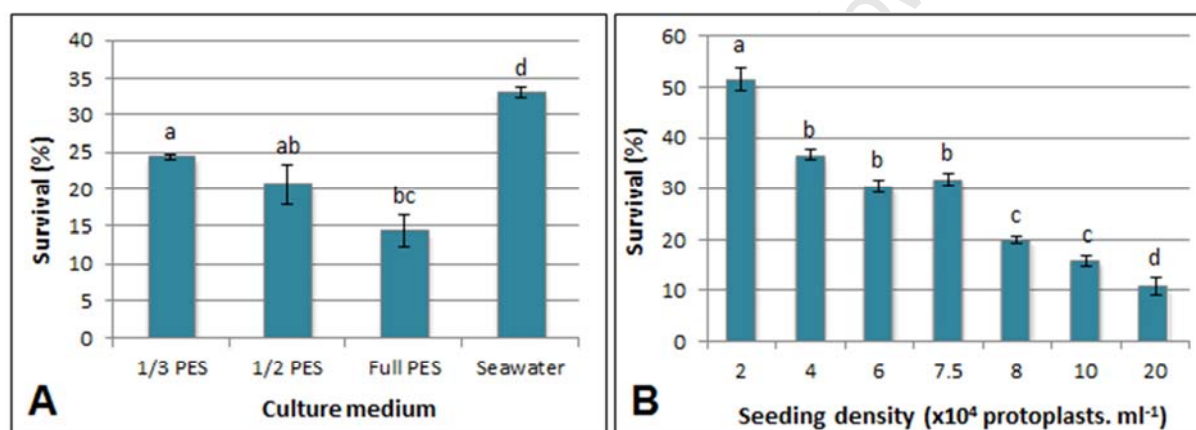


**Figure 3.9** Antibiotic supplementation of *G. gracilis* protoplast culture media (A) compared to culture media lacking antibiotics (B). Cultures are 4 days old. Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 25  $\mu$ m.

### 3.4.4 Optimization of factors affecting regeneration of whole plants

The effect of different culture media and seeding densities on protoplast survival over a one week period was tested in order to ascertain which medium and seeding density would be optimal for *G. gracilis* protoplast cultivation. Both these factors have previously been shown to affect protoplast regeneration potential (Reddy *et al.*, 2008a; Yeong *et al.*, 2008).

*G. gracilis* protoplasts showed decreased survival when increased PES supplementation was used in combination with filter-sterilized natural seawater (Fig. 3.10A), indicating sensitivity to the PES supplementation. In comparison, significantly ( $P < 0.05$ ) increased levels of survival ( $33.11 \pm 0.73\%$ ) were obtained when protoplasts were cultured in filter-sterilized natural seawater with no PES supplementation (Fig. 3.10A).



**Figure 3.10** Effect of different culture media (A) and seeding densities (B) on the survival of *G. gracilis* protoplasts over a one week period. Data represents the mean  $\pm$  standard error. Different postscripts indicate significant differences ( $P < 0.05$ ; one-way ANOVA) between sample means.

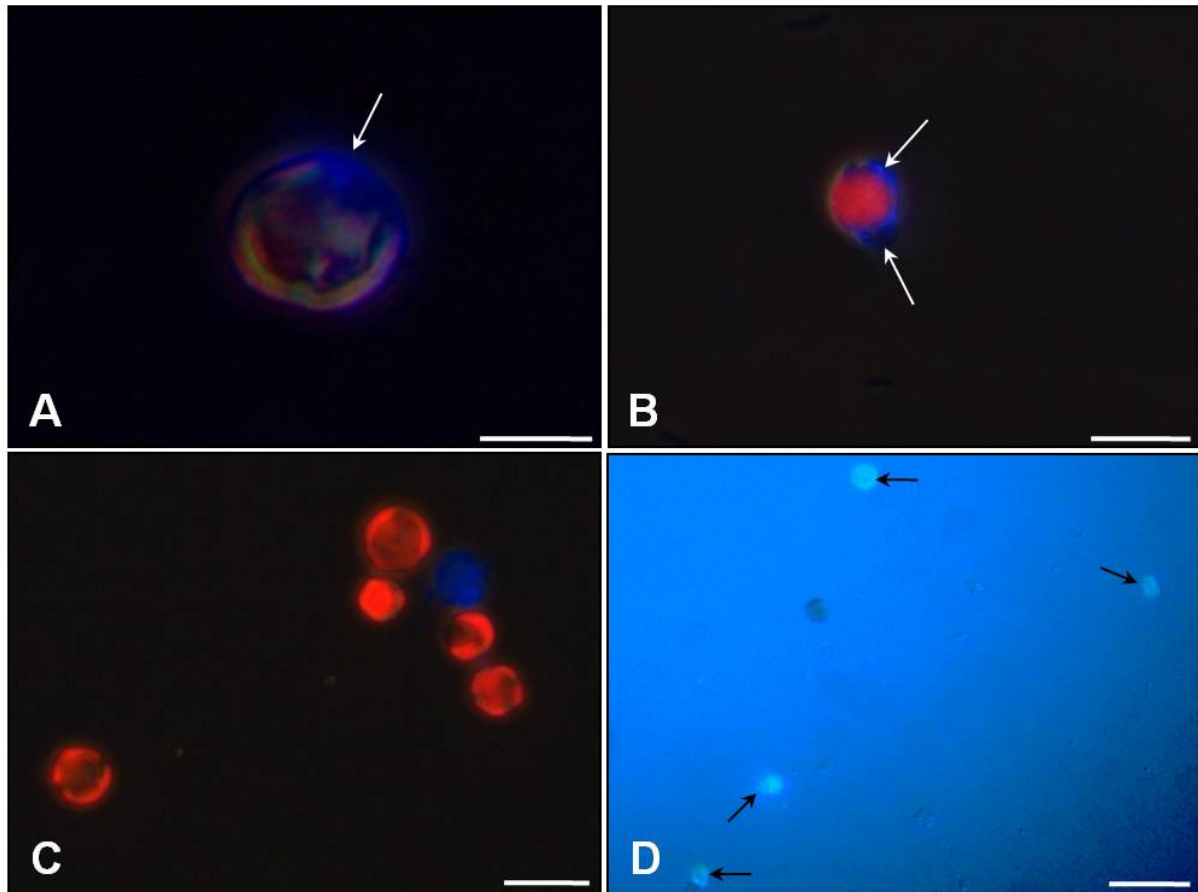
A general trend of decreased survival was observed in relation to increased *G. gracilis* protoplast seeding densities (Fig. 3.10B). A seeding density of  $2 \times 10^4$  cells  $\text{ml}^{-1}$  resulted in the highest ( $P < 0.05$ ) survival percentage ( $51.60 \pm 2.26\%$ ), however, protoplasts at this density did not develop further. There was no significant ( $P > 0.05$ ) difference in the survival percentages of protoplasts when seeded at densities of 4, 6 or  $7.5 \times 10^4$  cells  $\text{ml}^{-1}$  (Fig. 3.10B). However, protoplasts seeded at either 8, 10 or  $20 \times 10^4$  cells  $\text{ml}^{-1}$  displayed significantly ( $P < 0.05$ ) reduced survival percentages, when compared to those seeded at either 4, 6 or  $7.5 \times 10^4$  cells  $\text{ml}^{-1}$  (Fig. 3.10B). The plating efficiency, which refers to the number of surviving protoplasts after 9 days of culture, was highest for *G. gracilis* protoplasts seeded at

a density of  $7.5 \times 10^4$  cells  $\text{ml}^{-1}$ . Incidentally, protoplasts seeded at densities of  $7-8 \times 10^4$  cells  $\text{ml}^{-1}$  were also the only cultures that continued to cell division and whole plant regeneration (3.4.6). All future experiments thus employed a seeding density of  $7.5 \times 10^4$  cell  $\text{ml}^{-1}$ .

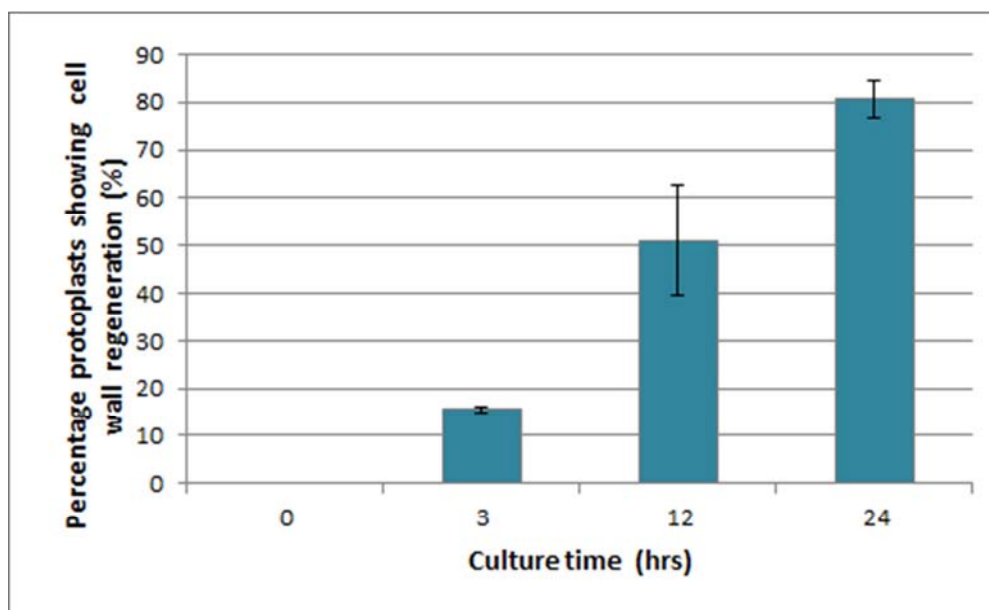
### 3.4.5 Cell wall regeneration

Cell wall resynthesis by protoplasts is the first step towards whole plant regeneration. This process was followed in *G. gracilis* protoplasts over a period of 24 hrs by two methods, namely calcofluor white staining and scanning electron microscopy.

Protoplasts were isolated using the method optimized in this study and cultured in medium containing 0.01% calcofluor white. The inclusion of calcofluor white in protoplast culture media is common, and is not known to have negative effects on protoplast survival (Chen and Chiang, 1994; Zablackis *et al.*, 1994; Matsumura *et al.*, 2000). *G. gracilis* protoplasts were examined for evidence of cell wall resynthesis after 0, 3, 12 and 24 hrs of culturing. Protoplasts undergoing cell wall resynthesis appear blue when examined under fluorescent light, while those without cell walls appear red (Chen and Chiang, 1994). Freshly isolated protoplasts showed only red autofluorescence, indicating they were free from cell wall material. Positive calcofluor staining could be seen after approximately 3 hrs of culturing (Fig. 3.11A & B). Staining began at one pole of the protoplast (Fig. 3.11A) and spread across the protoplast surface from this point (Fig. 3.11B). After 3 hrs, approximately  $15.57 \pm 0.69\%$  of the *G. gracilis* protoplasts showed blue fluorescence indicative of cell wall regeneration (Fig. 3.12). Whole protoplasts appeared positively stained by calcofluor white after 12 hrs of culturing (Fig. 3.11C). However, cell wall regeneration did not occur at an equal rate for all protoplasts, and some did not display cell wall deposition at all after 12 hrs (Fig. 3.11C). Indeed, approximately  $51.07 \pm 11.35\%$  of protoplasts were positive for cell wall regeneration at 12 hrs (Fig. 3.12). After 24 hrs of culturing, approximately  $80.56 \pm 4.00\%$  of protoplasts had regenerated their cell walls (Fig. 3.11D and Fig. 3.12). The fraction that had not regenerated cell walls at 24 hrs did not do so over the next 48 hrs. Over the next 48 hrs of culturing, the blue fluorescence of the calcofluor stained cells faded to pale green (data not shown), a phenomenon that is commonly observed during cell wall regeneration.

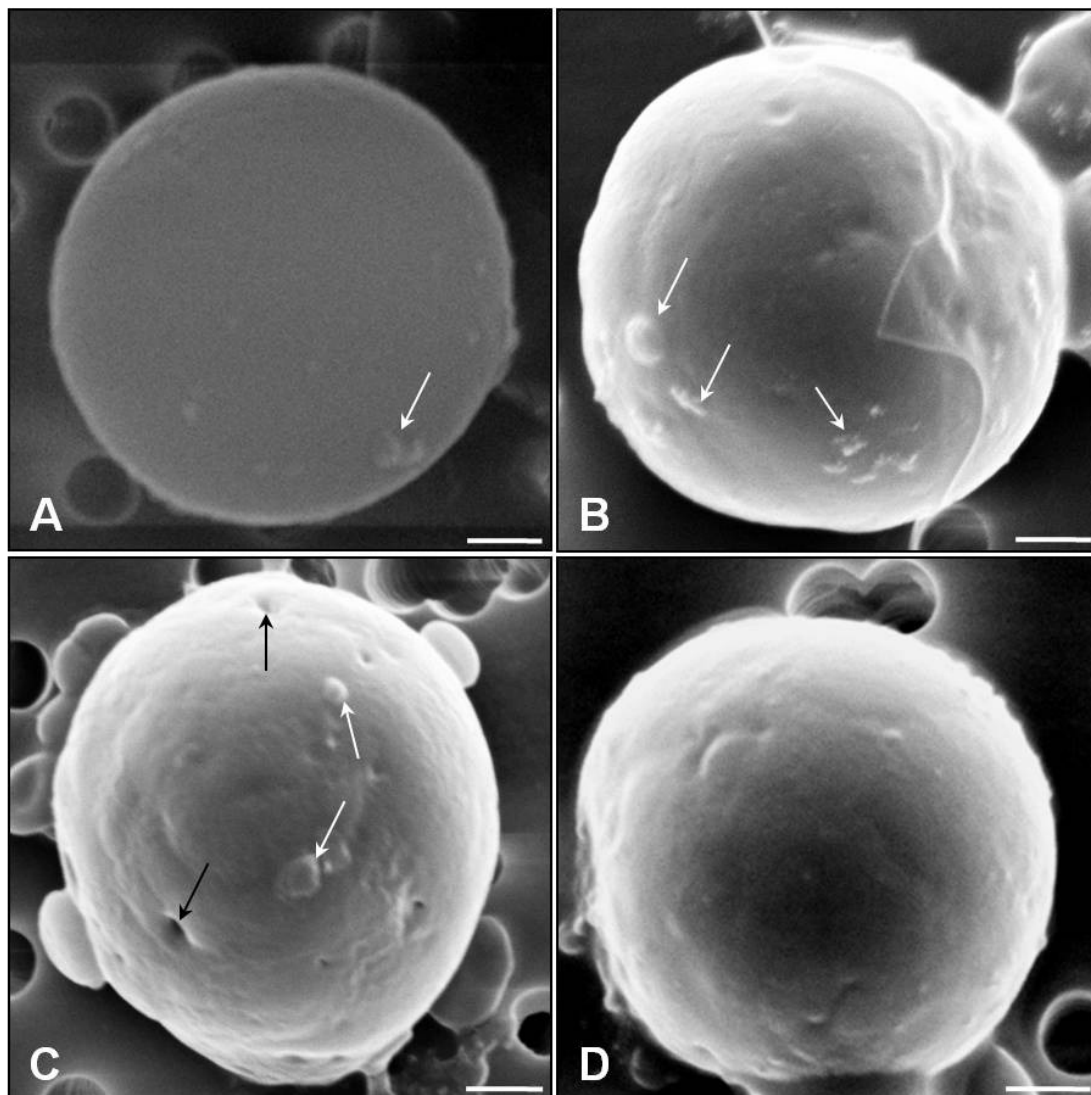


**Figure 3.11** Protoplasts showing cell wall regeneration following calcofluor white staining after 3 (A & B), 12 (C) and 24 hrs (D) of culture. Protoplasts were viewed under a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 400 nm emission filter, either with (A) or without (B-D) background lighting, on microscope slides (A-C) or in culture plates (D). Areas showing bright blue (A-C) or blue-white (D) fluorescence are areas in which cell wall regeneration has begun. Protoplasts or areas of protoplasts lacking cell walls appear red-orange (A-D). Cell wall deposition, detected 3 hrs after initiation of culture, began at one end of the cell ( $\rightarrow$ ) (A&B). Cell wall regeneration did not progress equally for all protoplasts, but some did appear completely covered in new wall material after 12 hrs of culture (C). The majority of protoplasts appeared completely covered in cell wall material after 24 hrs ( $\rightarrow$ ) (D). Scale bar = 5  $\mu$ m (A), 10  $\mu$ m (B&C) and 20  $\mu$ m (D).



**Figure 3.12** Rate of cell wall deposition by *G. gracilis* protoplasts over a period of 24 hrs. Protoplasts were stained with calcofluor white and examined 0, 3, 12 and 24 hrs after culture initiation for blue fluorescence indicating cell wall deposition. Any blue staining, even if only visible at one pole of a protoplast, was counted as positive staining. Data represents the mean  $\pm$  standard error.

SEM observation of newly isolated *G. gracilis* protoplasts (approximately 15 min old) showed that protoplasts had a relatively smooth surface and that an amorphous, mucilage-like material was already distributed on the surface of the protoplast (Fig. 3.13A). After 3 hrs of cultivation, spherical projections were distributed over the surface of the protoplast (Fig. 3.13B), and after 12 hrs of cultivation the protoplast surface appeared uneven with a few spherical projections still visible (Fig. 3.13C). After 24 hrs of culture, the protoplast surface appeared much smoother than at 12 hrs and there appeared to be a fairly uniform layer of cell wall material with few gaps (Fig. 3.13D).



**Figure 3.13** SEM observation of cell wall deposition by *G. gracilis* protoplasts over a 24 hr period. Freshly isolated protoplasts (approximately 15 min old) already show the presence of amorphous matrix material (white arrow; →) (A). After 3 hrs of culture excretion of the matrix material has increased and these form bulges on the protoplast surface (white arrow, →) (B). The protoplast surface after 12 hrs of culture appears rough with patchy cell wall deposition (black arrows; →) and bulges of matrix material are still visible (white arrow; →) (C). After 24 hrs, the protoplast surface appears smoother and continuously covered in cell wall material (D). Scale bar = 1  $\mu\text{m}$ .

### 3.4.6 Effect of temperature and light intensity on protoplast development

The effect of both low and high light intensities, in combination with either high or low culture temperature, on protoplast development was assessed.

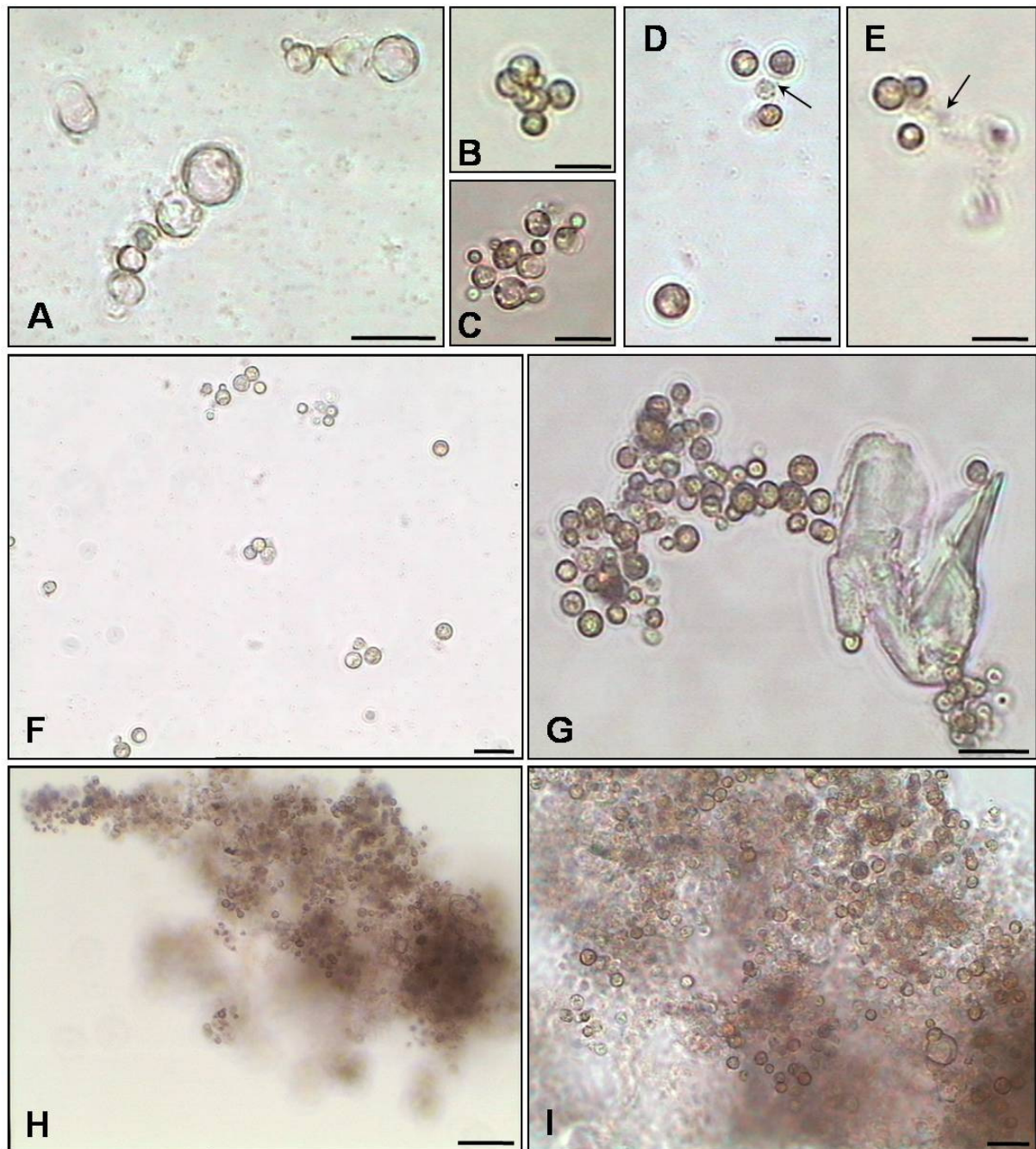
Protoplasts cultured under conditions of low light intensity ( $5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 16/8 hrs (day/night)) and a high incubation temperature ( $18 \pm 1 \text{ }^\circ\text{C}$ ) showed an interesting

pattern of cell division. After 4-5 days of culture, some of the cells began to elongate to form peanut-shaped cells (Fig. 3.14A). The peanut-shaped cells gradually constricted in the middle (Fig. 3.14B-E) to produce two cells (Fig. 3.14F). After division, the cells remained loosely associated, resulting in groups of cells which were strung together (Fig. 3.15A). The cultures were placed on an orbital shaker (30 rpm) after one week. Although agitation caused some cells to detach from the plate surface, they remained clumped (Fig. 3.15B-F). The clumps consisted of visibly distinct cells that were loosely associated with each other, possibly through some sort of matrix (Fig. 3.15D & E). Clumps of cells associated with debris in the culture medium and with other clumps of cells (Fig. 3.15G-I and Fig. 3.16A-B). The cell clumps could easily be separated by gentle shaking of the culture dishes.

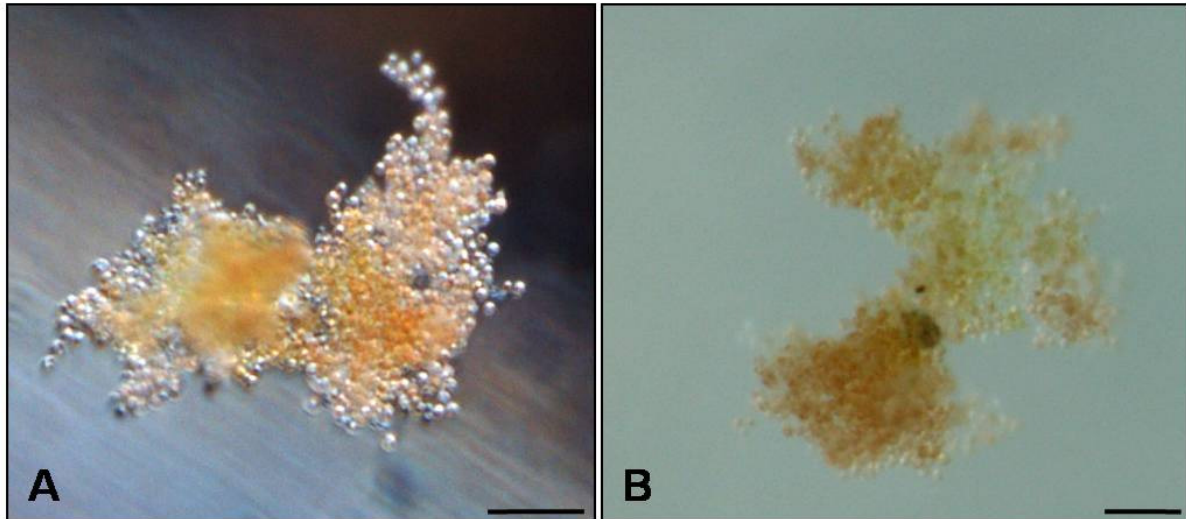


**Figure 3.14** Successive stages of *G. gracilis* protoplast cell division photographed in different cultures. Cell elongation to form a peanut-shaped cell (A). Elongated cells gradually constricted in the middle (→) (B-F) to produce 2 cells. Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 10  $\mu\text{m}$ .

Even in very large clumps of cells, division of the protoplasts continued to occur (Fig. 3.15I). However, the cells remained in this state of division and association, and could not be induced to regenerate whole plants even when the culture conditions (light intensity, temperature and/or agitation) were changed. These cell suspension cultures could only be maintained for a period of 5–6 weeks, after which all the cells died.

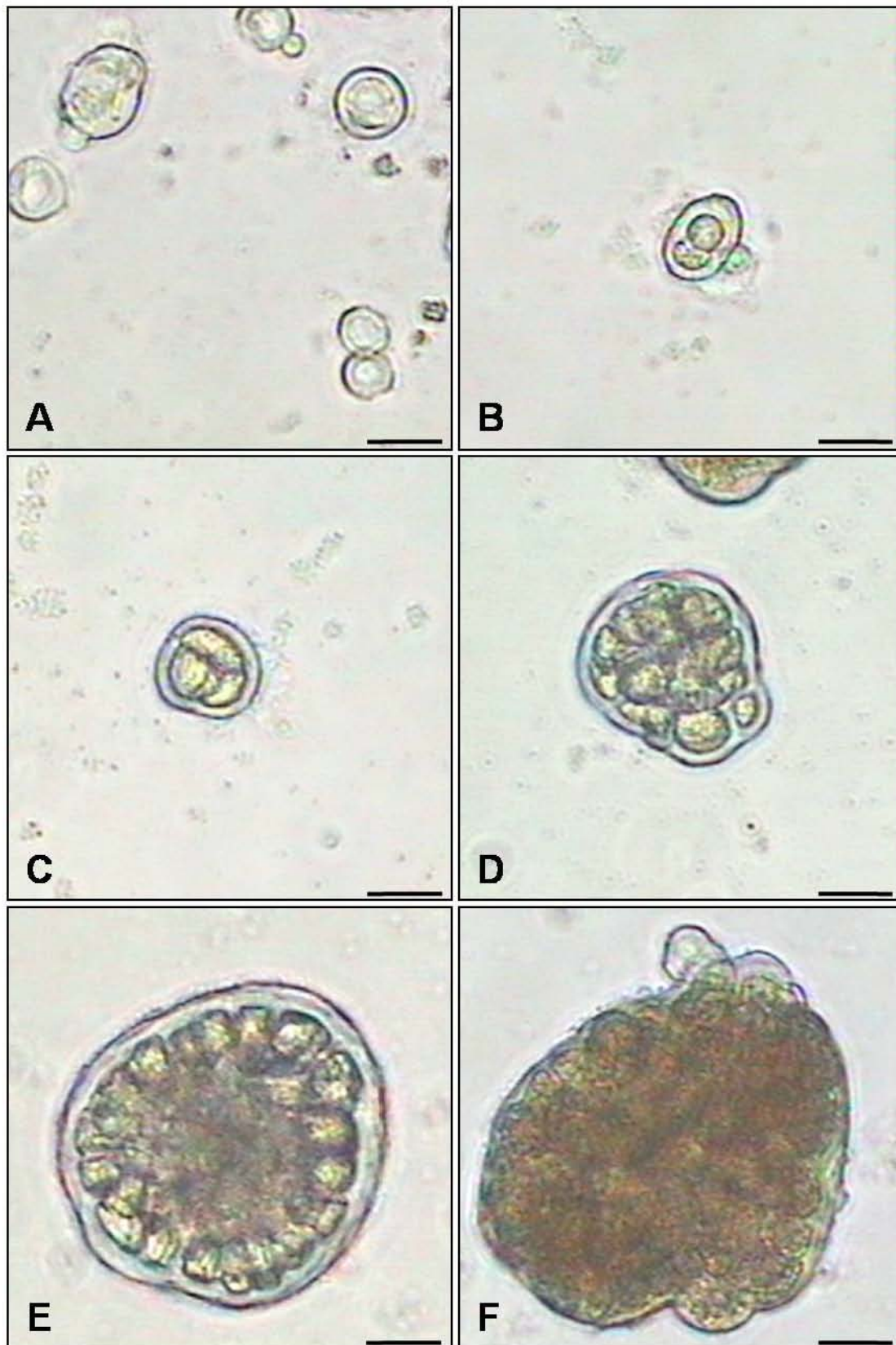


**Figure 3.15** Suspension culture of *G. gracilis* cells. Successive division resulted in cell clumping (A-I). Clumps consisted of clearly distinct cells that were loosely associated through some sort of matrix (→) (D & E). Cell clumps associated with debris in the media (G) and with each other to produce large clusters of cells (H & I). Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 25  $\mu\text{m}$  (A-G & I) and 150  $\mu\text{m}$  (H).

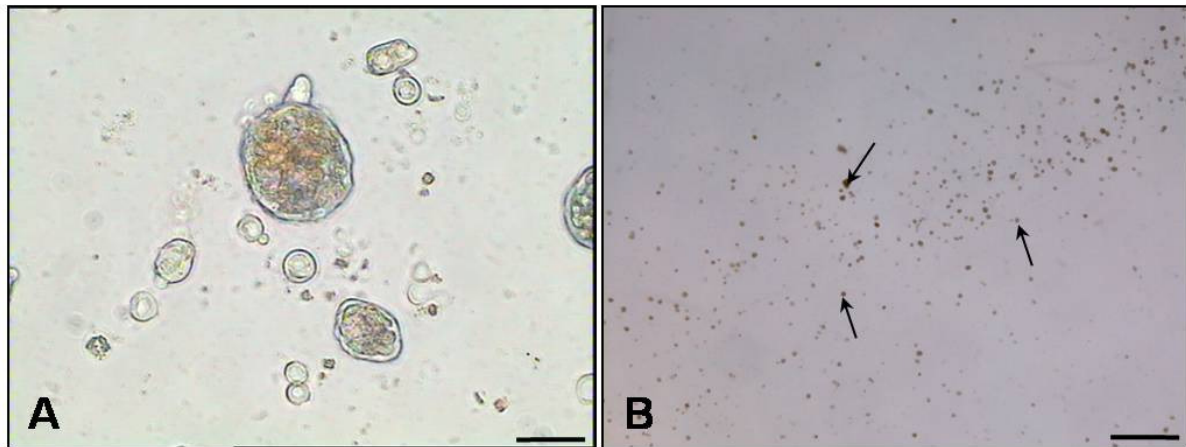


**Figure 3.16** Clumps of *G. gracilis* cells in suspension culture (A-B). Distinct cells can be seen making up the cell clusters. Cells are pigmented red-brown. Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 100  $\mu\text{m}$ .

Whole plants could be regenerated from protoplasts cultured under conditions of higher light intensity ( $10 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 1 week and then increased to  $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 16/8 hrs (day/night)) and either low or high incubation temperatures ( $14 \pm 1$  or  $18 \pm 1$   $^{\circ}\text{C}$ ). Successive cell division gave rise to a callus-like mass (referred to as a callus from here on in accordance with Aguirre-Lipperheide *et al.*, 1995; Reddy *et al.*, 2008b) (Fig. 3.17A-F). However, development did not progress at the same pace for all cells, and at any particular time, various stages of division and development were visible in the culture plates (Fig. 3.18). Approximately 90% of protoplasts produced calli. Many of the calli exhibited filament development (Fig. 3.19). Two types of filaments were visible: long filaments (in excess of  $300 \mu\text{m}$ ) without visible cell definitions (Fig. 3.19A & C), or shorter filaments (Fig. 3.19B & C) with visible cell borders (Fig. 3.19D). The callus filaments were no longer visible at the time of bud formation. Bud formation refers to the start of thallus growth and a bud is defined as a growing “point” from a callus (Fig. 3.20A & B).



**Figure 3.17** Successive division of *G. gracilis* protoplasts to produce calli (A-F). Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 20  $\mu\text{m}$ .



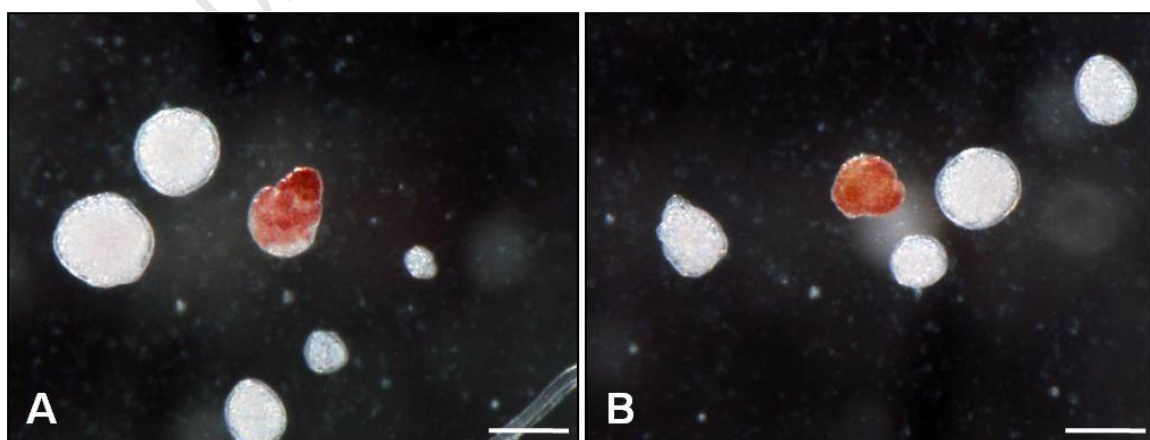
**Figure 3.18** Developing *G. gracilis* protoplasts. At any particular time there were cells at various stages of development present in a single culture (A). Many calli appeared as red-brown specs (→) in culture. Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 50  $\mu\text{m}$  (A) and 2000  $\mu\text{m}$  (B).

The colour intensity of the calli changed during the development process. There appeared to be a distinct change in colour intensity in particular areas of the callus at the onset of bud formation (Fig. 3.20A-B). The colour intensity appeared to be greatest at the “growing” point (bud) of the callus.

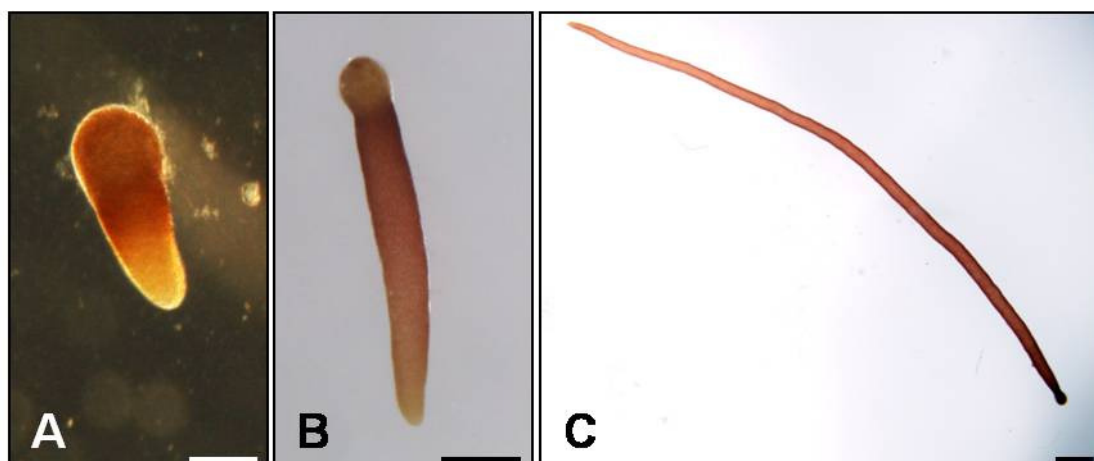
Whole *G. gracilis* plants were regenerated from the calli (Fig. 3.21, 3.22 and 3.23). Plants developed in one of two ways: i) either a single bud was produced, which gave rise to a single branching thallus (Fig. 3.21), or ii) many buds were produced, which gave rise to many branching thalli (Fig. 3.22 and 3.23). In most cases, the calli became detached from the culture plate surface at the stage of bud development. Even though most protoplasts (90% of protoplasts surviving at week 1) divided to produce calli, not all calli continued to produce whole plants. Plant regeneration rates were approximately 2-3 in  $10^4$  protoplasts (0.02% of protoplasts surviving at week 1). *G. gracilis* plant regeneration was relatively slow and generally took two or more months before actual budding and thallus regeneration was observed. Furthermore, not all plants grown from one culture plate began developing at the same time, and in some cases bud and thallus regeneration began 1–2 months apart. The change from callus to shoot development happened sporadically since culture conditions were kept constant during this period. However, we observed that once a *G. gracilis* plant developed, it appeared to stimulate budding and development of other plants within the same culture plate.



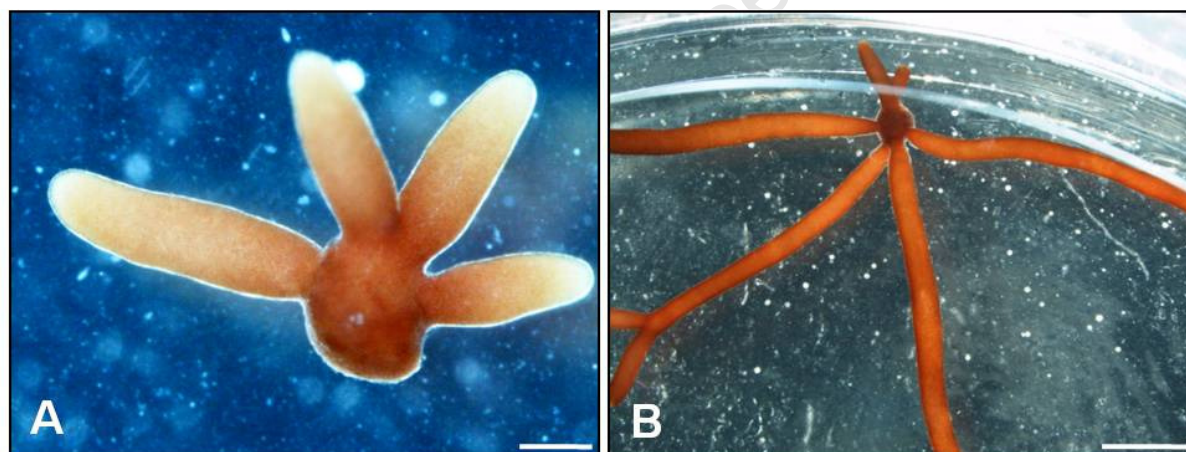
**Figure 3.19** Filaments present on some *G. gracilis* calli. Often more than one filament (→) was present on a single callus (A-D). Two types of filaments were visible: either long filaments lacking visible cell definitions (A & C), or shorter filaments (B & C) exhibiting visible cell borders (→) (D). These filaments disappeared from calli prior to bud formation. Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 50  $\mu\text{m}$  (A-C) and 20  $\mu\text{m}$  (D).



**Figure 3.20** Changes in colouring of *G. gracilis* calli. Colour appears to be most intense at the growing point or bud of the calli. Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 100  $\mu\text{m}$ .



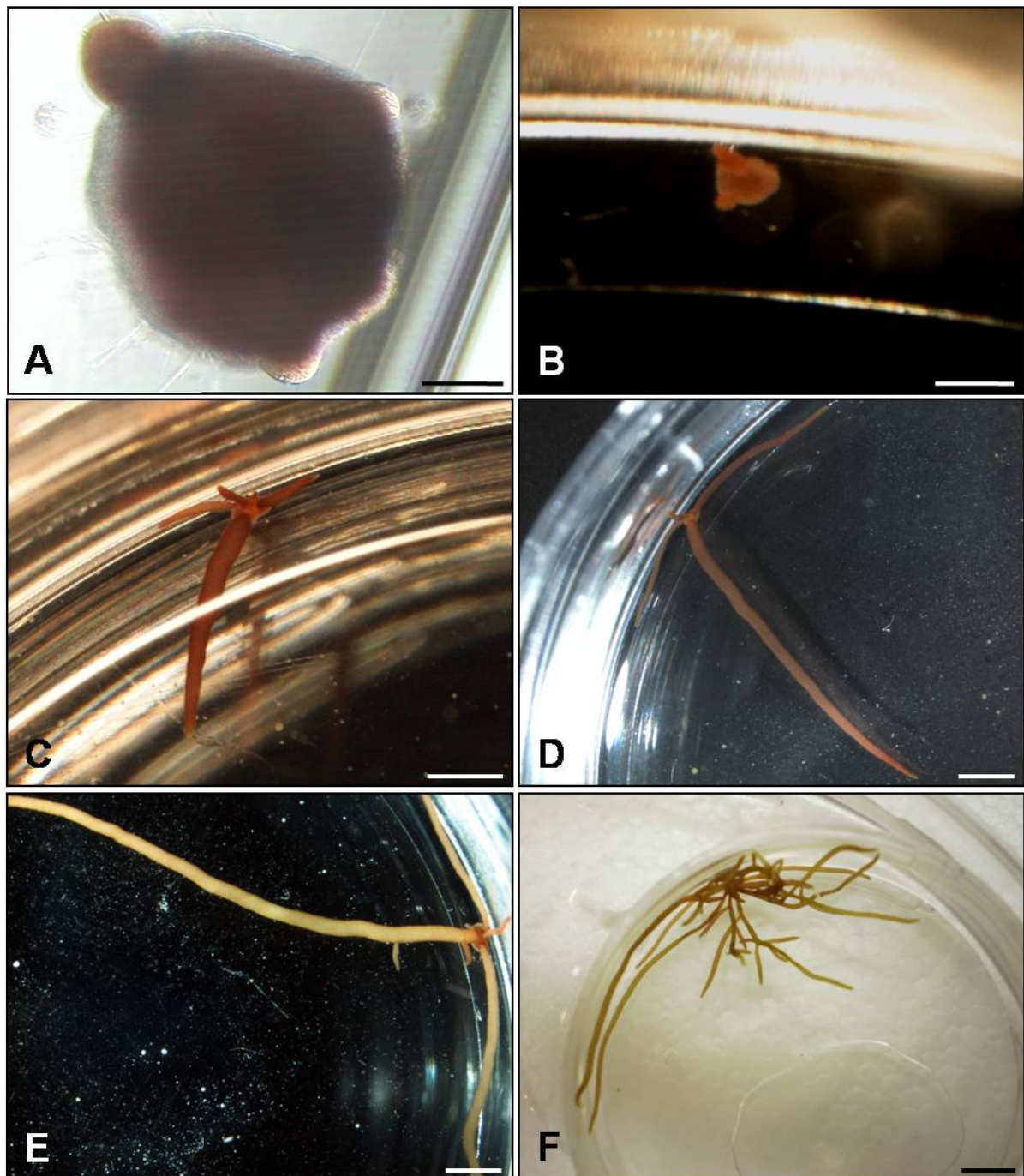
**Figure 3.21** Successive development of a whole *G. gracilis* plant from a protoplast cell (A-C). The developed bud detached from the culture plate surface 5 months after culture initiation (A). The same plant 6 days (B) and 3 weeks (C) later. Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 150  $\mu\text{m}$  (A), 300  $\mu\text{m}$  (B) and 1000  $\mu\text{m}$  (C).



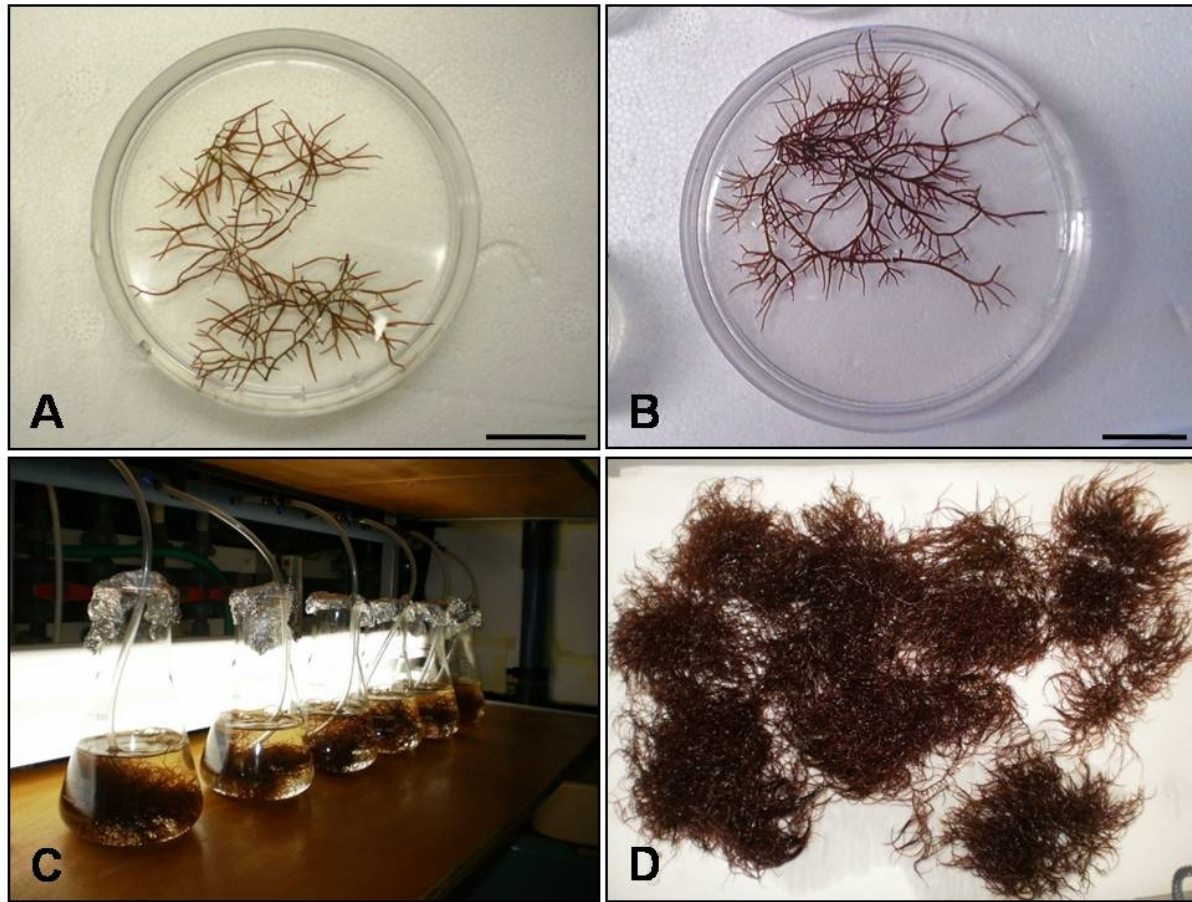
**Figure 3.22** Development of a whole *G. gracilis* plant from a single callus that gave rise to numerous thalli (A-B). The developing, budding plant detached from the culture plate surface 4 months after culture initiation (A). The same plant 7 weeks later (B). Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 200  $\mu\text{m}$  (A) and 2000  $\mu\text{m}$  (B).

Regenerated *G. gracilis* thalli were pooled together and cultured, firstly in culture dishes (Fig. 3.24A & B) and later in aerated culture flasks (Fig. 3.24C). Growth rates obtained from the regenerated seaweed ( $58.13 \pm 7.84 \text{ mg d}^{-1}$ ) were similar to that obtained for *G. gracilis* received from the supplier ( $58.44 \pm 1.98 \text{ mg d}^{-1}$ ) when cultured under the same conditions. The regenerated seaweed was later maintained in the same manner as described for the seaweed received from the supplier (Chapter 2.3.1). A total of 115 g of regenerated seaweed

was cultured from approximately 15 plants over a period of approximately 12 months (Fig. 3.24D).

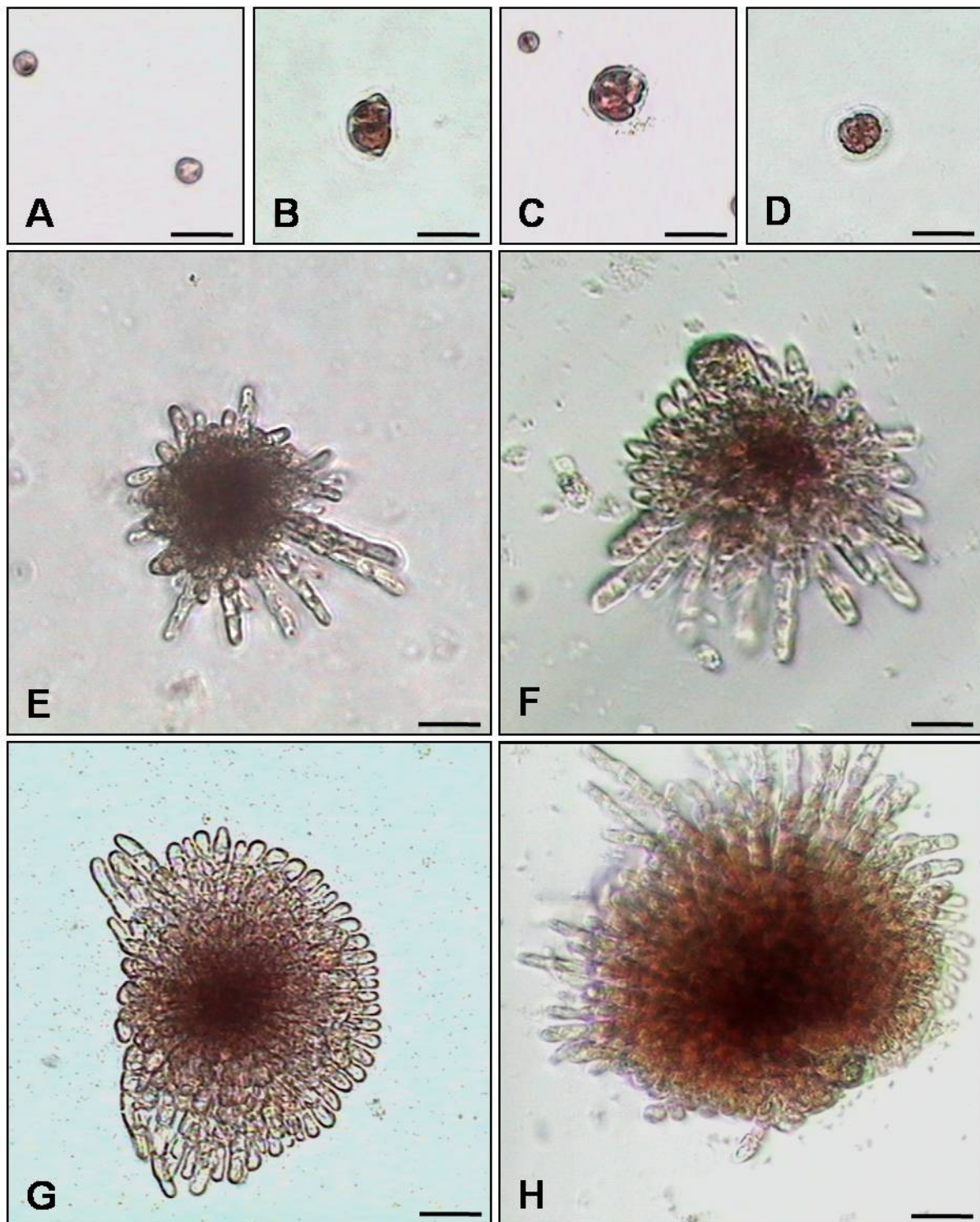


**Figure 3.23** Successive development of a whole *G. gracilis* plantlet from a single protoplast over a period of 5½ months (A-F). Initial budding was observed 4 months after culture initiation (A & B). The same plant 3 weeks (C), 7 weeks (D), 9 weeks (E) and 5½ months (F) after initial budding. Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 100 (A), 500 (B), 1000 (C), 2000 (D & E) and 5000  $\mu\text{m}$  (F), respectively.



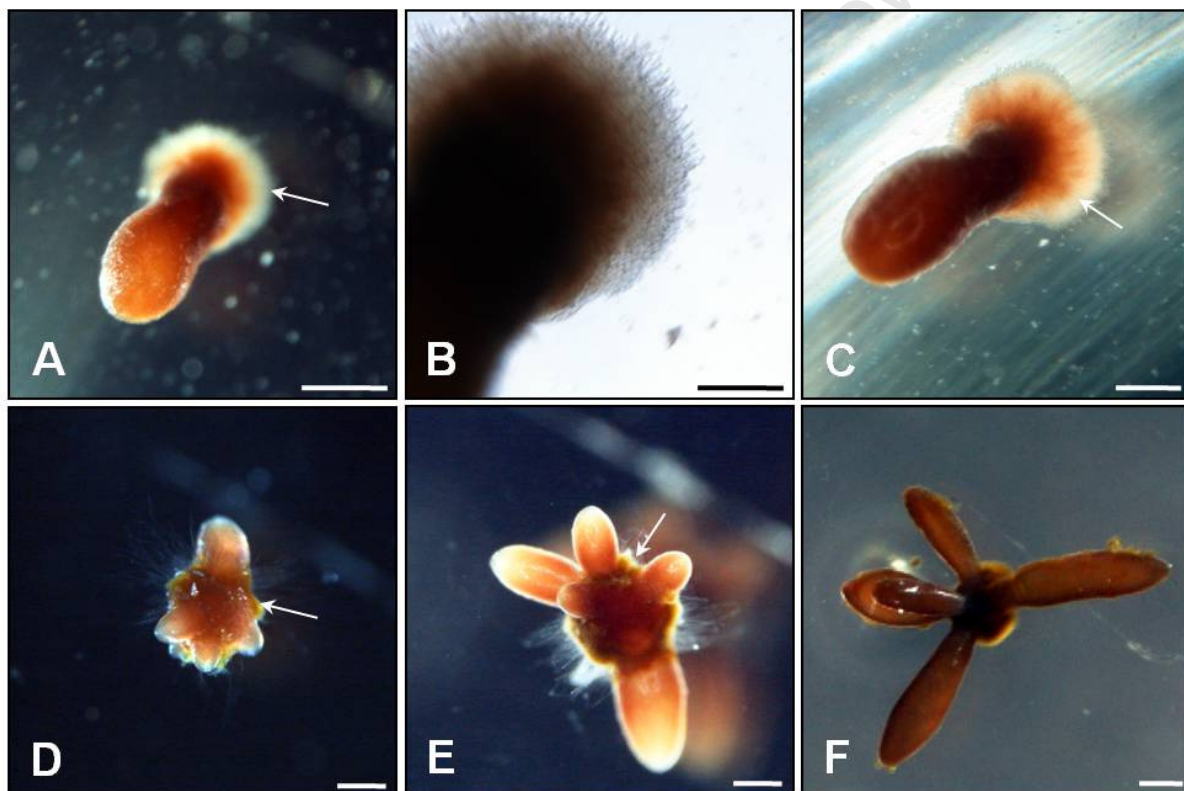
**Figure 3.24** Regenerated *G. gracilis* plantlets grown from individual protoplasts. Thalli were grown in culture dishes (A & B) until the seaweed biomass was large enough to culture in aerated flasks (C). Total mass of seaweed regenerated from 15 plants over the period of 1 year (D). Scale bar = 10 (A) and 20 mm (B), respectively.

A second, more rapid developmental pattern of whole plant regeneration from *G. gracilis* protoplasts which occurred spontaneously in the same cultures as the aforementioned developmental pattern was also observed. After 2–5 days of culture, protoplasts underwent division and rapidly produced calli (Fig. 3.25B-D). The calli remained attached to the bottom of the culture dishes and many uniseriate filaments were seen growing at the periphery of the calli after 10 days in culture (Fig. 3.25E-H). In some cases these filaments began to disappear after 20 days in culture, but in other cases these filaments remained present, even during bud development (Fig. 3.26).

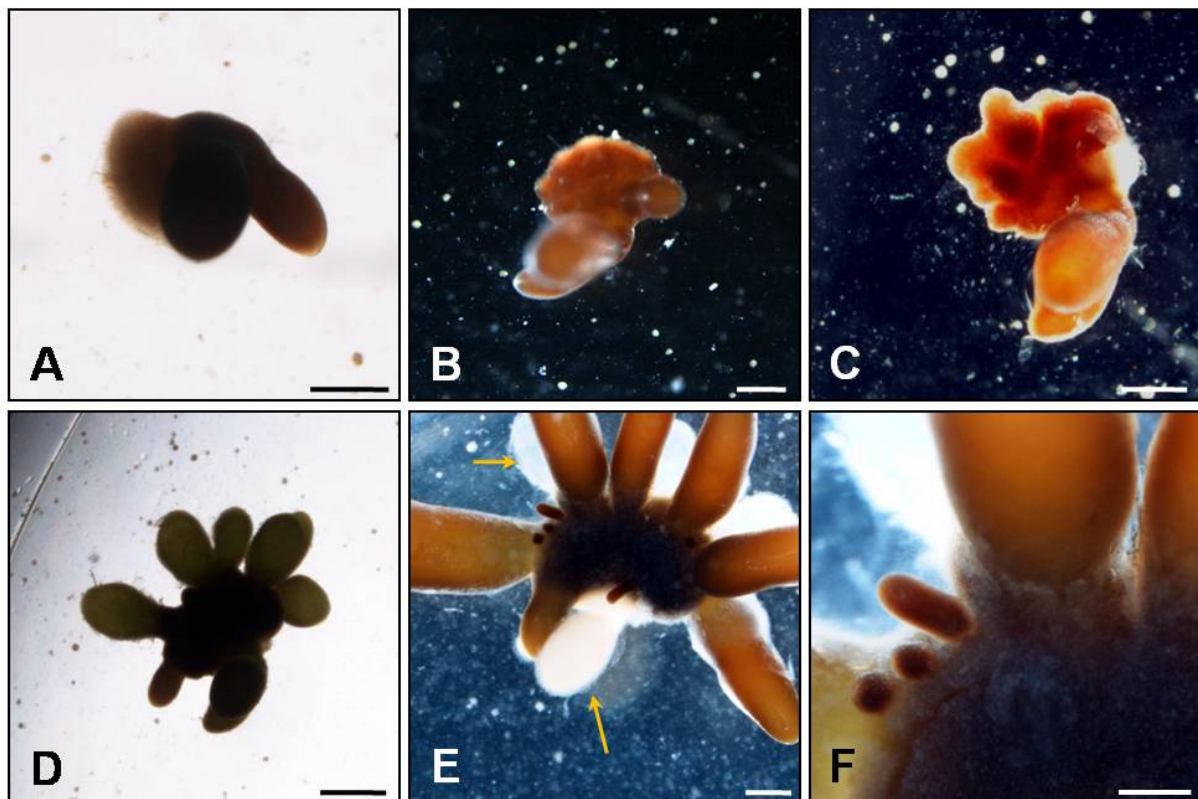


**Figure 3.25** *G. gracilis* protoplasts and developing calli. One-day old protoplasts (A). Dividing protoplast after 2 (B) and 5 (C & D) days culture, respectively. Uniseriate filaments produced from calli after 10–20 days in culture (E–H). Images were taken with an Olympus CK40 Inverted Microscope. Scale bar = 25  $\mu$ m.

Buds began to appear after 20 days in culture, and once the first bud appeared, many more buds began to develop from the same callus (Fig. 3.26D). In some instances a single plant was produced from a callus (Fig. 3.26A-C), but in many cases more than one bud grew from a single callus (Fig. 3.26D-E and Fig. 3.27). *G. gracilis* plants grown from this developmental pattern remained short with thick thalli that did not display branching (Fig. 3.26 and Fig. 3.27). The life span of these plants appeared to be limited, and once a bud reached a particular length (approximately 800–2000  $\mu\text{m}$ ) it died. This phenomenon was observed for all the plants that developed via this rapid regeneration process. In one particular case, in which many buds were produced from the same callus, the oldest buds died when they reached a particular size (Fig. 3.27E), and new buds regenerated to replace them (Fig. 3.27F).

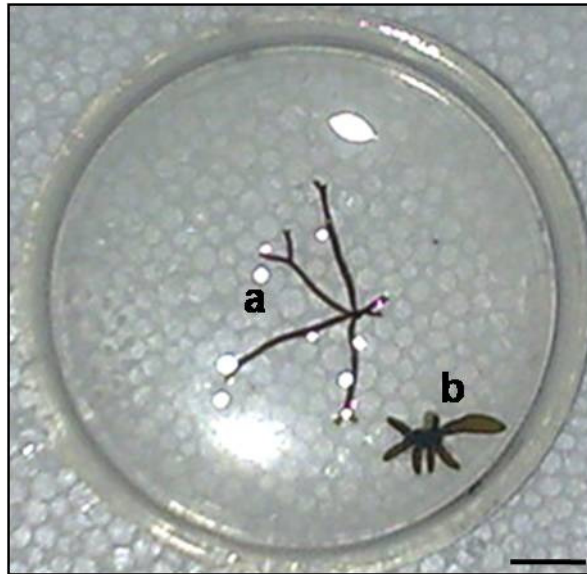


**Figure 3.26** Regenerating *G. gracilis* plantlets that retained uniseriate filaments ( $\rightarrow$ ) throughout development. Plantlet with uniseriate filaments ( $\rightarrow$ ) were still present after 8 (A) and 9 weeks (C) of development. A magnified view of the uniseriate filaments in A (B). Regenerating *G. gracilis* plantlet at 8 (D), 9 (E) and 11 weeks (F), respectively. Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 300 (A, C-E), 150 (B) and 500  $\mu\text{m}$  (F).



**Figure 3.27** Regenerating *G. gracilis* plant after 5 (A), 8 (B), 11 (C), 18 (D) and 26 weeks (E). Dying buds after 26 weeks of culturing are indicated by arrows ( $\rightarrow$ ) (E). Magnified view of E showing new buds being produced (F). Images were taken using a Nikon Stereoscopic Zoom Microscope SMZ1500. Scale bar = 300 (A & B), 500 (C & E), 1000 (D) and 200  $\mu$ m (F).

The contrast between the *G. gracilis* plantlets regenerated through the two different patterns can clearly be seen in Fig. 3.28. Plants regenerated by the first pattern resembled the parent plants and had slender thalli that branched, while *G. gracilis* plants regenerated through the second pattern remained small and the thalli were thick and unbranched (Fig. 3.28).



**Figure 3.28** *G. gracilis* plants regenerated from protoplasts via the two different developmental patterns identified in this study. The plant developed through the first pattern resembles the parent plant and has slender, branched thalli (a). *G. gracilis* plantlet developed through second pattern has short, thick, unbranched thalli (b). Scale bar = 5 mm.

### 3.5 Discussion

The success of transformation in macroalgae is reliant on the development of the algal tissue culture field (Stevens and Purton, 1997). Although induction of callus has been successfully achieved in many species of macroalgae, the problems with seaweed callus (discussed in Chapter 1.8) have led some to suggest that protoplasts offer a viable alternative (Aguirre-Lipperheide *et al.*, 1995; Baweja *et al.*, 2009). While there are numerous reports of protoplast isolation and culture for marine multicellular macrophytic algae, regeneration from protoplasts to complete thalli is a fairly recent development. Protoplasts have been isolated from 13 genera belonging to the Rhodophyta, but whole plant regeneration has been reported for only four species of *Gracilaria*; *G. asiatica*, *G. changii*, *G. chilensis* and *G. tikvahiae* (Cheney, 1990; Yan and Wang, 1993; Reddy *et al.*, 2008a; Yeong *et al.*, 2008).

Protoplasts are difficult to work with and numerous factors influence their yield and regeneration rates (Reddy *et al.*, 2008a). Therefore, prior to regeneration of protoplasts, we established a basic protocol for protoplast isolation from *G. gracilis* thalli. Subsequently, the various parameters influencing protoplast yields were optimized in order to ensure that large quantities of viable protoplasts could consistently be isolated from *G. gracilis* thalli.

The protoplast isolation method established in this study was modified from those of Mollet *et al.* (1995), Araki *et al.* (1998) and Yeong *et al.* (2008). This method was shown to successfully liberate  $5 \times 10^5$ – $1 \times 10^6$  protoplasts g<sup>-1</sup> f.wt protoplasts from mainly the apical tip and cut surfaces of *G. gracilis* thalli. This was expected since the cell wall degrading enzymes can potentially gain easier and/or better access to the cells within these parts of the thallus. Two main size classes of protoplasts were isolated, namely those which were smaller in size (5-15 µm diameter) and were derived from the cortical region, and those which were larger in size (20-40 µm diameter) and derived from the perimedullary region of the thallus. The smaller cortical protoplasts comprised approximately 80-90% of the protoplasts isolated. The isolation of two size classes of protoplasts has been previously reported for species of *Gracilaria* (Yan and Wang, 1993; Mollet *et al.*, 1995; Araki *et al.*, 1998; Yeong *et al.*, 2008; Gupta *et al.*, 2011).

Cell wall-degrading enzymes have previously been shown to yield protoplasts from macroalgae when used separately (Butler *et al.*, 1989; Coury *et al.*, 1993; Yeong *et al.*, 2008).

We showed that a concentration of 3% (w/v) cellulase Onozuka R-10 resulted in significantly ( $P<0.05$ ) increased protoplast yields in comparison to the other cellulase concentrations tested. However, algal cell walls are particularly complex in their composition and in addition to cellulose, and depending on the species of macroalgae, a wide variety of matrix polysaccharides are present in the cell wall in varying amounts (Reddy *et al.*, 2008a). Depending on their nature and complexity, the synergistic action of a number of cell wall degrading enzymes may be required for the efficient degradation and release of protoplasts from seaweed thalli.

Since the cell walls of *Gracilaria* are composed mainly of cellulose and agar, it was thought that the inclusion of agarase in the cellulase digestion medium would potentially result in more complete cell wall digestion and the liberation of more protoplasts. As expected, the inclusion of agarase in the enzymatic medium resulted in significantly ( $P<0.05$ ) higher yields of protoplasts, in comparison to yields obtained when cellulase was used on its own.

The use of macerozyme in combination with other cell wall degrading enzymes for protoplast isolation from red macroalgae is well reported (Chen and Chiang, 1994a; Araki *et al.*, 1998; Reddy *et al.*, 2006; Yeong *et al.*, 2008; Gupta *et al.*, 2011). The enhancing effect that macerozyme has on protoplast yields is attributed to its ability to aid in pectin digestion and thereby assist in cell separation (Yeong *et al.*, 2008). Similarly, in this study the inclusion of macerozyme, together with cellulase and agarase, resulted in significantly ( $P<0.05$ ) increased protoplast yields from *G. gracilis* thalli.

Pre-treatment of *G. gracilis* thalli with papain proved successful in increasing protoplast yields by up to 14.1-fold, when compared to an untreated control sample. Although pre-treatment with either 1 or 5% papain resulted in similar protoplast yields, the lower concentration was selected for further *G. gracilis* protoplast isolations as papain has been reported to have toxic effects on protoplasts (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a). Amano and Noda (1990) reported that papain pre-treatment of *P. yezoensis* resulted in protoplasts that were unable to regenerate cell walls. While protease treatments of thalli may very well have negative effects on protoplast survival and regeneration, steps are also taken to limit and prevent their toxicity. Papain, or protease treatments, are generally only applied as a pre-treatment step (Polne-Fuller and Gibor, 1990; Araki *et al.*, 1998; Dipakkore *et al.*, 2005). In this study, papain was also only applied as a pre-treatment step,

and a general protease inhibitor was included in the digestion medium to alleviate any toxic effects from possible carryover of papain into the enzymatic digestion medium. The general protease inhibitor employed in this study may not only protect *G. gracilis* protoplasts from any remaining papain, but may also inhibit proteases released during tissue digestion and therefore protect the applied cell wall degrading enzymes. Furthermore, *G. gracilis* protoplasts isolated in this study showed no detrimental effects following papain pre-treatment, and protoplasts continued to regenerate cell walls and develop into whole plants as has been the case for many other species in which papain treatment has been applied (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a; Chen and Chiang, 1995).

Optimal tissue digestion periods employed in the isolation of protoplasts from *Gracilaria* species are reported to be between 1.5-12 hrs (Cheney *et al.*, 1986; Bjork *et al.*, 1990; Yan and Wang, 1993; Mollet *et al.*, 1995; Araki *et al.*, 1998; Yeong *et al.*, 2008; Gupta *et al.*, 2011). Due to this large variation, the optimal time period for *G. gracilis* protoplast isolation was investigated in this study. An enzymatic digestion period of 3 hrs proved optimal in terms of both protoplast yield and viability. This time period is similar to that reported for *G. asiatica* (Yan and Wang, 1993) and *G. changii* (Yeong *et al.*, 2008). In contrast, Mollet *et al.* (1995) reported a time period of 12 hrs for *G. verrucosa*, while Araki *et al.* (1998) reported a time period of just 1.5 hrs and Gupta *et al.* (2011) a period of 6 hrs for the same species. While the protocols do vary slightly between these reports, it is likely that the algal material itself has a significant role to play in the variation of the time periods, since the physiological state of the plant used for protoplast isolation is known to affect protoplast yields (Reddy *et al.*, 2008a). It is therefore suggested that the incubation period should be assessed for each new source of a particular algal species, as the physiological state of the plants are likely to be different.

Enzyme activity of the cell wall degrading enzymes is influenced by both the pH of the enzymatic medium and the temperature at which the digestions are carried out (Yeong *et al.*, 2008). A pH of 6.15 and a temperature of 22 °C were shown to be optimal for *G. gracilis* protoplast isolation in this study. Optimal pH values for protoplast isolation from other species of macroalgae where similar cell wall degrading enzymes have been employed have also been reported to be in a comparable range (Polne-Fuller and Gibor, 1990; Yan and Wang, 1993; Mollet *et al.*, 1995; Araki *et al.*, 1998; Dipakkore *et al.*, 2005; Yeong *et al.*, 2008). Gupta *et al.* (2011) however report an optimal pH of 7.5 for the isolation of

protoplasts from *G. verrucosa* and *G. dura*. While the incubation temperature at which enzymatic digestion is carried out does affect enzyme activity, the optimal temperature employed for protoplast isolations is more closely related to that at which the species of macroalgae can be naturally found growing. Therefore, tissue digestions for protoplast isolations from the tropical *Gracilaria* species, *G. changii*, was optimal at 28 °C (Yeong *et al.*, 2008), while that of the more temperate species, *G. verrucosa*, was 22 °C (Araki *et al.*, 1998) and 25 °C (Gupta *et al.*, 2011), when similar enzyme combinations were used.

Protoplasts require the presence of an osmotic substance (e.g. mannitol or sorbitol) in order to counteract the turgor pressure exerted by the cytoplasm following cell wall removal (Davey *et al.*, 2005a). A concentration of 0.8 M mannitol was optimal for the isolation of *G. gracilis* protoplasts in this study. This is similar to that employed for the isolation of protoplasts from marine macroalgae in other studies (Polne-Fuller and Gibor, 1990; Yan and Wang, 1993; Araki *et al.*, 1998; Dipakkore *et al.*, 2005; Reddy *et al.*, 2006; Yeong *et al.*, 2008; Lafontaine *et al.*, 2011). The use of a low strength ionic medium (1 or 3% NaCl in deionised water) instead of ASW however, resulted in significantly decreased ( $P < 0.05$ ) protoplast yields. This is contrary to reports by Uppalapati and Fujita (2002), Dipakkore *et al.* (2005) and Reddy *et al.* (2006), who showed increased protoplast yields when low ionic strength media was used to replace ASW in the enzymatic medium. However, only one of these investigations was based on isolation of protoplasts from a red macroalga, *P. okhaensis*, while the other reports investigated the isolation of protoplasts from green macroalgae.

The method optimized for the isolation of protoplasts from *G. gracilis* thalli in this study consistently resulted in protoplast yields of 20–30 x 10<sup>5</sup> protoplasts g<sup>-1</sup> f.wt, but at times yields were as high as 1–2 x 10<sup>7</sup> protoplasts g<sup>-1</sup> f.wt. These overall protoplast yields are comparable with those reported for other *Gracilaria* species (Yan and Wang, 1993; Yeong *et al.*, 2008; Gupta *et al.*, 2011).

Protoplast yields did vary between batches of seaweed received from the supplier as the physiological state of the plants varied between batches. The age of the plant, the growth rate, the duration of laboratory culture and the season in which the plants were harvested have all previously been shown to affect protoplast yields (Mollet *et al.*, 1995, Reddy *et al.*, 2008a, Yeong *et al.*, 2008). An effort was made to compensate for these factors by using only the youngest thalli for protoplast isolation and using tissue that had been cultured under

laboratory conditions for 2 weeks or less during optimization experiments. In addition, *G. gracilis* tissue that did not visually appear to be healthy i.e. was light in colour, or that showed visible signs of disease, was not used for protoplast isolations, as this generally led to poor yields and protoplasts with decreased viability.

The continued presence of remnant cell wall material led to decreased protoplast survival in this study (data not shown). The use of a density gradient enabled improved protoplast purification, and resulted in protoplasts which were free of contaminating cell wall debris. The removal of the plant cell wall during the protoplast isolation procedure is a very stressful process, and it is highly likely that this process is perceived by the plant itself as a possible pathogen attack. *Laminaria* sporophytes have been shown to produce hydrogen peroxide in the presence of cell wall degrading enzymes (Benet *et al.*, 1997). This is because algae appear to recognize the degradation products of their own cell walls as defence signals (Vreeland and Kloareg, 2000; Weinberger *et al.*, 2005). Furthermore, a study on expressed sequence tags from a protoplast library of *Chondrus crispus* showed that there was enhanced expression of stress genes in protoplasts (Collén *et al.*, 2006). The presence of any residual cell wall material, after protoplast isolation, may be a source for the continued induction of stress and disease response genes within newly isolated protoplast cells. Therefore, any residual cell wall material should be removed, as was done in this study, through the use of a density gradient.

The use of density gradients employing sucrose for protoplast purification is well documented for higher plants (Rao and Prakash, 1995; Raquel and Oliveira, 1996; Jullien *et al.*, 1998; Umate *et al.*, 2005; Borgato *et al.*, 2007). Density gradients employing Percoll have also been used in the purification of macroalgal protoplasts (Polne-Fuller and Gibor, 1990; Corzo *et al.*, 1995; Mollet *et al.*, 1995). The OptiPrep<sup>®</sup> discontinuous density gradient designed and employed in this study was found to be the most suitable for obtaining pure fractions of *G. gracilis* protoplasts. The fact that increased densities had to be employed for the purification of *G. gracilis* protoplasts compared to what has been used for higher plant protoplasts was perhaps not surprising, since macroalgal protoplasts are likely to contain higher concentrations of intracellular solutes and, therefore, would have a higher buoyant density.

The method developed for thallus sterilization in Chapter 2.3.4 severely affected protoplast yields and had to be adapted for further use in protoplast production. Adaptations to the previous method included the addition of a liquid detergent wash and exclusion of the 3 hr incubation in sterile distilled water. The use of liquid detergents in the sterilization of macroalgal tissue has been well documented (Reddy *et al.*, 1989; Yokoya and Handro, 1996; Huang and Fujita, 1997a and 1997b; Yokoya, 2000; Yokoya *et al.*, 2004; Rajahrishna Kumar *et al.*, 2007). Although *G. gracilis* thalli were shown to be axenic by plate culture (data not shown), protoplast cultures occasionally began to show signs of bacterial contamination by the fourth day of culture. As previously discussed (Chapter 2.5), establishing axenic algal material is a difficult task and numbers of bacteria associated with seaweeds can vary throughout the year, and even from batch to batch (Baweja *et al.*, 2009). In this study microbial contamination appeared to be batch dependant and particularly problematic during the summer months, resulting in decreased protoplast survival (data not shown). Similar problems concerning bacterial contamination were noted by Benet *et al.* (1997). In cases where contamination was present, the inclusion of penicillin G (100  $\mu\text{g ml}^{-1}$ ) and amphotericin B (0.1  $\mu\text{g ml}^{-1}$ ) significantly reduced the load of bacterial contaminants within *G. gracilis* protoplast cultures. Neither of these two antimicrobial agents was observed to have any apparent negative effects on *G. gracilis* protoplast culture and therefore both were included in the initial protoplast culture media for a period of 2 weeks, after which they were diluted out. Cheney (1990) also reported the use of antibiotics following the fusion and subsequent regeneration of *G. tikvahiae* and *G. chilensis* protoplasts.

*G. gracilis* protoplasts showed sensitivity to culture medium containing PES supplementation, while significantly ( $P < 0.05$ ) increased survival levels were obtained when protoplasts were cultured in filter-sterilized natural seawater with no supplementation during the course of the first week. Yeong *et al.* (2008) reported that *G. changii* protoplasts did not regenerate when cultured in PES medium, but did so only when cultured in MES medium. They suggested that the protoplasts may have been sensitive to ammonium and nitrate within the PES medium, as the presence of 20 mM ammonium nitrate has been shown to be toxic to higher plant protoplasts (Yeong *et al.*, 2008). Benet *et al.* (1997) also reported sensitivity of *Laminaria* sporophyte protoplasts to culture medium supplementation and stated that a 2-3 week period of recovery, where metabolism is minimal, was required by the protoplasts. It is possible that *G. gracilis* protoplasts also exhibit sensitivity to ammonium and nitrate in the PES medium early in the culture process and therefore, may require a recovery period.

*G. gracilis* protoplasts also survived better when they were disturbed and handled less, as was also observed for *Laminaria* protoplasts (Benet *et al.*, 1997). However, since protoplast regeneration has been reported for a number of species that have been cultured in PES enriched media immediately after isolation (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a; Lafontaine *et al.*, 2011), protoplast sensitivity to culture media, as observed in this study, may be species specific.

*G. gracilis* protoplast survival tended to increase significantly ( $P < 0.05$ ) with decreased seeding densities. This is likely to be due to the higher cell densities leading to competition for resources in the surrounding medium. In addition, those protoplasts undergoing necrosis at the higher densities may also release toxic substances into the surrounding culture medium, which are detrimental to protoplast survival (Yeong *et al.*, 2008). Protoplasts seeded at a density of  $2 \times 10^4$  cells  $\text{ml}^{-1}$  showed the highest ( $P < 0.05$ ) survival percentage ( $51.60 \pm 2.26\%$ ), but never continued to develop any further and this is an important consideration. In comparison, *G. gracilis* protoplasts seeded at densities of  $7-8 \times 10^4$  cells  $\text{ml}^{-1}$  were the only cultures that displayed further development. Similarly, *G. changii* and *Grateloupia turuturu* protoplast regeneration has also been reported to be dependent on protoplast density (Yeong *et al.*, 2008; Lafontaine *et al.*, 2011). While *Enteromorpha* protoplast regeneration occurred at a wide range of densities ( $0.86-5.16 \times 10^3 \text{ cm}^{-2}$ ), the regeneration rate was culture dependant (Reddy and Fujita, 1991). A protoplast density of  $7.28 \times 10^4$  cells  $\text{ml}^{-1}$  was required for regeneration in *G. changii* protoplasts (Yeong *et al.*, 2008), which is similar to what was found in this study. However, the seeding density employed for regeneration of *G. asiatica* was lower at  $5-8 \times 10^3$  cells  $\text{ml}^{-1}$  (Yan and Wang, 1993). Optimal seeding densities for higher plants are species dependant and range from  $5 \times 10^2-1 \times 10^6$  cells  $\text{ml}^{-1}$  (Davey *et al.*, 2005b) and therefore, optimal seeding densities for macroalgal protoplasts may also be species dependant. In many published reports the culture densities employed are not clearly stated and should be given more attention.

The cell wall re-synthesis by *G. gracilis* protoplasts was followed by calcofluor white staining and SEM. Freshly isolated protoplasts stained with calcofluor white showed only red autofluorescence originating from the protoplasts' natural pigments, indicating they were free from cell wall material. Similarly, SEM observation of newly isolated *G. gracilis* protoplasts (approximately 15 min old) showed that the protoplasts had a relatively smooth surface and that amorphous, mucilage-like material could already be seen distributed on the

surface of the protoplast. This early-stage release of mucilage-like material has also been reported for the protoplasts of *Grateloupia sparsa* (Chen and Chiang, 1994a and 1995). Calcofluor staining of *G. gracilis* protoplasts was apparent after 3 hrs of culturing and was observed to begin at one end of the protoplast. A similar pattern of cell wall deposition, beginning at a single pole of the protoplast, was also noted for *K. alvarezii* (Zablackis *et al.*, 1993). Protoplasts of *Porphyra* and *Grateloupia* have reportedly shown calcofluor positive staining after 1–3 hrs and 2 hrs of culturing, respectively (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a). SEM observation of 3 hr old protoplasts showed spherical projections distributed over a still fairly smooth cell surface. These spherical projections were also noted on the surfaces of *P. palmata* and *G. sparsa* protoplasts undergoing cell wall re-synthesis (Liu *et al.*, 1992; Chen and Chiang, 1995). While the exact chemical nature of this substance is unknown, Chen and Chiang (1995), with the aid of TEM observations, suggested that this amorphous substance was produced by endoplasmic reticula (ER). After 12 hrs of culturing, whole protoplasts appeared positively stained by calcofluor white. SEM appearance of *G. gracilis* protoplasts of the same age showed a surface which appeared uneven, but covered in cell wall material with a few spherical projections still visible. The patchy appearance (visible ‘holes’) is likely to be due to cell wall re-synthesis not yet being complete in these areas of the cell surface. This uneven and patchy appearance is similar to the appearance of both regenerating *P. palmata* and *G. sparsa* protoplasts (Liu *et al.*, 1992; Chen and Chiang, 1995). After 12 hrs of culturing, approximately  $51.07 \pm 11.35\%$  of the protoplasts showed signs of cell wall regeneration although some protoplasts still showed no cell wall deposition. A similarly unequal rate of protoplast development has been previously observed for *P. palmata* protoplasts (Liu *et al.*, 1992). The cell surface of regenerated *G. gracilis* protoplasts appeared much smoother at 24 hrs than at 12 hrs, and there seemed to be a fairly continuous layer of cell wall material with few gaps. The smoother appearance of the cell surface at this stage of cell wall regeneration was once again in agreement with what had been observed for *P. palmata* and *G. sparsa* protoplasts (Liu *et al.*, 1992; Chen and Chiang, 1995). After 24 hrs of culturing,  $80.56 \pm 4.00\%$  of *G. gracilis* protoplasts had regenerated their cell walls. The fact that not all protoplasts regenerated cell walls is not surprising, as this fraction possibly reflects the proportion of the population that was damaged during the isolation and purification steps. Previous studies have also reported that a small fraction of the protoplast populations, in each case, did not regenerate cell walls (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a).

The fact that the fluorescent appearance of the protoplasts changed further over the following 48 hrs suggests that perhaps cell wall deposition is not complete by 24 hrs. This change in fluorescence was also noted for *Porphyra* and *Grateloupia* protoplasts stained with calcofluor white (Polne-Fuller and Gibor, 1990; Chen and Chiang, 1994a). In both cases the authors suggest that this change in fluorescent appearance could be as a result of further cell wall materials being deposited on the surface of the cells. Cell wall regeneration of macroalgal protoplasts has been reported to take 24 hrs to 15 days depending on the species, although in most cases cell regeneration has been reported to take place within the first 24–48 hrs of culture (Cheney *et al.*, 1986; Chen, 1987; Polne-Fuller and Gibor, 1990; Reddy and Fujita, 1991; Liu *et al.*, 1992; Yan and Wang, 1993; Zablackis *et al.*, 1993; Chen and Chiang, 1994a and 1995; Mollet *et al.*, 1995; Matsumura *et al.*, 2000; Dipakkore *et al.*, 2005; Yeong *et al.*, 2008; Lafontaine *et al.*, 2011). The cell wall regeneration rate obtained for *G. gracilis* protoplasts in this study is comparable with those obtained for *G. changii* protoplasts (Yeong *et al.*, 2008). However, these rates are faster than those reported for *G. verrucosa* (4 days) (Mollet *et al.*, 1995), *G. asiatica* (3–5 days; although in this case cell wall regeneration was only confirmed visually and not through calcofluor staining, (Yan and Wang, 1993)) and, *G. tikvahiae* and *G. lemaneiformis* (2–6 days) (Cheney *et al.*, 1986). It is not unexpected that cell wall regeneration rates vary between different seaweed species, or even the same species under different culture conditions, since protoplast regeneration is known to be affected by so many factors (Reddy *et al.*, 2008a).

During the course of this investigation, it was noted that *G. gracilis* protoplasts cultured under low light intensity conditions and higher incubation temperature, underwent cell division that resulted in the formation of cell clumps. This pattern of protoplast development has not previously been reported for *Gracilaria*, but appears similar to cell suspension culture seen in *Porphyra* cultures (Chen, 1989; Polne-Fuller and Gibor, 1990). Culture temperature has also been shown to control the regeneration of *L. japonica* protoplasts (Matsumura *et al.*, 2000). The initiation of cell division through the formation of a peanut-shaped cell is also not unlike that shown for the red seaweed, *Grateloupia* (Chen and Chiang, 1994a; Lafontaine *et al.*, 2011). When *G. gracilis* cultures were allowed to continue to divide under these culture conditions with no agitation, a ‘string’ of cells was produced through repeated cell division. However, these cell filaments never extended beyond six cells. Lafontaine *et al.* (2011) reported a similar regeneration pattern of *G. turuturu* protoplasts into cell filaments of 2–6 cells, after which no further development was visualised.

Agitation was initially tried with *G. gracilis* protoplasts in an attempt to separate divided cells from each other. However, this did not prove successful as cells appeared to be associated with each other through some sort of matrix, as was seen in *P. linearis* cultures (Chen, 1989). Cell clumps also associated with each other, to form large clusters of closely associated cells. The application of orbital shaking to *Porphyra* cultures did not inhibit cell division but was thought to inhibit the development of polarity in protoplasts (Polne-Fuller and Gibor, 1990). Polne-Fuller and Gibor (1990) also noted that cells that were initially attached and then detached from the plate surface as a result of agitation, lost their polarity and only cells which remained attached developed polarity. The resulting non-polarized calli that were formed in these cultures, could however be induced to regenerate into normal plants when they were returned to non-agitated cultures (Polne-Fuller and Gibor, 1990). It is unknown whether the agitation of the *G. gracilis* cultures also resulted in a loss of polarity of the cells, however, returning the cell clumps to stationary culture did not result in plant regeneration.

*Porphyra* suspension cultures generated under conditions of low light intensity ( $10\text{-}20 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ), higher incubation temperatures ( $20 \text{ }^\circ\text{C}$ ) and shorter photoperiods (10/14 hr or 8/16 hr) could be induced to regenerate whole plants through reversing the culture conditions (Chen, 1989). However, this was not the case with *G. gracilis* cells. Cultures of suspended cells could only be maintained for a period of 5-6 weeks before they died. It is likely that this pattern of cell division is induced in *G. gracilis* protoplast cultures as a result of non-optimal incubation conditions, and that these conditions result in a permanent change in the cells.

Whole *G. gracilis* plants could be regenerated from protoplasts cultured under conditions of a higher light intensity and either low or high incubation temperatures ( $14 \pm 1$  or  $18 \pm 1 \text{ }^\circ\text{C}$ ). However, two patterns of development were observed which gave rise to plants that differed in appearance. Plants regenerated by the first pattern resembled the parental plants, with slender, branched thalli, while plants regenerated through the second pattern remained small with thalli that were thick and unbranched.

In both developmental patterns, successive cell divisions gave rise to calli. However, calli that developed via the second, rapid developmental pattern, showed a greater number of uniseriate filaments at their periphery. In higher plants, a callus is described as disorganized cell growth in differentiated tissue (Yeoman, 1987), but since seaweeds lack a high degree of

organization and differentiation, the idea of callus within seaweeds is a contentious topic (Aguirre-Lipperheide *et al.*, 1995). Therefore, the term “callus-like” has been applied to the filamentous outgrowths seen in seaweeds (Garcia-Reina *et al.*, 1991; Yokoya *et al.*, 1993; Reddy *et al.*, 2008b). The term callus-like is used since the cells are organised into filaments and hence cannot be termed a true callus, but the filamentous growth does indeed represent callus growth in seaweeds (Yan and Wang, 1993). Yan and Wang (1993) also report the presence of these filaments at the periphery of *G. asiatica* calli developed from regenerating protoplasts. Images by Cheney (1990) also show the presence of filaments at the periphery of *G. tikvahiae* callus. Development of filamentous structures was also reported during the regeneration of protoplasts of *Grateloupia* (Chen and Chiang, 1994a).

In both cases, development did not progress at an equal pace for all cells, and at any particular time there were various stages of division and development present in the same culture plate. This variation in the developmental pace of cells was also noted during cell wall regeneration and is likely to be an inherent property of the individual cells. In both cases, about 90% of surviving protoplasts went on to produce a callus and when culture plates were viewed, the surface was covered in these cell-masses. In the first developmental pattern, filaments disappeared from calli prior to bud formation. The disappearance of filaments prior to bud formation was also noted during the regeneration of *G. asiatica* plants (Yan and Wang, 1993). In contrast, the filaments remained present even during plant growth in some cases during the second developmental pattern of *G. gracilis* protoplasts.

It was interesting to note that the colour of the calli changed during the development process. There appeared to be a clear colour change in particular areas of the callus at the onset of bud formation and the colour intensity appeared to be highest at this “growing” point of the callus, indicating a change in the distribution of “growth-substances” as suggested by Yan and Wang (1993) during the development of *G. asiatica* calli

The first developmental pattern gave rise to plants in which either a single bud developed into a single branching thallus, or in which many buds were produced to develop into many branching thalli. Plant regeneration through this manner was slow, and generally, actual budding and thallus regeneration was only visible after 2 or more months. However, development occurred rapidly in these plants once buds began to grow. In contrast, the second developmental pattern gave rise to buds after just 20 days of culture. This pattern of

development gave rise to either a single plant from a callus, or in most cases, multiple buds that grew from a single callus. The development of many plants or shoots from a single callus was also reported during the development of whole plants from both *G. changii* (Yeong *et al.*, 2008) and *G. asiatica* (Yan and Wang, 1993).

It was noted that while most protoplasts (90%) divided to produce calli, regardless of the developmental pattern, not all calli produced whole plants. In fact plant regeneration rates were only around 0.02%. In addition, bud and thallus regeneration often occurred at various times for calli within the same culture plate. The change from callus to shoot development occurred spontaneously and seemed more dependent on the actual calli than on any media or culture conditions, since these were kept constant during this period. This appeared to be similar to previous reports regarding callus induction in some seaweed species; that the occurrence of callus within a species appears to be sporadic and more dependent on internal factors of the explants themselves, rather than on the external culture conditions applied (Aguirre-Lipperheide *et al.*, 1995).

It was interesting to note that the development of one *G. gracilis* plant seemed to stimulate the budding of others in the same culture plate. The budding calli may have produced growth stimulating substances which were released into the surrounding culture medium where they could have a stimulatory effect on other calli. While such details are not reported in literature concerning protoplast regeneration in macroalgae, Yan and Wang (1993) did state that the presence of algal segments were necessary to stimulate cell division and plant regeneration in *G. asiatica*. The authors concluded that the segments which were co-cultured with the protoplasts released growth substances which stimulated cell division (Yan and Wang, 1993). Similarly, regenerating heterokaryons generated through the fusion of *G. chilensis* and *G. tikvahiae* protoplasts were cultured in the presence of *Gracilaria* callus nurse cultures (Cheney, 1990). While the presence of algal segments or calli was not required for cell division of protoplasts in this study, budding calli may have played a similar role to that of the algal segments included in *G. asiatica* protoplast culture. It is a pity that so little is known about the physiological role that plant growth regulators (PGRs) play in the growth and differentiation of seaweeds (Reddy *et al.*, 2008b), since their application to protoplast culture may well benefit the field. However, until more rigorous research on these PGRs is available, their current role in protoplast culture may be debatable, as it is in callus stimulation for seaweed explants (Reddy *et al.*, 2008b).

Plants grown from the second developmental pattern remained short with thick thalli that never branched. This was also the case for plants regenerated from *G. asiatica* protoplasts (Yan and Wang, 2003). Unfortunately there is not much detail regarding the appearance of adult plants regenerated from *G. changii* (Yeong *et al.*, 2008), so a comparison cannot be drawn between plant development in this study and that of *G. changii*. Similarly, Cheney (1990) only reports on the appearance of hybrid plants resulting from the fusion of *G. tikvahiae* and *G. chilensis* protoplasts, and therefore comparisons between that and the current study are also not pertinent. The apparent limited life span of the plants regenerated through the second developmental pattern has not been reported in other cases of regenerated *Gracilaria*. The cause of plant death is unknown, but the aberrant appearance of these plants may suggest a developmental flaw. Plant regeneration may well have been premature in this case, with protoplasts either not having recovered properly from the stress of isolation or with rapid bud stimulation leading to the abnormal appearance of adult plants. Adult plants regenerated from *Laminaria saccharina* sporophyte protoplasts have also been reported to be abnormal and show necrosis at the distal part of their laminae, however the ultimate reason for this is not known (Benet *et al.*, 1997).

The first developmental pattern however, gave rise to plants which resembled the parent plants and when cultured under the same conditions, could grow at a similar rate to the seaweed received from the supplier. Plants regenerated through this pattern grew rapidly to produce a large biomass under laboratory conditions and to date, plants have been maintained for two years.

The reason for the two developmental patterns observed for *G. gracilis* in this study is still unknown, but both developmental patterns occurred in a single culture plate and therefore, these patterns were not batch dependant. Different morphologies of regeneration have been noted to arise from protoplasts isolated from *Porphyra perforata* blades and these patterns have been suggested to be related to the level of differentiation of cells within the area from which the protoplasts arose (Polne-Fuller and Gibor, 1984). It is unknown whether this is the case for regenerating protoplasts in this study, since *Gracilaria* do not show a high degree of differentiation. However, it is likely that developmental patterns are protoplast-specific and possibly due to a trait inherited, or entrained, prior to cell treatment and which may ultimately be related to origin of the protoplasts themselves.

Regenerated plants in this study are unlikely to be the result of spore contamination since protoplasts gave rise to calli distinguished through the presence of uniseriate filaments. There is no mention of uniseriate filament development or callus development from spores in any literature relating to *G. gracilis* spore development (De Oliveira and Plastino, 1994; Kain and Destombe, 1995; Polifrone *et al.*, 2006). Instead, previously reported literature shows that discs form from germinating spores which give rise to holdfasts and plantlets (De Oliveira and Plastino, 1994; Kain and Destombe, 1995; Engel *et al.*, 2001; Polifrone *et al.*, 2006). We were not able to grow whole *G. gracilis* plants from spores since only male gametophytic plants were available and tetrasporophytic and female plants have never been observed in our source of *G. gracilis* over a period of 3 years. Microscopic examination of *G. gracilis* plants was routinely carried out and plants always showed the presence of spermatangial conceptacles. Furthermore, plants received from the supplier and plants regenerated in this study (derived through the first developmental pattern) were microscopically examined and both shown to be gametophytic (male). This substantiates the conclusion that the plants regenerated in this study originated from protoplasts and not from contaminating spores.

While the isolation of protoplasts has previously been reported from other species of *Gracilaria*, the culture and regeneration of whole plants from *G. gracilis* protoplasts has never before been reported. The results obtained in this work suggest not only that whole plant regeneration from protoplasts of *G. gracilis* is possible, given the correct conditions, but that protoplasts themselves could be employed as a cell system for molecular studies in this commercially important species.

In conclusion, we established a protocol for generating large quantities of viable protoplasts by optimizing the various parameters (enzyme constituents and concentrations, the pre-treatment of thalli, the incubation period and temperature, the pH of the enzymatic medium and the osmoticum in the enzymatic medium) which are known to influence protoplast yields. Furthermore, an OptiPrep<sup>®</sup> discontinuous density gradient was designed and employed for improved protoplast purification. Cell wall regeneration of *G. gracilis* protoplasts was monitored by calcofluor white staining and by SEM. Finally, factors affecting whole plant regeneration were assessed.

## **CHAPTER 4**

### **TRANSFECTION OF *GRACILARIA GRACILIS* PROTOPLASTS**

## Contents

<b>4.1</b>	<b>Summary</b> .....	121
<b>4.2</b>	<b>Introduction</b> .....	122
4.2.1	Aims of this chapter.....	125
<b>4.3</b>	<b>Materials and Methods</b> .....	126
4.3.1	Algal material and culture conditions.....	126
4.3.2	General molecular techniques .....	126
4.3.3.	Bacterial strains, plasmids, media and culture conditions.....	126
4.3.4	PEG-mediated <i>G. gracilis</i> protoplast transfection.....	127
4.3.5	Optimization of <i>G. gracilis</i> protoplast transfection efficiency.....	130
4.3.6	Testing the effects of targeted HR and MARs on transgene expression.....	131
4.3.6.1	Construction of expression constructs for assessing the effects of HR and MARs on transgene expression.....	131
4.3.6.2	Protoplast transfection.....	135
4.3.6.3	Sampling and assessment EGFP levels.....	137
4.3.6.4	PCR detection of <i>egfp</i> in transfected protoplasts .....	137
4.3.6.5	Confirmation of chromosomal integration of foreign DNA .....	137
4.3.7	Testing of <i>G. gracilis</i> protoplast sensitivity to antibiotics and BASTA <sup>®</sup> .....	139
4.3.7.1	Effect of chloramphenicol and kanamycin on protoplast survival.....	139
4.3.7.2	Effect of BASTA <sup>®</sup> on protoplast survival.....	140
4.3.8	BASTA <sup>®</sup> selection of transfected <i>G. gracilis</i> protoplasts .....	140
4.3.8.1	Construction of expression constructs for assessing the use of BASTA <sup>®</sup> as a selective agent in transfected protoplasts .....	140
4.3.8.2	Transfection of p18S-SV-bar/ <i>egfp</i> and pSV-bar/ <i>egfp</i> into <i>G. gracilis</i> protoplasts .....	142
4.3.8.3	Monitoring protoplast survival.....	144
4.3.8.4	PCR detection of <i>bar</i> in transfected protoplasts.....	144
<b>4.4</b>	<b>Results</b> .....	145
4.4.1	Transfection of <i>G. gracilis</i> protoplasts .....	145
4.4.2	Optimization of parameters affecting <i>G. gracilis</i> protoplast transfection efficiency .....	146
4.4.3	Effect of HR and MARs on transgene expression.....	149
4.4.4	<i>G. gracilis</i> protoplast sensitivity to antibiotics and BASTA <sup>®</sup> .....	156
4.4.5	BASTA <sup>®</sup> selection of transfected protoplasts .....	158
<b>4.5</b>	<b>Discussion</b> .....	162

## 4.1 Summary

The possibility of transforming *G. gracilis* protoplasts by PEG-mediated transfection was investigated. Initially, a transfection protocol was optimized by investigating the effects of the transfected DNA concentration, PEG concentration, concentration of divalent cations in the transfection medium, length of transfection period and the protoplast concentration on EGFP expression. Optimization of the various critical parameters for PEG-mediated transfection of *G. gracilis* protoplasts resulted in increased transfection efficiencies of 89 in  $10^5$  protoplasts. The optimized transfection method was then used to investigate the effects of targeted homologous recombination (HR) and matrix attachment regions (MARs) on transgene expression, and in so doing identify possible means of increasing transgene expression. A suite of expression plasmids was designed, constructed and transfected into *G. gracilis* protoplasts after which EGFP levels and the presence of *egfp* were monitored for nine days post-transfection. The presence of tobacco Rb7 MARs and 18S rDNA regions on plasmid DNA (p18S-SV-egfpMARs) resulted in the most significant increases in relative fluorescence and therefore EGFP levels 3 and 4 days post-transfection ( $6.65 \pm 0.75$  and  $3.15 \pm 0.52$  fold change in relative fluorescence at 3 and 4 days, respectively). Furthermore, a PCR amplification strategy was employed to show that targeted HR had in fact taken place in transfected *G. gracilis* protoplasts. In an effort to find a suitable selectable marker for *G. gracilis* transformation, protoplasts were assessed for their sensitivity to the antibiotics kanamycin and chloramphenicol, and the herbicide BASTA<sup>®</sup>. While *G. gracilis* protoplasts showed a high level of resistance to both the antibiotics tested, protoplasts were shown to be sensitive to BASTA<sup>®</sup>. Therefore plasmids containing the *bar* gene, conferring resistance to the herbicide, under the influence of the SV40 promoter/enhancer were constructed and transfected into protoplasts. Transfected protoplasts were then assessed for their ability to survive BASTA<sup>®</sup> selection. *G. gracilis* protoplasts transfected with either pSV-bar/egfp or p18S-SV-bar/egfpMARs exhibited significantly ( $P < 0.01$ ) increased survival percentages 5-7 days post-transfection (2-4 days post-BASTA<sup>®</sup> supplementation) in comparison to the negative control protoplasts. In addition, 4-5% of protoplasts survived a second round of selection 21 days post-transfection. After a period of 2 months, surviving cells had stopped dividing and had not continued to produce calli.

## 4.2 Introduction

Reports of macroalgal transformation are still fairly limited, and much of the pioneering research has focused on establishing transformation methods for various commercially important species of macroalgae (Reddy *et al.*, 2008a). In particular, research on species such as *Laminaria* and *Porphyra* has been successful, and on-going efforts have begun to explore methods for increasing expression of transgenes as well as for the production of seaweed strains with biotechnological applications (He *et al.*, 2001; Jiang *et al.*, 2002; Liu *et al.*, 2003; Bernasconi *et al.*, 2004; Mizukami *et al.*, 2004; Gao *et al.*, 2005; Gong *et al.*, 2007; Zuo *et al.*, 2007; Fukuda *et al.*, 2008; Mikami *et al.*, 2011). *Laminaria* transformation has made use of microparticle bombardment since gametophytes have been targeted for transformation (Qin *et al.*, 2005). In contrast, procedures for *Porphyra* protoplast isolation and regeneration are well developed (Reddy *et al.*, 2008a; Wang *et al.*, 2010b), and since protoplasts lack cell walls, techniques such as electroporation and PEG-mediated transfection have been applied to a number of *Porphyra* species (Table 4.1). Furthermore, successful transformation with a variety of reporter genes has been accomplished in *Porphyra* protoplasts (Table 4.1).

The successful application of recombinant gene technology requires stable and reliable transgene expression (Liu *et al.*, 2003). However, transgenes are generally expressed at low levels and are often subject to gene silencing. In order to overcome these issues concerning transgene expression, homologous recombination (HR) technology has been successfully employed in animals, plants, microorganisms (Gorman and Bullock, 2000; Vasquez *et al.*, 2001; Puchta, 2002) and most recently in two species of red alga, *P. yezoensis* (Liu *et al.*, 2003) and *P. haitanensis* (Zuo *et al.*, 2007). HR enables the targeted integration of foreign genes into specific sites of the host chromosome(s) (Hohe and Reski, 2003). In both the *Porphyra* species studies, the 18S rRNA gene was used as the HR target site (Liu *et al.*, 2003; Zuo *et al.*, 2007). The use of a rRNA gene as the target for HR is advantageous as it is highly conserved and exists in multiple copies within an organism (Liu *et al.*, 2003). Furthermore, since rRNA genes comprise multiple copies per cell, they potentially provide multiple target sites for integration and consequently, the likelihood that HR would have a lethal effect on the host organism due to disruption of a single copy is very low (Amador *et al.*, 2000). This strategy has also been successfully employed in *Arabidopsis thaliana* (Zhang *et al.*, 2000

referenced in Liu *et al.*, 2003), yeast (Wery *et al.*, 1997), rice (Xu *et al.*, 1999 referenced in Liu *et al.*, 2003) and the bacterium *Brevibacterium lactofermentum* (Amador *et al.*, 2000).

**Table 4.1** Macroalgal species for which transformation has been reported by methods other than microparticle bombardment.

Species <sup>a</sup>	Reporter gene <sup>b</sup>	Promoter <sup>c</sup>	Transfection method	Reference
<i>Porphyra haitanensis</i>	<i>cat</i> <sup>†</sup>	SV40	electroporation	Zuo <i>et al.</i> (2007)
(conchosporos)	<i>lacZ</i> / <i>egfp</i>	SV40	glass bead agitation	Wang <i>et al.</i> (2010b)
<i>P. leucosticta</i> (archaeosporos)	<i>lacZ</i>	CaMV 35S	electroporation	Lin <i>et al.</i> (2001) (abstract) & Reddy <i>et al.</i> (2008a)
<i>P. miniata</i>	<i>uidA</i>	CaMV 35S	electroporation	Kübler <i>et al.</i> (1994)
<i>P. tenera</i>	<i>uidA</i>	CaMV 35S	electroporation	Okauchi and Mizukami (1999)
<i>P. yezoensis</i>	<i>uidA</i>	CaMV 35S	electroporation / PEG	Kuang <i>et al.</i> (1998)
	<i>uidA</i> / <i>gfp</i>	ns	<i>Agrobacterium</i>	Cheney <i>et al.</i> (2001) (abstract)
	<i>uidA</i> / <i>cat</i> / <i>gluc</i>	ns	PEG / electroporation	He <i>et al.</i> (2001) (abstract)
	<i>uidA</i>	CaMV 35S	electroporation	Hado <i>et al.</i> (2003)
	<i>uidA</i> <sup>‡</sup>	CaMV 35S	electroporation	Liu <i>et al.</i> (2003)
	<i>nfsI</i>	ns	<i>Agrobacterium</i>	Bernasconi <i>et al.</i> (2004) (abstract)
	S65T-GFP / <i>uidA</i> / <i>luc</i>	Rubisco	electroporation	Mizukami <i>et al.</i> (2004)
	<i>uidA</i>	CaMV 35S / beta-tubulin	electroporation	Gong <i>et al.</i> (2007)
<i>Ulva lactuca</i>	<i>uidA</i> <i>NPTII</i>	CaMV 35S NOS	electroporation	Huang <i>et al.</i> (1996)

<sup>a</sup> Unless otherwise stated, research was carried out on protoplasts of the indicated species.

<sup>b</sup> *gluc*, glucose oxidase; *cat*, chloramphenicol acetyl transferase; *luc*, luciferase; *nfsI*, bacterial nitroreductase gene; *NPTII*, bacterial neomycinphosphotransferase

<sup>†</sup> Either *cat*, or *cat* flanked by portions of 18S rDNA of *P. haitanensis*, or *cat* flanked by 1 or 2 matrix attachment regions (MARs) of silkworm and portions of 18S rDNA of *P. haitanensis*

<sup>‡</sup> Either *uidA*, or *uidA* flanked by portions of 18S rDNA of *P. yezoensis*

<sup>c</sup> ns, not stated by authors; Rubisco, *P. yezoensis* Rubisco gene promoter; beta-tubulin, *P. yezoensis* beta-tubulin 5' flanking sequence; NOS, nopaline synthase promoter

Matrix attachment regions (MARs) and/or scaffold attachment regions (SARs) have both been shown to increase transgene expression levels (Allen *et al.*, 2000; Cheng *et al.*, 2001; Mankin *et al.*, 2003; Butaye *et al.*, 2004; Kim *et al.*, 2004; Zuo *et al.*, 2007) and reduce the variations observed in transgene expression between individual transformants (Holmes-Davis and Comai, 1998). MARs are DNA sequences that bind to a cell's proteinaceous nuclear matrix at the bases of chromatin loop domains, the highest level of chromatin organisation (Spiker and Thompson, 1996; Han *et al.*, 1997; Eivazova *et al.*, 2009). These sequences are thought to act as boundaries between neighbouring transcriptionally active loop domains, insulating the active loop domains from the influence of neighbouring sequences (Spiker and Thompson, 1996). It has been suggested that they also function in facilitating DNA replication and augmentation of transcription (Bode *et al.*, 2000; Fiorini *et al.*, 2006; Linnemann *et al.*, 2009). MARs are AT-rich DNA sequences, typically with an AT-content of more than 70%, that are highly conserved in their binding affinity across all organisms investigated to date (Spiker and Thompson, 1996; Allen *et al.*, 2000; Cheng *et al.*, 2001). A silkworm MAR has been shown to increase the transient expression of chloramphenicol acetyl transferase (CAT) in *P. haitanensis* protoplasts (Zuo *et al.*, 2007), suggesting that these sequences could be used in macroalgal transformation with the same success as in higher plants.

The recovery of successfully transformed organisms generally requires the use of selectable markers (Hallmann, 2007). Selectable markers are often antibiotic resistance genes which are dominant markers as they confer a new genotypic trait to the transformed organism (Hallmann, 2007). However, very little research has been conducted on macroalgal susceptibility to antibiotics and herbicides which are commonly employed in higher plants, and extensive studies have only been done on *Laminaria* and *Undaria* (Qin *et al.*, 1998 referenced in Reddy *et al.*, 2008a). Although kanamycin and hygromycin resistance are two of the most widely used selectable markers in plant systems (Huang *et al.*, 1996; Reddy *et al.*, 2008a; Wang *et al.*, 2010c), both *Laminaria* and *Undaria* have been shown to be resistant to kanamycin (Qin *et al.*, 1998 referenced in Reddy *et al.*, 2008a). Similarly, *Ulva* and *Monostroma* have been reported to be resistant to both kanamycin and hygromycin (Reddy *et al.*, 2008a). Therefore, the choice and use of selectable markers for macroalgal selection cannot simply be based on those commonly employed for higher plants. Instead, the

sensitivity of each macroalgal species to selective agents should be investigated before employing these agents for selection purposes.

#### **4.2.1 Aims of this chapter**

The aim of this chapter was initially to establish and optimize a protocol for *G. gracilis* protoplast transfection. This method was then employed to investigate the effects of targeted HR and MARs on transgene expression using the *egfp* gene as a test example, and in so doing identify a possible means for increasing transgene expression. Furthermore, *G. gracilis* protoplast sensitivity to kanamycin, chloramphenicol and BASTA<sup>®</sup> were investigated in an effort to find a suitable selective marker for use in *G. gracilis* transformation.

University of Cape Town

### 4.3 Materials and Methods

All media and solutions used in this study are listed in Appendix A.

#### 4.3.1 Algal material and culture conditions

As described in Chapter 2.3.1.

#### 4.3.2 General molecular techniques

General molecular techniques were carried out as described in Chapter 2.3.2, unless otherwise stated.

Restriction fragments were blunt end-repaired using Klenow Fragment (Fermentas), as described by Ausubel *et al.* (1989) (Appendix B.6).

Dephosphorylation of vector DNA was carried out as described by Coyne *et al.* (2002) (Appendix B.7).

*E. coli* DH5 $\alpha$  identified as containing desired recombinant constructs following colony PCR, were inoculated into 5 ml LB (Appendix A.1.5) supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin (Appendix A.2.2) and incubated overnight with agitation at 37 °C for the preparation of overnight cultures.

#### 4.3.3. Bacterial strains, plasmids, media and culture conditions

*E. coli* strains and plasmids used in this chapter are listed in Table 4.2 and 4.3, respectively.

*E. coli* strains were either grown in LB or on LA (Appendix A.1.6) at 37 °C. *E. coli* strains harbouring plasmid vectors listed in Table 4.2 were cultured in LB or on LA containing 100  $\mu\text{g ml}^{-1}$  ampicillin or 100  $\mu\text{g ml}^{-1}$  kanamycin (Appendix A.2.2) at 37 °C.

**Table 4.2** Bacterial strains used in this study.

Strain	Genotype/ relevant feature(s) <sup>a</sup>	Reference
<i>Escherichia coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F'(traD36 proAB' lacI<sup>q</sup> lacZΔM15)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>Escherichia coli</i> DH5α	<i>[(φ80lacZΔM15) recA1 endA1 gyrA96 thi-1 hsdR17(r<sub>K</sub><sup>-</sup>m<sub>K</sub><sup>+</sup>) supE44 (Nal<sup>r</sup>) relA1 Δ(lacZYA-argF)<sub>U169</sub> phoA</i>	Hanahan (1983)
<i>Escherichia coli</i> K12	<i>ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) Tet<sup>S</sup> endA1 rspL136 (Str<sup>R</sup>) dam13::Tn9 (Cam<sup>R</sup>) xylA-5 mtl-1 thi-1 mcrB1 hsdR2</i>	New England Biolabs

<sup>a</sup> Nal<sup>r</sup>, naladixic acid resistant; Str<sup>r</sup>, streptomycin resistant

#### 4.3.4 PEG-mediated *G. gracilis* protoplast transfection

*G. gracilis* protoplasts were isolated as described in Chapter 3.3.4 and purified similar to 0 hr samples described in Chapter 3.3.6. After collection of *G. gracilis* protoplasts from the OptiPrep<sup>®</sup> discontinuous density gradient, the protoplasts were rinsed in transfection medium (TFM; Appendix A.2.10.21). Thereafter, protoplasts were resuspended in 1 ml TFM and protoplast numbers determined as described in Chapter 3.3.2.1. The final *G. gracilis* protoplast concentration was adjusted to 1 x 10<sup>6</sup> cells ml<sup>-1</sup>.

*G. gracilis* protoplasts were transfected by a method modified from Kuang *et al.* (1998), Zelazny *et al.* (2007) and Kim *et al.* (2002). Protoplasts (1 x 10<sup>5</sup> cells), resuspended in 100 µl TFM, were pipetted into a microfuge tube and 10 µg of pSV-egfp (Table 4.3) was added. The suspension was gently mixed before drop-wise addition of PEG medium (Appendix A.2.10.22) to a final concentration of 15%. The suspension was gently mixed, before being incubated for 10 min at room temperature (RT). Following this incubation period, 1 ml of 0.125 M NaCl - filter-sterilized natural seawater (Appendix A.2.10.23) was added to the suspension and the protoplasts collected by centrifugation (160x *g* for 10 min at 22 °C). Protoplasts were resuspended in 2 ml of 0.125 M NaCl - filter-sterilized natural seawater and dispensed into 35 x 10 mm tissue culture dishes (Falcon) together with penicillin G (100 µg ml<sup>-1</sup>) and amphotericin B (0.1 µg ml<sup>-1</sup>) (Appendix A.2.2). Protoplasts

were cultured in the presence of NaCl as an osmoticum for the first 2 days as transfected protoplasts had shown better survival rates when additional NaCl was included in the culture medium. Culture plates containing *G. gracilis* protoplasts were incubated for 2 days at 14-15 °C in the dark. After 2 days the entire culture volume was replaced with 2 ml of filter-sterilized natural seawater supplemented with penicillin G (100 µg ml<sup>-1</sup>) and amphotericin B (0.1 µg ml<sup>-1</sup>). This was done in order to remove the NaCl used as an osmoticum during the initial stages of culturing. The protoplast cultures were then incubated at 14 °C under a light intensity of 10 µmol photons m<sup>-2</sup>s<sup>-1</sup> for 1 week and thereafter at 30 µmol photons m<sup>-2</sup>s<sup>-1</sup>, while being maintained on a 16/8 hrs day/night cycle. Half the culture volume was replaced with fresh culture medium every five days for the duration of the experiment.

The presence of enhanced green fluorescent protein (EGFP) inside transfected *G. gracilis* protoplasts was confirmed by examining transfected protoplasts under a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 510 nm emission filter. *G. gracilis* protoplast transfection efficiencies were determined by counting the number of EGFP-expressing cells and representing this as a fraction of the total number of cells counted.

In addition, protoplast fluorescence levels were used as a measure of EGFP. Briefly, transfected *G. gracilis* protoplast cultures were harvested 2 days post-transfection, collected by centrifugation (160x *g* for 10 min at 22 °C) and rinsed in 0.125 M NaCl-filter-sterilized natural seawater. EGFP values were quantified based on fluorescence units per cell using a fluorometer (GloMax<sup>TM</sup> 96 Microplate Luminometer, Turner BioSystems using the fluorometer function of the reader) after excitation at 490 nm and the emission maximum at 510-570 nm (the blue optical kit supplied with the reader). Protoplast numbers were determined by Neubauer improved bright-line Haemocytometer cell count. Fluorescence readings were always performed in duplicate for each protoplast culture. Due to variations in the natural fluorescence of *G. gracilis* protoplasts between different batches and with culture time, data was always normalized to a negative control and, therefore, was presented as fold changes in fluorescence per cell (relative fluorescence). Untransfected *G. gracilis* protoplasts served as the negative control for both microscopy and fluorometer readings.

**Table 4.3** Plasmids used in this study.

Plasmid	Relevant feature(s) <sup>a</sup>	Reference
pSV-egfp	Derivative of pSV- $\beta$ -Galactosidase with the <i>egfp</i> gene substituted for the <i>lacZ</i> gene; Amp <sup>r</sup>	This study
pTRAc	CaMV 35S promoter, 2 copies of the tobacco Rb7 matrix attachment region, Amp <sup>r</sup>	Maclean <i>et al.</i> (2007)
pSV-egfpMAReco	Derivative of pSV-egfp with the Rb7 MAR cloned upstream of the SV40 promoter; Amp <sup>r</sup>	This study
pSV-egfpMARs	Derivative of pSV-egfpMAReco with a second Rb7 MAR cloned downstream of the <i>egfp</i> gene; Amp <sup>r</sup>	This study
p18S-21	Derivative of pSV-egfp containing a 1615 bp fragment of the <i>G. gracilis</i> 18S rRNA gene; Amp <sup>r</sup>	This study
p18S-SV-egfp	Derivative of p18S-21 with the SV40 promoter and <i>egfp</i> gene interrupting the 18S rRNA gene; Amp <sup>r</sup>	This study
p18S-SV-egfpMARs	Derivative of p18S-21 with the two MAR regions, SV40 promoter and <i>egfp</i> gene interrupting the 18S rRNA gene; Amp <sup>r</sup>	This study
pBluescript KS	Amp <sup>r</sup> , $\beta$ -galactosidase	Short <i>et al.</i> (1988)
pEarleyGate201 $\Delta$ <i>ccdB</i>	Km <sup>r</sup> , <i>bar</i> , CaMV 35S promoter, Gateway (Earley <i>et al.</i> , 2006), lacks <i>ccdB</i> cytotoxic gene	Smart (2011); Earley <i>et al.</i> (2006)
pKS- <i>bar</i>	Derivative of pBluescript KS with the <i>bar</i> gene cloned into the MCS; Amp <sup>r</sup>	This study
pSV- <i>bar</i>	Derivative of pSV-egfp with the <i>bar</i> gene substituted for the <i>egfp</i> gene; Amp <sup>r</sup>	This study
pSV- <i>bar/egfp</i>	Derivative of pSV-egfp with a second SV 40 promoter driving the expression of the <i>bar</i> gene; Amp <sup>r</sup>	This study
p18S-SV- <i>bar/egfp</i> MARs	Derivative of p18S-SV-egfpMARs with a second SV 40 promoter driving the expression of the <i>bar</i> gene; Amp <sup>r</sup>	This study

<sup>a</sup> Amp<sup>r</sup>, ampicillin resistant; Km<sup>r</sup>, kanamycin resistant

### 4.3.5 Optimization of *G. gracilis* protoplast transfection efficiency

Several parameters have been shown to affect transformation efficiency during PEG-mediated transfection (Nicolaisen and Poulsen, 1993). These parameters include the transfected DNA concentration, PEG concentration, concentration of divalent cations in the transfection medium, length of transfection period and the protoplast concentration.

The aforementioned parameters were optimized sequentially. During the optimization experiments, all *G. gracilis* thalli were pre-treated and all protoplasts isolated and collected as described in 4.3.4. Each optimization experiment employed three independent transfections per variable. *G. gracilis* protoplasts were harvested 2 days post-transfection, washed with culture medium and fluorescent readings along with cell numbers determined as described in 4.3.4. Due to variations in the natural fluorescence of protoplasts between batches and with culture time, data was always normalized to the negative control and presented as fold changes in fluorescence per cell (relative fluorescence). One-way ANOVA was used to analyze the optimization data. When the results of the ANOVA were significant, the Tukey Test was used to determine the significant differences due to various treatments using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at  $P < 0.05$ .

The effect of different amounts of plasmid DNA on transfection efficiency was assessed by varying the amount of pSV-egfp DNA (5, 10, 15, 20, 25 and 30  $\mu\text{g}$ ) added to the *G. gracilis* protoplast suspension during transfection. The volume of plasmid added to the protoplast suspension was kept constant (15  $\mu\text{l}$ ), while the concentration was altered. A volume of 15  $\mu\text{l}$  of TE buffer (Appendix A.2.1) was added to the negative control samples during the transfection process.

In order to assess which concentration of PEG (10, 13 or 15%) was optimal for the transfection of *G. gracilis* protoplasts, the volume of PEG medium added to the protoplast suspension was varied. Negative control samples received TFM instead of PEG medium.

The effect of divalent cation ( $\text{Mg}^{2+}/\text{Ca}^{2+}$ ) concentration on *G. gracilis* protoplast transfection efficiency was assessed by varying the concentrations of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  to 10 and 15 mM in the transfection medium (TFM; Appendix A.2.10.21). Negative control protoplasts received no plasmid DNA.

The length of the PEG medium incubation step was varied (2, 5, 10, 15, 20 and 30 min), in order to assess its effect on transfection efficiency and *G. gracilis* protoplast survival. Negative control protoplasts received TFM and no PEG medium.

The effect of *G. gracilis* protoplast concentration ( $1 \times 10^6$  and  $2 \times 10^6$  cells  $\text{ml}^{-1}$ ) on transformation efficiency was assessed. The plasmid/protoplast ratio was maintained at the previously determined optimal level of 20  $\mu\text{g}$  plasmid DNA :  $1 \times 10^5$  protoplasts. Control protoplasts received no plasmid DNA.

#### **4.3.6 Testing the effects of targeted HR and MARs on transgene expression**

A suite of expression plasmids was designed and constructed to test whether the tobacco Rb7 MAR and 18S rDNA-targeted HR could increase EGFP levels in *G. gracilis*. Protoplasts were transfected with one of four plasmids, or were untransfected (negative control) and the relative fluorescence of protoplasts was monitored over a period of 9 days as a measure of EGFP expression.

##### **4.3.6.1 Construction of expression constructs for assessing the effects of HR and MARs on transgene expression**

Four different plasmids were constructed to test the effect of HR and MARs on transgene expression: pSV-egfp (Chapter 2.3.6.3), pSV-egfpMARs, p18S-SV-egfpMARs and p18S-SV-egfp. Diagrams of the cloning strategies used to construct these plasmids can be found in Appendix D.

##### Construction of pSV-egfpMARs

The plasmid pSV-egfp DNA was prepared using a large-scale plasmid isolation protocol (Chapter 2, Table 4.3). pSV-egfp was linearised with the restriction enzyme *EcoRI*, dephosphorylated and resolved on a 0.8% (w/v) TAE agarose gel before being gel purified (Appendix D Fig. D1a).

A 1062 bp fragment, containing the tobacco Rb7 matrix attachment region (MAR), was amplified (Appendix B.15.5) from pTRAc (Maclean *et al.*, 2007; Table 4.3) using the high fidelity *Pfu* Polymerase (Fermentas) and oligonucleotide primers MAReco-F and MAReco-R

(Table 4.4; Appendix D Fig. D1b). The PCR primers MAReco-F and MAReco-R were both designed to include *EcoRI* restriction sites. Additionally, MAReco-F was designed to include a *BclI* restriction site downstream and adjacent to the *EcoRI* restriction site. The *EcoRI* restriction enzyme recognition sequences were included in order to simplify cloning into the destination vector pSV-egfp. Similarly, the *BclI* restriction site was included for simplified sub-cloning into p18S-21 (Table 4.3). The amplified PCR product was subjected to restriction digestion with *EcoRI* and gel purified. The MAR-containing fragment was subsequently sub-cloned into linearised and dephosphorylated pSV-egfp. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures and screened for the presence of the 1038 bp MAR-containing inserts by *EcoRI* restriction enzyme analysis. Recombinant constructs containing the 1038 bp DNA fragment were identified and subjected to further restriction enzyme analysis with *NdeI* to verify the orientation of the inserted DNA. The recombinant construct containing the correct insert was designated pSV-egfpMAReco (Table 4.3; Appendix D Fig. D1c).

In order to construct pSV-egfpMARs, the vector pSV-egfpMAReco (Table 4.3) was digested with the restriction enzymes *PstI* and *XbaI* (Appendix D Fig. D2a). The resulting DNA fragments were resolved on a 0.8% (w/v) TAE agarose gel and the 5051 bp DNA fragment excised and gel purified.

A 1065 bp DNA fragment, containing the tobacco Rb7 matrix attachment region (MAR), was PCR amplified (Appendix B.15.6) from pTRAc (Maclean *et al.*, 2007) (Table 4.3) using the high fidelity *Pfu* Polymerase (Fermentas) and oligonucleotide primers MARxba-F and MARpst-R (Table 4.4; Appendix D Fig. D2b). The PCR primers MARxba-F and MARpst-R were designed to include *XbaI* and *PstI* restriction sites, respectively. Additionally, MARpst-R was designed to include a *BclI* restriction site upstream and adjacent to the *PstI* restriction site. The *XbaI* and *PstI* restriction enzyme recognition sequences were included in order to simplify cloning into the destination vector pSV-egfpMAReco. The *BclI* restriction site was included for simplified sub-cloning into p18S-21. The PCR product was subjected to restriction enzyme digestion with *XbaI* and *PstI* and gel purified. The MAR-containing DNA fragment was subsequently sub-cloned into the restricted pSV-egfpMAReco. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures and screened for the presence of the 1046 bp MAR-

containing insert by *Xba*I and *Pst*I restriction enzyme analysis. The recombinant construct containing the correct insert was designated pSV-egfpMARs (Table 4.3; Appendix D Fig. D2c).

**Table 4.4** Oligonucleotide primers used in this study.

Primer	Sequence (5' – 3') <sup>a</sup>	Reference
18S-F	GAGTGAATTGTACAACGAAACTGC	This study
18S-R	GATCTGAATAATCAGTTCATCTAGC	This study
MAReco-F	ATATCCATTGAATTCCA <b>TGATCA</b> TTCCGCC	This study
MAReco-R	CTTTGGACCGAATTCGCCCGCGCC	This study
MARxba-F	ATATCCAT <b>TCTAGA</b> GCAAGTCAATTCCGCC	This study
MARpst-R	GGTCTTTGGCTGCAG <b>TGATCA</b> CCGCGCC	This study
egfp-F	ATGGTGAGCAAGGGCGAGG	This study
egfp-R	CACGAACTCCAGCAGGACC	This study
pat-f	CCAGAAACCCACGTCATGCCAGTT	Smart (2011)
pat-r	CTACATCGAGACAAGCACGGTCAACTT	Smart (2011)
E18R	CTACGGAAACCTTGTTACGACTTCTCC	Iyer <i>et al.</i> (2005)
R18F	CCTGGTTGATCCTGCCAGTGG	Iyer <i>et al.</i> (2005)
egfp-2R	CCTCGCCCTTGCTCACCAT	This study
TSS-R	GGTGGCGACCGGTACCAGACC	This study

<sup>a</sup> Sequences appearing in underlined lettering (GAATTC) indicate the inclusion of an *Eco*RI restriction site, those in red lettering (**TGATCA**) indicate the inclusion of a *Bcl*I restriction site, those in blue lettering (**TCTAGA**) indicate the inclusion of a *Xba*I restriction site and those in doubly underlined lettering (CTGCAG) indicate the inclusion of a *Pst*I restriction site.

#### Construction of p18S-SV-egfpMARs

Plasmid pSV-egfp was digested with the restriction enzyme *Pvu*II, dephosphorylated (Appendix D Fig. D3a) and the resulting DNA fragments were resolved on a 0.8% (w/v) TAE agarose gel. The desired 2364 bp DNA fragment containing the  $\beta$ -lactamase (*bla*) gene encoding for ampicillin resistance and the origin of replication (*ori*) was gel purified.

Genomic DNA was isolated from *G. gracilis* samples using a modified method of Wattier *et al.* (2000) (Appendix B.9.1). A 1615 bp fragment of the 18S rRNA gene was PCR amplified (Appendix B.15.7) using the primers 18S-F and 18S-R (Table 4.4) and high fidelity *Pfu*

Polymerase (Fermentas) (Appendix D Fig. D3b). The amplified PCR product was resolved on a 0.8% (w/v) TAE agarose gel excised and gel purified. The 1615 bp *G. gracilis* 18S rDNA fragment was subsequently ligated to the 2364 bp fragment from pSV-egfp and the resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  capable of growth on ampicillin were screened for the desired recombinant construct by a plate pool screening method (Appendix B.14; B.15.8), followed by colony PCR (Appendix B.15.8). *E. coli* DH5 $\alpha$  identified as containing the desired recombinant construct by colony PCR were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and digested with *Xba*I and *Nde*I to screen for clones containing the desired insert in the correct orientation in relation to the vector. The recombinant construct with the correct insert was designated p18S-21 (Table 4.3; Appendix D Fig. D3c).

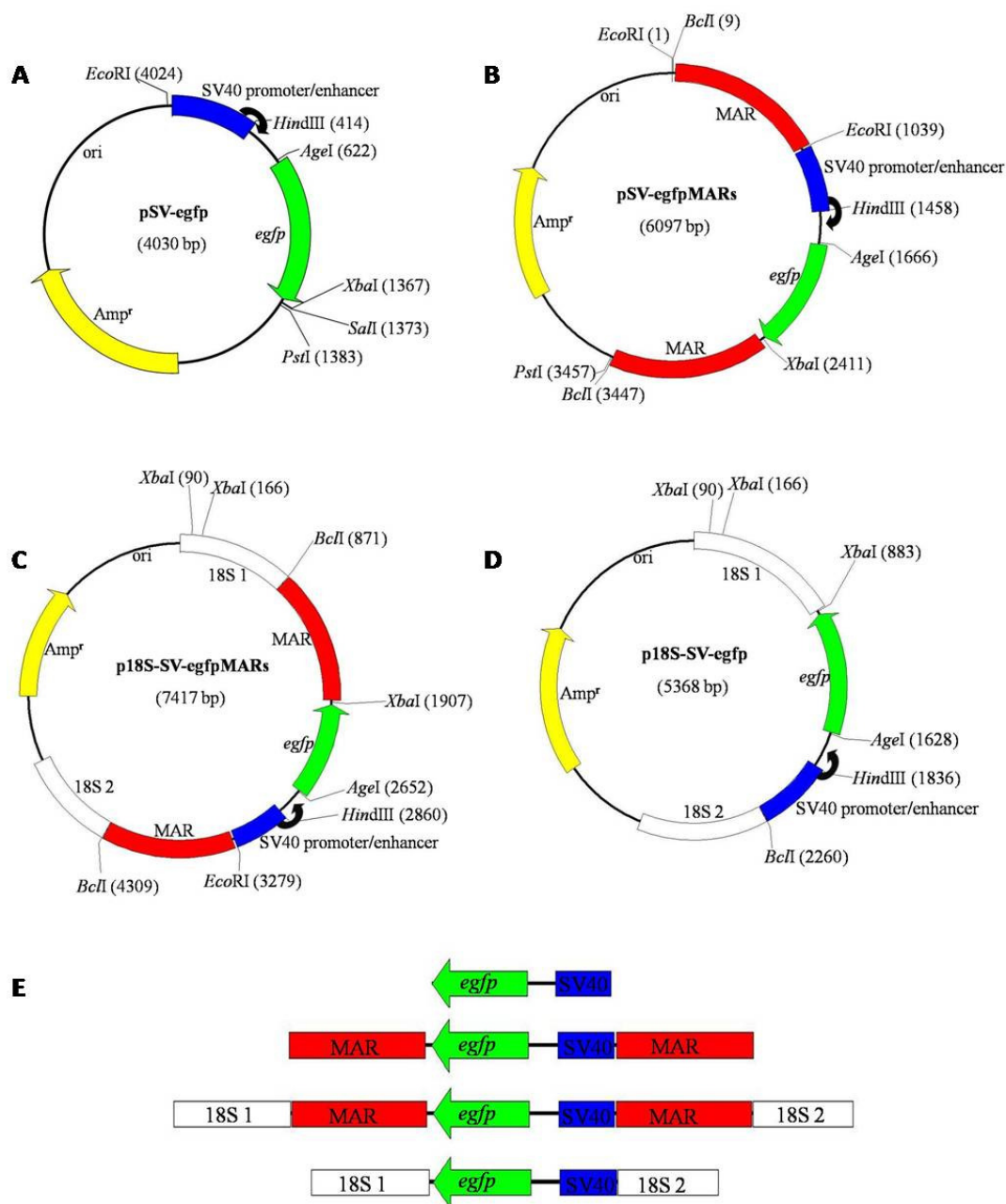
Plasmids pSV-egfpMARs and p18S-21 (Table 4.3) were transformed separately into *dam/dcm* competent *E. coli* K12 (Table 4.2; New England Biolabs). It was necessary to transform the constructs into a *dam*<sup>-</sup> strain of *E. coli*, since the restriction enzyme to be used for the next step of the cloning strategy, *Bcl*II, is sensitive to *dam*. Plasmid DNA was isolated from overnight cultures and both constructs were subsequently subjected to restriction enzyme digestion with *Bcl*II (Appendix D Fig. D4a-b). The resulting DNA fragments from the pSV-egfpMARs restriction digestion were resolved on 0.8% (w/v) TAE agarose gel before the 3438 bp fragment, containing the SV40 promoter/enhancer, *egfp* gene and MARs, was gel purified. The linearised p18S-21 vector was dephosphorylated (Appendix D Fig. D4b) prior to being resolved on 0.8% (w/v) TAE agarose gel and gel purified. The 3438 bp fragment from pSV-egfpMARs was ligated to the linearised, dephosphorylated p18S-21. The resulting constructs were transformed into competent *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  capable of growth on ampicillin were screened for the desired recombinant construct by colony PCR analysis using oligonucleotide primers egfp-F and egfp-R (Table 4.4; Appendix B.15.9). *E. coli* DH5 $\alpha$  that were shown to harbour the desired recombinant construct were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and screened by *Pvu*II restriction enzyme analysis to confirm the presence of the desired insert and its orientation in relation to the vector backbone. A recombinant construct displaying the correct restriction digestion pattern was identified and designated p18S-SV-egfpMARs (Table 4.3; Appendix D Fig. D4c).

### Construction of p18S-SV-egfp

Plasmid pSV-egfp was digested with the restriction enzymes *EcoRI* and *PstI*, blunt end-repaired (Appendix D Fig. D5a) and the resulting DNA fragments were resolved on a 1% (w/v) TAE agarose gel. The desired 1389 bp fragment containing the SV40 promoter and *egfp* gene was gel purified. The 1389 bp fragment was sub-cloned into p18S-21 that had been linearised with *BclI*, blunt end-repaired, dephosphorylated (Appendix D Fig. D5b) and gel purified. The resulting recombinant constructs were transformed into competent *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  capable of growth on ampicillin were screened for the desired recombinant construct by colony PCR analysis using oligonucleotide primers egfp-F and egfp-R (Table 4.4; Appendix B.15.9). *E. coli* DH5 $\alpha$  identified as harbouring the desired recombinant construct were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and screened by *PvuII* restriction enzyme analysis to determine both the presence of the desired insert and its orientation in relation to the vector backbone. A recombinant construct displaying the correct restriction digest pattern was identified and designated p18S-SV-egfp (Table 4.3; Appendix D Fig. D5c).

#### **4.3.6.2 Protoplast transfection**

*G. gracilis* thalli were pre-treated and protoplasts isolated and collected as described in 4.3.4. *G. gracilis* protoplasts were transfected using the optimized conditions determined in 4.3.5. Briefly, protoplasts ( $2 \times 10^5$  cells), resuspended in 100  $\mu$ l TFM (containing 15 mM Ca<sup>2+</sup>), were pipetted into a microfuge tube and 40  $\mu$ g of one of the four plasmids pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs or p18S-SV-egfp (Table 4.3, Fig. 4.1) or TE buffer (negative control) was added. The suspension was gently mixed before the drop-wise addition of PEG medium to a final concentration of 13%. The suspension was gently mixed, before being incubated for 2 min at RT. Three *G. gracilis* protoplast samples were transfected per treatment and sampling point. Following transfection, the protoplast samples were treated and cultured as described in 4.3.4, and each protoplast culture was considered a biological repeat.



**Figure 4.1** Expression constructs transfected into *G. gracilis* protoplasts for assessment of the effects of homologous recombination (HR) and MARs on transgene expression (A - D). The recombinant plasmids pSV-egfpMARS (B), p18S-SV-egfpMARS (C) and p18S-SV-egfp (D) are derivatives of the vector pSV-egfp (A). All plasmids contain the *egfp* reporter gene under the influence of the SV40 viral promoter (E). In addition, plasmids pSV-egfpMARS and p18S-SV-egfpMARS have Rb7 MARs flanking the reporter gene and promoter cassette (B, C, E). Plasmids p18S-SV-egfpMARS and p18S-SV-egfp have 5' and 3' portions of the *G. gracilis* 18S rRNA gene flanking the MARs or the promoter/reporter cassette, respectively (C, D, E). Relevant restriction sites are shown. Elements of plasmids are as follows: ➔, *egfp*; ➔, Amp<sup>r</sup>; ➔, indicates direction of transcription from the promoter; ■, SV40 promoter/enhancer; ■, Rb7 MAR; , 18S 1/2, where 1 and 2 represent the 5' and 3' portions of the gene, respectively.

#### 4.3.6.3 Sampling and assessment EGFP levels

EGFP was assessed 2, 3, 4, 5, 6 and 9 days post-transfection by measuring protoplast fluorescence, as described in 4.3.5. Fluorescence data was normalized where relevant, and analyzed using one-way ANOVAs. When the results of the ANOVA were significant, the Tukey Test was used to determine the significant differences due to various treatments using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at  $P < 0.05$ .

EGFP-expressing *G. gracilis* cells were also visually detected by fluorescence microscopy. Following sample processing, *G. gracilis* protoplasts from each treatment and time point were rapidly frozen in liquid nitrogen and stored for later DNA isolation and PCR analysis.

#### 4.3.6.4 PCR detection of *egfp* in transfected protoplasts

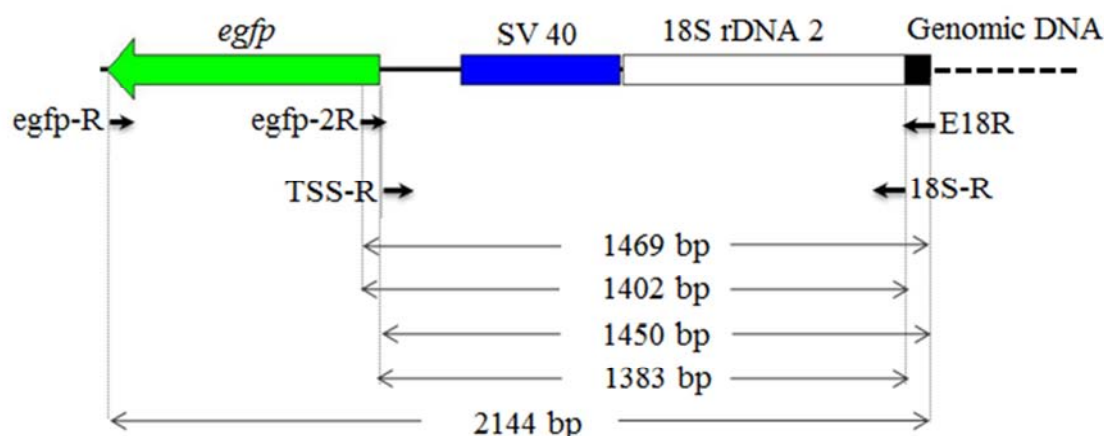
Total DNA was isolated from *G. gracilis* protoplasts transfected with plasmid DNA and from negative control samples at the time points described in 4.3.6.3, using a modified method of Wattier *et al.* (2000) (Appendix B.9.2). Oligonucleotide primers *egfp*-F and *egfp*-R (Table 4.4) were used to PCR amplify (Appendix B.15.10) a 675 bp DNA fragment of the *egfp* gene, while the primers 18S-F and 18S-R (Table 4.4) were used to PCR amplify (Appendix B.15.11) a 1615 bp DNA fragment of the *G. gracilis* 18S rRNA gene from both negative control and plasmid DNA transfected samples at each time point. The 18S rRNA control PCR confirmed the presence of intact and amplifiable *G. gracilis* genomic DNA in each of the samples. Amplified products for both sets of PCR primers were analysed by electrophoresis through a 1% (w/v) TAE agarose gel.

#### 4.3.6.5 Confirmation of chromosomal integration of foreign DNA

Genomic DNA extracted from *G. gracilis* protoplasts 9 days post-transfection was used as a template to amplify relevant sections of the plasmid that had integrated into the genomic DNA. This PCR amplification was used to confirm *G. gracilis* chromosomal integration of p18S-SV-*egfp*.

The oligonucleotide primers *egfp*-R and E18R (Table 4.4; Fig. 4.2) were used in a first round of PCR amplification (Appendix B.15.12) to amplify a 2144 bp DNA product from total DNA extracted from *G. gracilis* protoplasts 9 days post-transfection with either

p18S-SV-egfp or pSV-egfp (HR negative control). A 2  $\mu$ l volume of amplification product from the first round of PCR amplification was then employed as the template in a second round of PCR amplification (Appendix B.15.12) employing the primers E18R and egfp-2R (Table 4.4; Fig. 4.2). The plasmid p18S-SV-egfp was employed as a plasmid negative control in the above PCR strategy, while the primer 18S-R (Table 4.4; Fig. 4.2) was substituted for E18R in the plasmid positive control employing p18S-SV-egfp as the template. E18R and R18F (Table 4.4) were used to PCR amplify (Appendix B.15.12) a 1745 bp DNA fragment of the *G. gracilis* 18S rRNA gene from both the HR negative control and the sample transfected with p18S-SV-egfp. The latter control confirmed the presence of intact and PCR amplifiable chromosomal DNA in each of the samples. Primers E18R and R18F are located outside the region of homology of the 18S rDNA included in p18S-SV-egfp. Amplified PCR products were analysed by electrophoresis through a 1% (w/v) TAE agarose gel.



**Figure 4.2** Schematic representation (not to scale) of PCR strategy employed to confirm HR in *G. gracilis* protoplasts following transfection with p18S-SV-egfp. Primer positions ( $\blacktriangleright$ ) and expected product sizes are indicated. Elements are as follows:  $\blacktriangleright$ , *egfp*;  $\blacksquare$ , SV40 promoter/enhancer;  $\square$ , 18S rDNA 2 (where 2 represents the 3' portion of the 18S rRNA gene also present in p18S-SV-egfp);  $\blacksquare$ , 3' portion of the 18S rRNA gene not present in p18S-SV-egfp; - - -, *G. gracilis* genomic DNA.

The 1469 bp product amplified from *G. gracilis* protoplast samples transfected with p18S-SV-egfp was gel purified and analysed further to confirm sequence identity. The amplified DNA fragment was sequenced. Sequencing reactions were performed using the Big Dye terminator v3.1 Cycle Sequencing kit according to manufacturer's instructions. Sequencing was done by MacroGen Inc. (1001 World Meridian Center 60-24, Gasan-dong

Geumchun-gu Seoul Korea 153-021). Sequence data was edited using CHROMAS version 2.01 software (Technelysium Pty Ltd., Australia) and analyzed using DNAMAN for windows version 4.13 (LynnonBiosoft, Canada) software.

The 1469 bp gel-purified DNA fragment was further amplified (Appendix B.15.12) using primers E18R and TSS-R or 18S-R and TSS-R (Table 4.4; Fig. 4.2). Amplified products were analysed by electrophoresis through a 1.2% (w/v) TAE agarose gel.

#### **4.3.7 Testing of *G. gracilis* protoplast sensitivity to antibiotics and BASTA<sup>®</sup>**

*G. gracilis* protoplasts isolated and purified as described in Chapter 3.3.4, were dispensed into 2 ml of filter sterilized natural seawater supplemented with penicillin G and amphotericin B, in 35 x 10 mm tissue culture dishes (Falcon) at a density of  $7.5 \times 10^4$  cells ml<sup>-1</sup>. Culture plates containing *G. gracilis* protoplasts were incubated at 14–15 °C in the dark for 2 days to allow protoplasts to settle. Protoplasts were then supplemented with either an antibiotic (chloramphenicol or kanamycin) or the herbicide BASTA<sup>®</sup>. Following supplementation, protoplasts were cultured at 14–15 °C under a light intensity of 10  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 1 week and thereafter, 30  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . A photoperiod of 16/8 hrs (day/night) was applied to the cultures. Half the culture volume was replaced with fresh culture medium every five days with the antibiotic or herbicide concentrations, respectively, being maintained.

Three biological repeats were conducted per concentration of antibiotic or herbicide tested. Control cultures of *G. gracilis* protoplasts receiving no selective agent supplementation were also included in all tests.

##### **4.3.7.1 Effect of chloramphenicol and kanamycin on protoplast survival**

*G. gracilis* protoplast cultures were supplemented either with 25, 50, 100, 200, 300, 400 or 500  $\mu\text{g ml}^{-1}$  chloramphenicol (Sigma) (Appendix A.2.2), or 10, 20, 40, 80, 100, 200, 300 or 500  $\mu\text{g ml}^{-1}$  kanamycin (Sigma) (Appendix A.2.2) 2 days after protoplast isolation (day 0). In addition, control protoplast cultures that did not receive any chloramphenicol or kanamycin supplementation were maintained under the same culture conditions as the test protoplast cultures. In order to assess whether the ethanol in which the chloramphenicol was

dissolved had any effect on protoplast survival, control protoplast cultures were supplemented with ethanol (etOH).

*G. gracilis* protoplasts were monitored and counted 7 days after antibiotic supplementation by selecting 10 random fields of view under a microscope and counting the number of protoplasts visible within each particular field of view. In each case protoplast survival was calculated as a percentage of the untreated controls.

#### **4.3.7.2 Effect of BASTA<sup>®</sup> on protoplast survival**

*G. gracilis* protoplast cultures were supplemented with BASTA<sup>®</sup> (Bayer CropScience) (Appendix A.2.10.24) at concentrations of 0.5, 1, 2, 3, 4, 5 and 10  $\mu\text{g ml}^{-1}$  after 2 days of settling (day 0) and again three days later (day 3). Control protoplast cultures did not receive BASTA<sup>®</sup>.

Protoplast survival was monitored over a period of 7 days and counts were done at 2 and 7 days following BASTA<sup>®</sup> supplementation, as described in 4.3.7.1. Protoplast survival was determined as described in 4.3.7.1.

#### **4.3.8 BASTA<sup>®</sup> selection of transfected *G. gracilis* protoplasts**

Since *G. gracilis* protoplasts were shown to be sensitive to BASTA<sup>®</sup> the possibility of employing this herbicide as a selective agent was tested. Plasmids containing the *bar* gene, which confers resistance to the herbicide, under the influence of the SV40 promoter/enhancer, were constructed and transfected into protoplasts. Transfected protoplasts were then assessed for their ability to survive BASTA<sup>®</sup> selection.

##### **4.3.8.1 Construction of expression constructs for assessing the use of BASTA<sup>®</sup> as a selective agent in transfected protoplasts**

Two different plasmids were constructed to test whether BASTA<sup>®</sup> could be used as a selective agent for transformed *G. gracilis* protoplasts: pSV-bar/egfp and p18S-SV-bar/egfpMARs. Diagrams of the cloning strategies used to construct these plasmids can be found in Appendix D.

### Construction of pSV-bar/egfp

Large-scale plasmid isolation was performed in order to isolate the plasmids pBluescript KS (Table 4.3; Stratagene) and pEarleyGate201 $\Delta$ *ccdB* (Table 4.3; Earley *et al.*, 2006; Smart, 2011). The plasmid pBluescript KS was linearised with the restriction enzyme *EcoRV* (Fermentas), dephosphorylated (Appendix D Fig. D6b) and resolved on a 0.8% (w/v) TAE agarose gel before being gel purified.

The plasmid pEarleyGate201 $\Delta$ *ccdB* was digested with the restriction enzymes *SacI* and *ClaI* (Fermentas) and blunt end-repaired. The resulting DNA fragments were resolved on a 1% (w/v) TAE agarose gel. The desired 823 bp fragment (Appendix D Fig. D6a), containing the *bar* gene, was gel purified and sub-cloned into the linearised pBluescript KS. The resulting recombinant constructs were transformed into competent *E. coli* DH5 $\alpha$ , plated on LA containing 100  $\mu$ g ml<sup>-1</sup> ampicillin and 40  $\mu$ g ml<sup>-1</sup> X-gal (Appendix A.2.1), and incubated overnight at 37 °C. *E. coli* DH5 $\alpha$  capable of growth on ampicillin and showing a white colour were screened for the desired recombinant construct by colony PCR analysis, using oligonucleotide primers pat-f and pat-r (Table 4.4; Appendix B.15.13). *E. coli* DH5 $\alpha$  harbouring the desired recombinant construct were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and screened for both the presence of the desired insert and its orientation in relation to the vector backbone, by *PstI* (Fermentas) restriction enzyme analysis. A recombinant construct displaying the correct restriction digest pattern was identified and designated pKS-bar (Table 4.3; Appendix D Fig. D6c).

Plasmid pSV-egfp was digested with the restriction enzymes *HindIII* and *XbaI* (Fermentas) (Appendix D Fig. D7a) and the resulting DNA fragments were resolved on a 1% (w/v) TAE agarose gel. The desired 3077 bp fragment containing the  $\beta$ -lactamase (*bla*) gene, encoding ampicillin resistance, the origin of replication (*ori*) and the SV40 promoter/enhancer region, was gel purified.

Plasmid pKS-bar was digested with the restriction enzymes *HindIII* and *XbaI* (Appendix D Fig. D7b), resolved on a 1% (w/v) TAE agarose gel and the 871 bp fragment containing the *bar* gene was gel purified. The 871 bp *bar* fragment was sub-cloned into the 3077 bp pSV-egfp fragment and the resulting construct was transformed into competent *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  capable of growth on ampicillin were screened for the desired recombinant

construct by a plate pool screening method (Appendix B.14; B.15.13), followed by colony PCR using oligonucleotide primers pat-f and pat-r (Table 4.4; Appendix B.15.13). *E. coli* DH5 $\alpha$  harbouring the desired recombinant construct were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and screened by *Eco*RI restriction enzyme analysis. A recombinant construct displaying the correct restriction digest pattern was identified and designated pSV-bar (Table 4.3; Appendix D Fig. D7c).

#### Construction of p18S-SV-bar/egfpMARs

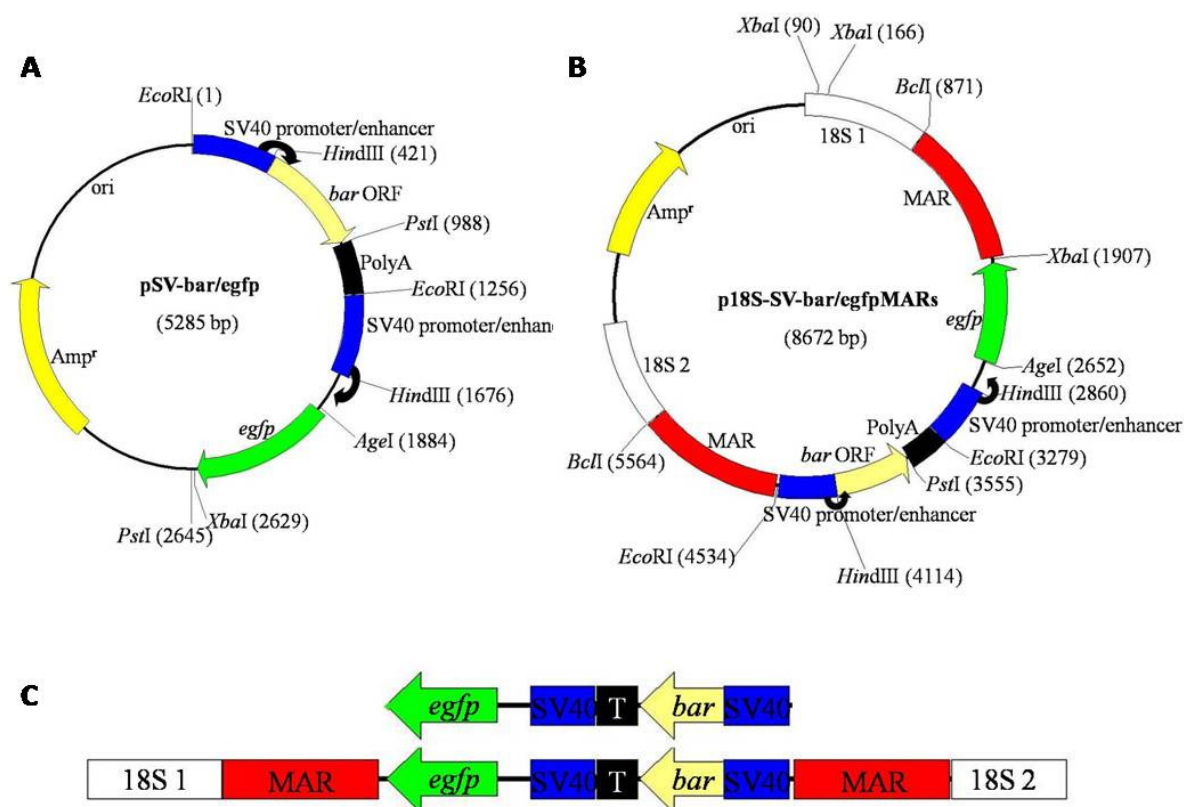
Plasmid pSV-bar was digested with the restriction enzyme *Eco*RI (Appendix D Fig. D8a and Fig. D9a), resolved on a 1% (w/v) TAE agarose gel and the 1255 bp fragment containing the SV40 promoter/enhancer and *bar* gene was gel purified.

The pSV-egfp and p18S-SV-egfpMARs were linearised with the restriction enzyme *Eco*RI, dephosphorylated (Appendix D Fig. D8b and Fig. D9b respectively) and resolved on a 0.8% (w/v) TAE agarose gel before being gel purified. The 1255 bp fragment from pSV-bar was sub-cloned into linearised pSV-egfp and p18S-SV-egfpMARs. Resulting constructs for each of the ligation reactions were transformed into competent *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  capable of growth on ampicillin were screened for the presence of the *bar* gene by a plate pool screening method (Appendix B.14; B.15.13), followed by colony PCR using oligonucleotide primers pat-f and pat-r (Table 4.4; Appendix B.15.13). *E. coli* DH5 $\alpha$  harbouring the desired recombinant constructs were inoculated for overnight culture. Plasmid DNA was isolated from the overnight cultures and screened by *Hind*III restriction enzyme analysis. Recombinant constructs displaying the expected restriction digest pattern were identified and designated pSV-bar/egfp (Table 4.2; Appendix D Fig. D8c) and p18S-SV-bar/egfpMARs (Table 4.2; Appendix D Fig. D9c), respectively.

#### **4.3.8.2 Transfection of p18S-SV-bar/egfp and pSV-bar/egfp into *G. gracilis* protoplasts**

*G. gracilis* thalli were pre-treated and, protoplasts isolated and collected as described in 4.3.4. Protoplasts were transfected under the optimal conditions determined in 4.3.5 with either pSV-bar/egfp (Table 4.3, Fig. 4.3), p18S-SV-bar/egfpMARs (Table 4.3, Fig. 4.3) or without any plasmid DNA (negative control). Six *G. gracilis* protoplast samples were transfected per treatment. Following transfection, protoplast samples were treated and cultured as described

in 4.3.4. Three protoplast cultures from each of the treatment groups were supplemented either with  $5 \mu\text{g ml}^{-1}$  BASTA<sup>®</sup> (final) as described in 4.3.7.2, or with no BASTA<sup>®</sup> (survival controls) 3 days post-transfection. *G. gracilis* protoplast cultures were cultured and treated further as described in 4.3.7.2. Once all the protoplasts in the negative control samples had died, BASTA<sup>®</sup> supplementation of the transfected protoplasts was stopped. This experiment was repeated a further two times on protoplast samples isolated from different batches of *G. gracilis* thalli.



**Figure 4.3** Plasmids employed in the transfection of *G. gracilis* protoplasts for the assessment of BASTA<sup>®</sup> as a selective agent (A & B). The recombinant plasmids pSV-egfp/bar (A) and p18S-SV-egfp/barMARS (B) are derivatives of the vector pSV-egfp. Both constructs contain the *egfp* reporter gene and *bar* gene under the influence of SV40 viral promoters (A-C), in addition, plasmid p18S-SV-bar/egfpMARS has Rb7 MARs flanking the gene and promoter cassette (B & C). Plasmid p18S-SV-bar/egfpMARS also has 5' and 3' portions of the *G. gracilis* 18S rRNA gene flanking the MARs (B & C). Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, *Amp*<sup>r</sup>; ➔, indicates direction of transcription from the promoter; ■, SV40 promoter/enhancer; ➔, *bar*; ■, PolyA; ■, Rb7 MAR; □, 18S 1/2, where 1 and 2 represent the 5' and 3' portions of the gene, respectively.

#### 4.3.8.3 Monitoring protoplast survival

Protoplast survival was monitored 2, 3 and 4 days post BASTA<sup>®</sup> supplementation, as described in 4.3.7.1. Protoplast survival was determined as a percentage of the untreated controls (survival controls) for each treatment. Survival data was natural log transformed and analyzed using one-way ANOVAs. When the results of the ANOVA were significant, the Tukey Test was used to determine the significant differences due to various treatments using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at  $P < 0.05$ .

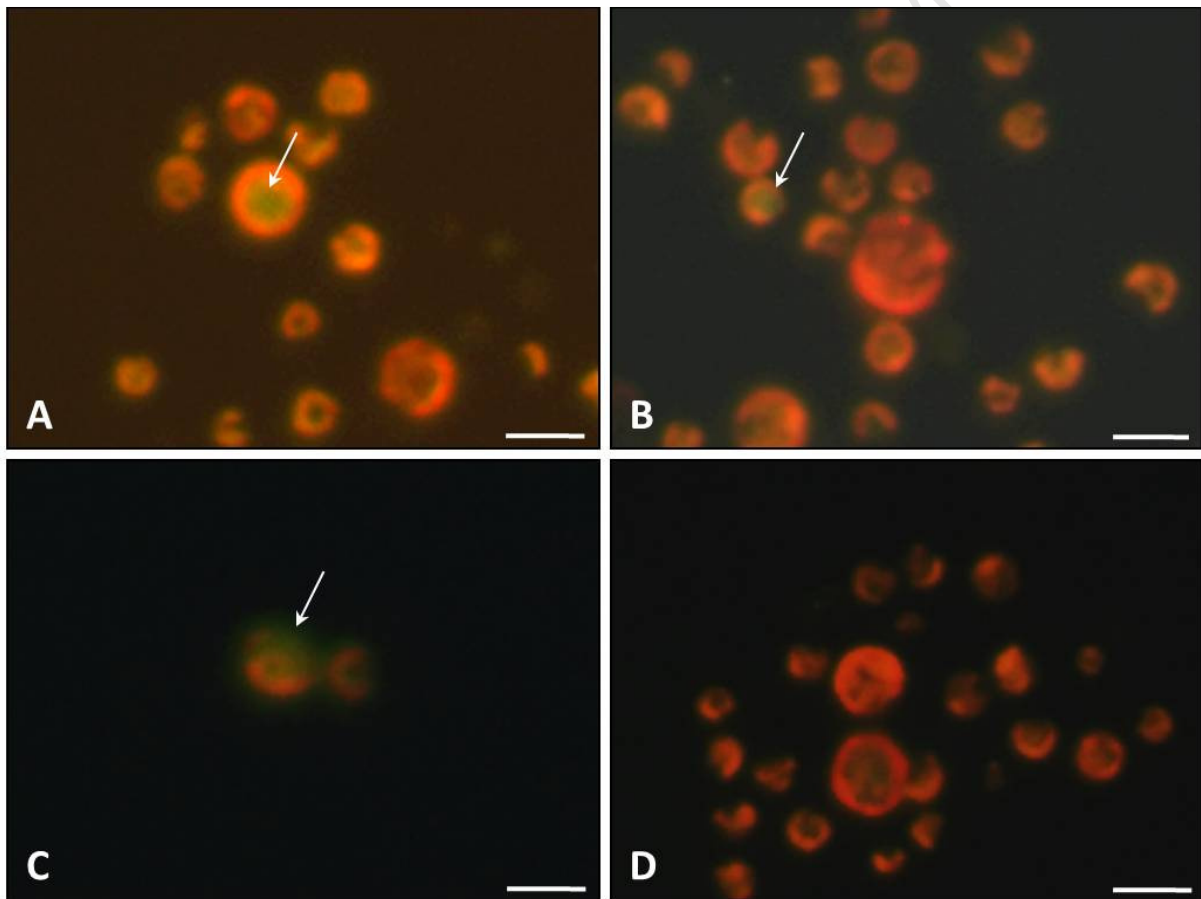
#### 4.3.8.4 PCR detection of *bar* in transfected protoplasts

Total DNA was isolated from *G. gracilis* protoplasts transfected with plasmid DNA and from the negative control samples using a modified method of Wattier *et al.* (2000) (Appendix B.9.2), 3 days post-transfection. Primers pat-f and pat-r (Table 4.4) were used to PCR amplify (Appendix B.15.14) a 412 bp fragment of the *bar* gene, while primers 18S-F and 18S-R (Table 4.4) were used to PCR amplify (Appendix B.15.11) a 1615 bp fragment of the *G. gracilis* 18S rRNA gene from both the negative control samples and the samples transfected with plasmid DNA. The latter control confirmed the presence of intact and PCR amplifiable chromosomal DNA in each of the *G. gracilis* samples. Amplified products for both sets of primers were analysed by electrophoresis through either a 1.5 or 1% (w/v) TAE agarose gel, respectively.

## 4.4 Results

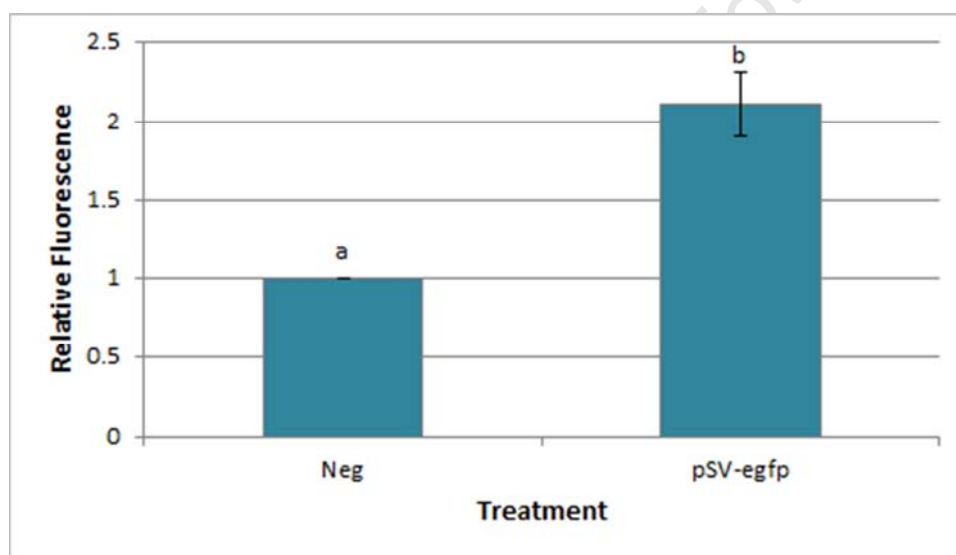
### 4.4.1 Transfection of *G. gracilis* protoplasts

The protoplast transfection method modified from those of Kuang *et al.* (1998), Zelazny *et al.* (2007) and Kim *et al.* (2002) proved successful for transforming *G. gracilis* protoplasts. Protoplasts transfected with pSV-egfp displayed visible green fluorescence 2 days post-transfection (Fig. 4.4A-C), while negative control (untransfected) protoplasts showed no green fluorescence (Fig.4.4D). Entire protoplasts did not appear green. Instead EGFP fluorescence could be observed within the protoplasts along with the natural orange-red fluorescence of the chloroplasts (Fig. 4.4A-C).



**Figure 4.4** Protoplasts of *G. gracilis* showing EGFP expression (→, arrow) (A-C) 2 days post-transfection with pSV-egfp. Negative control protoplasts show no green fluorescence (D). Images were taken with a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 510 nm emission filter. Scale bar = 15  $\mu$ m.

Although EGFP was visible in protoplasts following transfection with pSV-egfp, an alternative method for measuring EGFP expression was also assessed. A fluorometer was employed to quantify protoplast fluorescence which could then be normalized to cell number and, in so doing, provide an accurate measure of fluorescence in each sample. Plasmid-transfected *G. gracilis* protoplasts showed significantly ( $P < 0.05$ ) higher fluorescence in comparison to untransfected protoplasts (Fig. 4.5), indicating that relative fluorescence of protoplast samples could be used to determine EGFP expression. This approach enabled simpler sample processing and analysis compared to counting samples under a fluorescent microscope. Therefore it was employed for the further quantitative determinations of EGFP expression within transfected *G. gracilis* protoplasts. However, EGFP expression was also always visually confirmed in protoplast samples through microscopy.



**Figure 4.5** Relative fluorescence of *G. gracilis* protoplasts 2 days post-transfection with no plasmid DNA (Neg, negative control) or pSV-egfp. Data represents the mean  $\pm$  standard error. Different postscripts indicate a significant difference ( $P < 0.05$ ; Student *t*-test) between sample means.

#### 4.4.2 Optimization of parameters affecting *G. gracilis* protoplast transfection efficiency

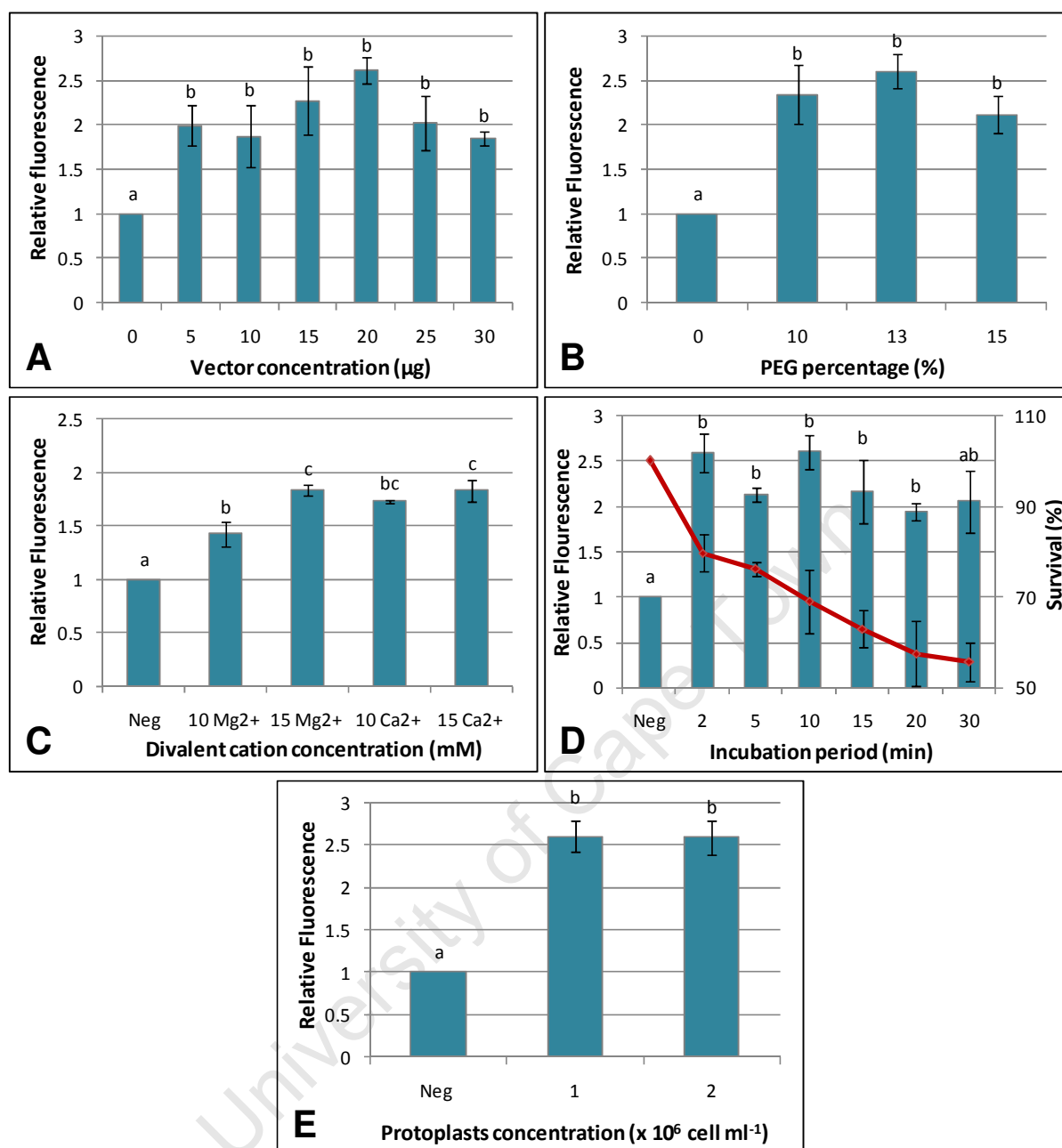
The modified method originally employed to transfect protoplasts isolated from *G. gracilis* thalli was successful, resulting in transfection efficiencies of approximately 10 in  $10^5$  protoplasts. However, previous studies pertaining to PEG-mediated protoplast transfection from both macroalgae and higher plant species show that transfection efficiencies can be increased through the optimization of transfection parameters (Maas and Werr, 1989;

Nicolaisen and Poulsen, 1993; Kuang *et al.*, 1998). These factors include the vector concentration, PEG concentration, transfection buffer constituents, transfection period and protoplast concentration (Kuang *et al.*, 1998). We tested different conditions affecting these parameters to develop optimal conditions for *G. gracilis* protoplast transfection using pSV-egfp as the test DNA.

Transfection of *G. gracilis* protoplasts with pSV-egfp at concentrations varying from 5 to 30  $\mu\text{g}$  resulted in relative fluorescence values which ranged between 1.9 and 2.6 and were significantly ( $P < 0.05$ ) higher when compared to the negative control protoplasts (Fig. 4.6A). Although there was no significant difference ( $P > 0.05$ ) between protoplast samples transfected with the various amounts of plasmid DNA, there was a general trend of increased fluorescence with increasing concentrations of plasmid DNA (5-20  $\mu\text{g}$ ) (Fig. 4.6A). Maximum fluorescence was observed when protoplasts were transfected with 20  $\mu\text{g}$  of pSV-egfp (Fig. 4.6A). Relative fluorescence decreased when either 25 or 30  $\mu\text{g}$  of plasmid was used for transfection (Fig. 4.6A).

Transfection with pSV-egfp in the presence of PEG (10-15% (w/v)) resulted in significant ( $P < 0.05$ ) increases in relative fluorescence of protoplasts when compared to the negative control samples which received no PEG treatment (Fig. 4.6B). Although there was no significant difference ( $P > 0.05$ ) between samples transfected in the presence of different concentrations of PEG, maximum fluorescence was observed when protoplasts were transfected with 13% (w/v) PEG (Fig. 4.6B).

The effect of divalent cations ( $\text{Mg}^{2+}/\text{Ca}^{2+}$ ) on transfection efficiency was assessed by varying their concentrations in the transfection medium (TFM). Transfection was successful in the presence of both cations at concentrations of either 10 or 15 mM with all exhibiting a significant ( $P < 0.05$ ) increase in relative fluorescence when compared to negative control samples (Fig. 4.6C). A concentration of 15 mM of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  proved optimal for transfection.



**Figure 4.6** Effects of varying plasmid concentrations (A), PEG concentrations (B), divalent cation concentrations in the transfection medium (TFM) (C), PEG incubation periods (D) and protoplast concentrations (E) on the relative fluorescence (■, A-E) and viability (—, D) of *G. gracilis* protoplasts 2 days post-transfection with pSV-egfp. Data represents the mean  $\pm$  standard error. Different postscripts indicate a significant difference ( $P < 0.05$ ; one-way ANOVA) between sample means. Neg, negative control.

A transfection period from 2-20 mins resulted in significant ( $P < 0.05$ ) increases in relative fluorescence of *G. gracilis* protoplasts when compared to the negative control samples which received no PEG treatment (Fig. 4.6D). While there was no significant difference ( $P > 0.05$ ) between samples transfected for various periods of time, longer transfection periods appeared

to have a negative effect on protoplast survival (Fig. 4.6D). A transfection period of 20 and 30 min resulted in only  $57.5 \pm 7.2$  and  $55.6 \pm 4.4\%$  protoplast survival, respectively, while a transfection period of 2 and 5 min resulted in  $79.7 \pm 4.0$  and  $76.2 \pm 1.6\%$  protoplast survival, respectively (Fig. 4.6D). Therefore, a transfection period of 2 min was selected for all further *G. gracilis* protoplast transfections.

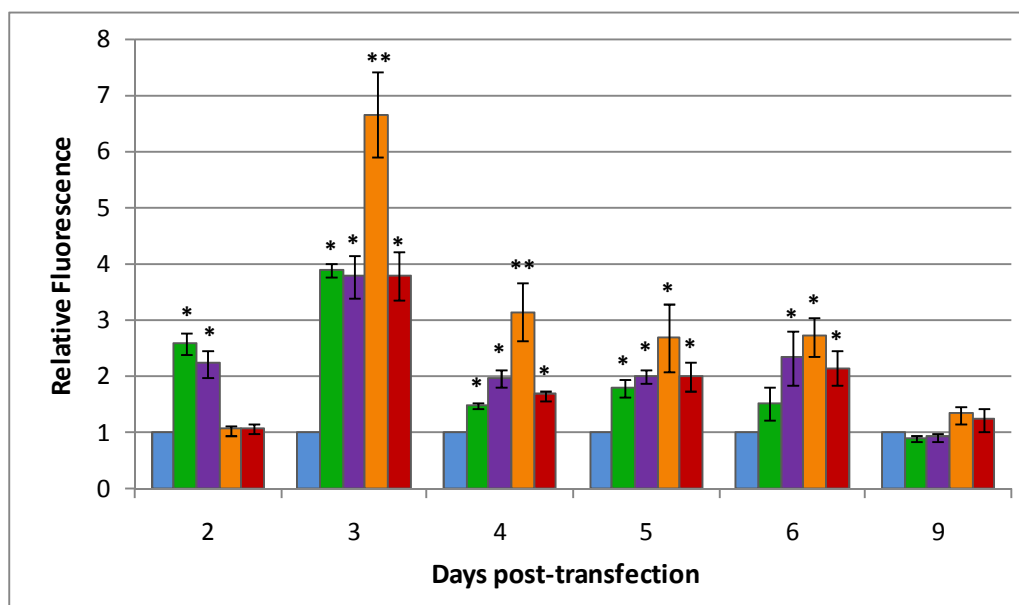
Varying the protoplast concentrations between 1 and  $2 \times 10^6$  cells  $\text{ml}^{-1}$  had no significant effect ( $P > 0.05$ ) on relative fluorescence (Fig. 4.6E). However, increased protoplast concentration allowed more sample material to be available for downstream processing. Furthermore, a protoplast concentration of  $2 \times 10^6$  cells  $\text{ml}^{-1}$  also translated to a plating concentration of  $7-8 \times 10^4$  cells  $\text{ml}^{-1}$  which was determined to be the optimal seeding density in Chapter 3.4.4.

Optimization of the various critical parameters for PEG-mediated transfection of *G. gracilis* protoplasts resulted in increased transformation efficiencies of approximately 89 in  $10^5$  protoplasts.

#### 4.4.3 Effect of HR and MARs on transgene expression

The occurrence of 18S rDNA-targeted HR and the presence of MARs on transfected DNA have been shown to improve transgene expression in *Porphyra* (Liu *et al.*, 2003; Zuo *et al.*, 2007). Therefore, four constructs were designed and constructed to test whether the tobacco Rb7 MARs and *G. gracilis* 18S rDNA-targeted HR could have a positive effect on EGFP expression in transfected *G. gracilis* protoplasts. Constructs were designed in such a way that Rb7 MARs flanked the SV40 promoter and *egfp*, and so would be able to exert their influence on EGFP expression. Furthermore, the expression plasmid p18S-SV-*egfp*MARs was specifically designed with regions of the *G. gracilis* 18S rDNA flanking the Rb7 MARs, so that if targeted HR took place, the Rb7 MARs would still be flanking the promoter and reporter gene. Protoplasts were transfected with one of the four expression plasmids and EGFP levels were monitored 2, 3, 4, 5, 6 and 9 days post-transfection in the cells through fluorescence readings and compared to untransfected samples (negative control samples). The results are represented graphically in Fig. 4.7 and summarized in Table 4.5.

There was a significant difference ( $P<0.01$ ) in the relative fluorescence of *G. gracilis* protoplasts transfected with either pSV-egfp or pSV-egfpMARs, when compared to the negative control and protoplasts transfected with either 18S-pSV-egfpMARs or 18S-pSV-egfp 2 days post-transfection (Fig. 4.7). The presence of pSV-egfp and pSV-egfpMARs resulted in a  $2.59 \pm 0.21$  and  $2.22 \pm 0.24$  fold change in relative fluorescence 2 days post-transfection, respectively (Table 4.5). *G. gracilis* protoplasts transfected with either 18S-pSV-egfpMARs or 18S-pSV-egfp showed no statistical difference ( $P>0.05$ ) when compared to the control protoplasts.



**Figure 4.7** The effect of HR and MARs on EGFP expression, measured as relative fluorescence of *G. gracilis* protoplasts, over a period of 9 days. Data represents the mean  $\pm$  standard error. All values are represented as fold change relative to the negative control sample. Different postscripts (\*, \*\*) indicate a significant difference ( $P<0.05$ ) between sample means within time points (one-way ANOVA). ■, Negative control samples; protoplasts transfected with: ■, pSV-egfp; ■, pSV-egfpMARs; ■, p18S-SV-egfpMARs; and ■, p18S-SV-egfp.

Three days post-transfection, *G. gracilis* protoplasts transfected with plasmid DNA (pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs or p18S-SV-egfp) displayed significantly ( $P<0.01$ ) higher relative fluorescence when compared to the control sample (Fig. 4.7, Table 4.5). The presence of all of the constructs resulted in EGFP expression (Fig. 4.8) 3 days post-transfection. There was no significant difference between transfection efficiencies of protoplast samples transfected with the different constructs. There was no significant

difference ( $P>0.05$ ) in relative fluorescence between *G. gracilis* protoplasts transfected with either pSV-egfp, pSV-egfpMARs or p18S-SV-egfp. Protoplasts transfected with p18sSV-egfpMARs however, exhibited the highest ( $P<0.01$ ) level of relative fluorescence (Fig. 4.7). The presence of pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs and p18S-SV-egfp resulted in a  $3.88 \pm 0.13$ ,  $3.78 \pm 0.38$ ,  $6.65 \pm 0.75$  and  $3.78 \pm 0.43$  fold change in relative fluorescence 3 days post-transfection, respectively (Table 4.5).

**Table 4.5** Effect of HR regions and MARs on EGFP expression in *G. gracilis* protoplasts.

Days post-transfection <sup>†</sup>	Relative fluorescence (fold change) <sup>‡</sup>				<i>P</i>
	pSV-egfp	pSV-egfpMARs	p18S-SV-egfpMARs	p18S-SV-egfp	
2	$2.59 \pm 0.21^b$	$2.22 \pm 0.24^b$	$1.05 \pm 0.08^a$	$1.06 \pm 0.09^a$	**
3	$3.88 \pm 0.13^b$	$3.78 \pm 0.38^b$	$6.65 \pm 0.75^c$	$3.78 \pm 0.43^b$	**
4	$1.50 \pm 0.05^b$	$1.97 \pm 0.14^b$	$3.15 \pm 0.52^c$	$1.68 \pm 0.08^b$	*
5	$1.81 \pm 0.16^b$	$2.00 \pm 0.11^b$	$2.70 \pm 0.60^b$	$2.01 \pm 0.26^b$	*
6	$1.53 \pm 0.30^{ab}$	$2.33 \pm 0.47^b$	$2.71 \pm 0.35^b$	$2.15 \pm 0.31^b$	*
9	$0.91 \pm 0.05^a$	$0.92 \pm 0.07^a$	$1.33 \pm 0.15^a$	$1.23 \pm 0.21^a$	
Average	$2.04 \pm 0.43^b$	$2.21 \pm 0.38^b$	$2.93 \pm 0.82^b$	$1.99 \pm 0.40^b$	*

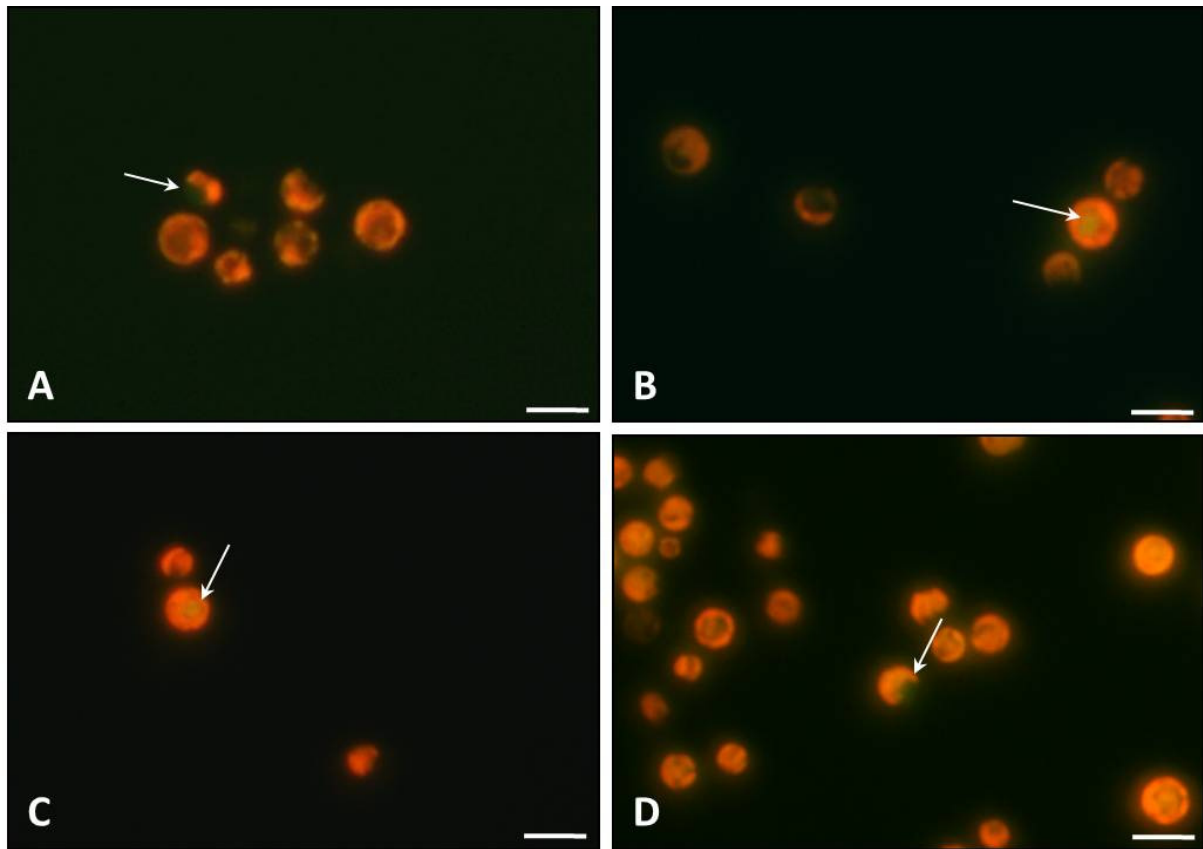
<sup>†</sup> Sampling time point given as days post-transfection.

<sup>‡</sup> Relative fluorescence represented as fold change relative to the negative sample. Values are means  $\pm$  SEM of three independent samples. Relative fluorescence with different superscripts are significantly different ( $P<0.05$  \*;  $P<0.01$  \*\*) (one-way ANOVA within time points). A superscript <sup>a</sup> indicates not different to the negative control sample (not shown in the table).

The pattern of relative fluorescence 4 days post-transfection was as described for day 3 samples, with p18S-SV-egfpMARs resulting in the highest relative fluorescence ( $P<0.05$ ) (Fig. 4.7, Table 4.5). The presence of pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs and p18S-SV-egfp resulted in a  $1.50 \pm 0.05$ ,  $1.97 \pm 0.14$ ,  $3.15 \pm 0.52$  and  $1.68 \pm 0.08$  fold change in expression 4 days post-transfection, respectively (Table 4.5).

Significantly higher ( $P<0.05$ ) levels of relative fluorescence were observed for plasmid-transfected samples 5 days post-transfection, when compared to the control sample (Fig. 4.7, Table 4.5). The presence of pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs and

p18S-SV-egfp resulted in a  $1.81 \pm 0.16$ ,  $2.00 \pm 0.11$ ,  $2.70 \pm 0.60$ ,  $2.01 \pm 0.26$  fold change in relative fluorescence 5 days post-transfection, respectively (Table 4.5).



**Figure 4.8** *G. gracilis* protoplasts showing EGFP expression ( $\rightarrow$ , arrow) 3 days post-transfection with pSV-egfp (A), pSV-egfpMARs (B), p18S-SV-egfpMARs (C) or p18S-SV-egfp (D). Images were taken with a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 510 nm emission filter. Scale bar = 15  $\mu$ m.

*G. gracilis* protoplasts transfected with either pSV-egfpMARs, p18S-SV-egfpMARs or p18S-SV-egfp and sampled 6 days later, showed significantly higher ( $P < 0.05$ ) relative fluorescence levels in comparison to the control sample protoplasts (Fig. 4.7). Transfection with pSV-egfpMARs, p18S-SV-egfpMARs and p18S-SV-egfp resulted in a  $2.33 \pm 0.47$ ,  $2.71 \pm 0.35$ ,  $2.15 \pm 0.31$  fold change in relative fluorescence of *G. gracilis* protoplasts, respectively (Table 4.5). A slightly higher proportion of EGFP-expressing cells (0.09-0.11%) were observed for p18S-SV-egfpMARs and p18S-SV-egfp transfected samples in comparison to pSV-egfpMARs transfected samples (0.05-0.07%). Samples transfected with pSV-egfp showed a  $1.53 \pm 0.30$  fold change in relative expression (Table 4.5). However, this did not

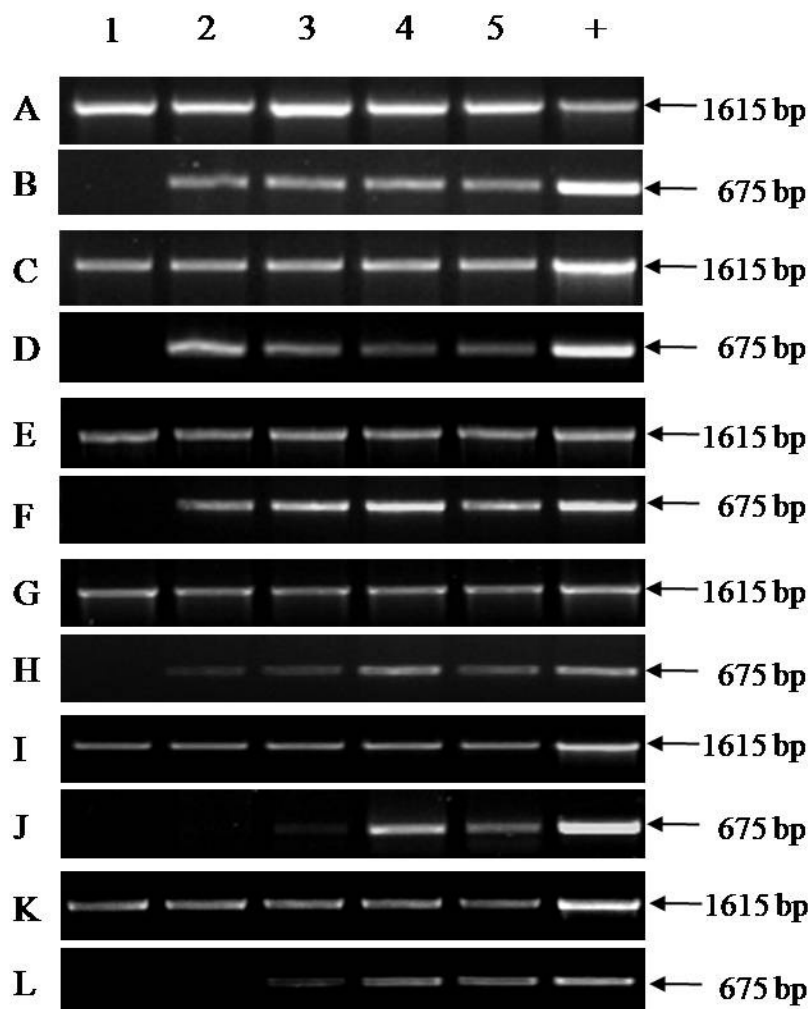
represent a significant difference ( $P>0.05$ ) in relative fluorescence when compared to the control sample (Fig. 4.7).

There was no significant difference ( $P>0.05$ ) between the relative fluorescence of the control protoplasts and the protoplasts transfected with any of the constructs 9 days post-transfection (Fig. 4.7, Table 4.5).

On average, pSV-egfp, pSV-egfpMARs, p18S-SV-egfpMARs and p18S-SV-egfp resulted in a  $2.04 \pm 0.43$ ,  $2.21 \pm 0.38$ ,  $2.93 \pm 0.82$  and  $1.99 \pm 0.40$  fold change in relative fluorescence over the 9 day sampling period, respectively, all of which are significant when compared to the control samples (Table 4.5). *G. gracilis* protoplasts exhibited the highest relative fluorescence ( $P<0.01$ ) 3 days post-transfection when transfected with any of the constructs.

PCR analysis of genomic DNA extracted from *G. gracilis* protoplasts sampled at different time points post-transfection (days 2, 3, 4, 5, 6 and 9) was conducted to confirm the presence of the *egfp* gene in the host cells (Fig. 4.9). A 675 bp *egfp* PCR product was amplified from DNA samples isolated from *G. gracilis* protoplasts 2-5 days post-transfection with each of the four expression constructs. The *egfp* PCR product was not amplified from any of the untransfected *G. gracilis* protoplast samples at any of the sampling time points. Furthermore, no *egfp* PCR product was obtained from protoplasts transfected with pSV-egfp at 6 and 9 days post-transfection. However, an *egfp* PCR product was amplified from protoplasts at 6 and 9 days post-transfection with the remaining three constructs. PCR amplification of a *G. gracilis* 18S rDNA PCR product from all the protoplast samples tested confirmed the presence of intact *G. gracilis* DNA.

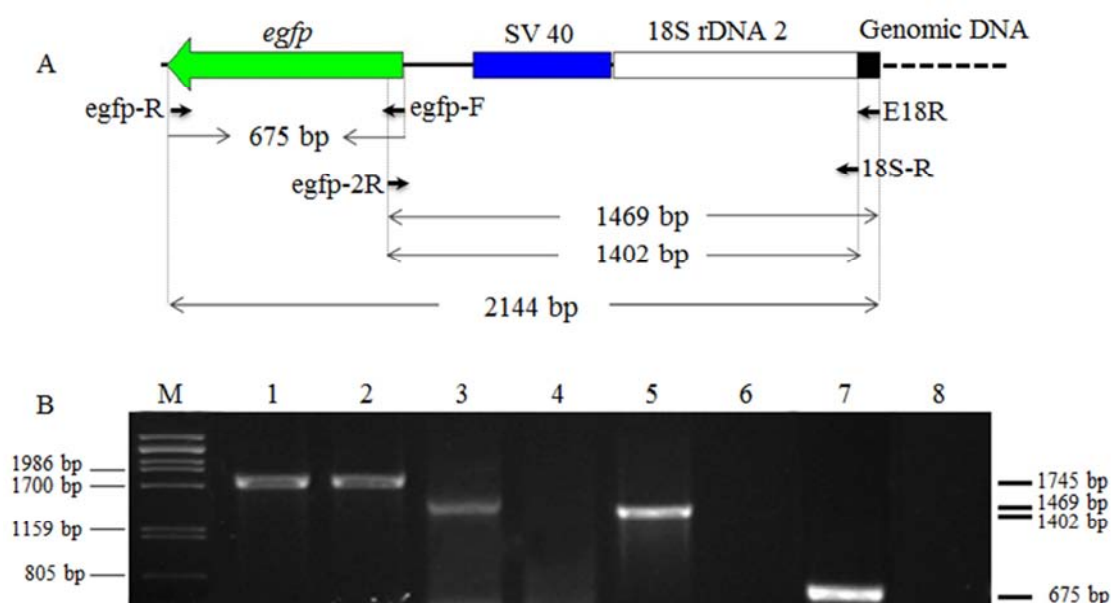
PCR analysis of genomic DNA extracted from protoplasts transfected with either p18S-SV-egfp or pSV-egfp (to serve as a negative control) 9 days post-transfection was carried out to confirm whether HR had taken place within the *G. gracilis* host cells (Fig. 4.10). The 9 days post-transfection samples were tested, as *G. gracilis* protoplasts transfected with pSV-egfp, lacking HR regions, no longer showed the presence of the transfected *egfp* gene while protoplasts transfected with p18S-SV-egfp still displayed the presence of the transfected *egfp* gene (Detected by PCR; 4.4.3). A similar PCR strategy to that described by Liu *et al.* (2003) was employed to detect HR that had taken place between the transfected plasmid DNA and the homologous 3'-portion of the *G. gracilis* 18S rRNA gene.



**Figure 4.9** PCR analysis of total DNA isolated from transfected *G. gracilis* protoplasts. Lanes 1-5: untransfected protoplasts (1), protoplasts transfected with pSV-*egfp* (2), pSV-*egfp*MARs (3), p18S-SV-*egfp*MARs (4), p18S-SV-*egfp* (5), respectively; +, positive control. Panels A, C, E, G, I and K: DNA fragments amplified with *G. gracilis* 18S rRNA gene specific primers for protoplasts sampled 2, 3, 4, 5, 6 and 9 days post-transfection, respectively. Panels B, D, F, H, J and L: DNA fragments amplified with *egfp* specific primers for protoplasts sampled 2, 3, 4, 5, 6 and 9 days post-transfection, respectively. The arrows indicate the approximate sizes of the amplified PCR products in base pairs (bp).

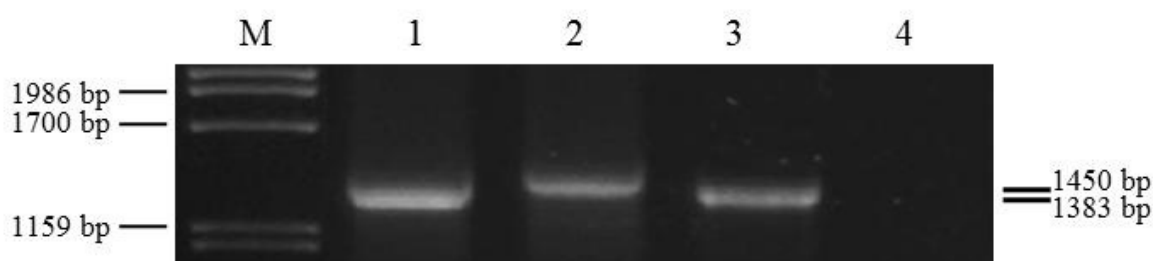
This strategy makes use of a pair of primers, one which is located outside the region of homology of the 18S rDNA included in p18S-SV-*egfp* (E18R) and, one which is located within the plasmid DNA and would not be present in the chromosomal DNA unless integration had taken place (*egfp*-R) (Fig. 4.10A). Thus, PCR products resulting from amplification of plasmid or chromosomal DNA with such a set of primers would not be visible on an agarose gel unless targeted HR had taken place. The plasmid negative control, employing a combination of the primers E18R and *egfp*-R/*egfp*-2R in consecutive rounds of

PCR, showed no PCR amplification product (lane 6), while the plasmid positive control, in which primer 18S-R was substituted for E18R, resulted in the amplification of a 1402 bp product (lane 5) (Fig. 4.10B). The expected 1469 bp PCR product (Fig. 4.10A) was amplified from *G. gracilis* protoplasts transfected with p18S-SV-egfp (lane 3), but not from protoplasts transfected with pSV-egfp (lane 4) (Fig. 4.10B). Therefore, there was a 67 bp size difference between the HR product and the PCR product amplified from the plasmid positive control. PCR amplification of a *G. gracilis* 18S rRNA PCR product confirmed the presence of intact *G. gracilis* DNA in all the samples tested (lanes 1&2; Fig. 4.10B).



**Figure 4.10** (A) Schematic representation of the PCR strategy employed to confirm HR in *G. gracilis* protoplasts. Primer positions (➡) and expected product sizes are indicated. Elements are represented as follows: ➡, *egfp*; ■, SV40 promoter/enhancer; □, 18S 2, where 2 represents the 3' portion of the 18S rRNA gene also present in p18S-SV-egfp; ■, 3' portion of the 18S rRNA gene not present in p18S-SV-egfp; - - -, *G. gracilis* genomic DNA. (B) PCR analysis of foreign DNA incorporation. Lanes 1 and 2: DNA fragments amplified with 18S rRNA gene specific primers (E18R and R18F) from protoplasts transfected with p18S-SV-egfp (1) or pSV-egfp (2), respectively; lanes 3 and 4: DNA fragments amplified with primer pairs designed to only amplify templates which have undergone HR in protoplasts transfected with p18S-SV-egfp (3) or pSV-egfp (4), respectively; lanes 5 and 6: Plasmid positive (5) and negative (6) control, respectively; lanes 7 and 8: DNA fragments amplified with *egfp* specific primers from protoplasts transfected with p18S-SV-egfp (7) or pSV-egfp (8), respectively; lane M: Marker,  $\lambda$ PstI MW marker with sizes indicated on the left. Sizes of the amplified products are indicated on the right.

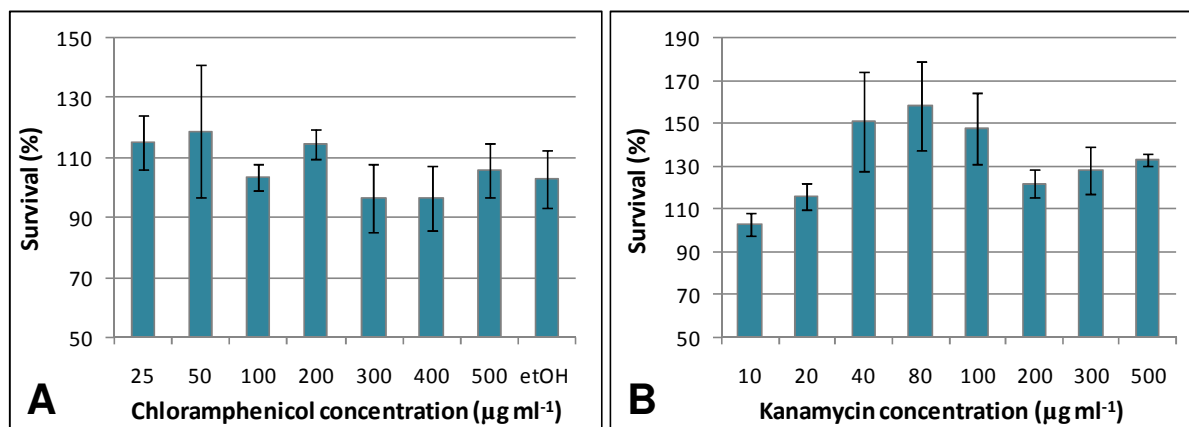
Sequencing and further PCR analysis using primers 18S-R and TSS-R (Fig. 4.11) confirmed the identity of the 1469 bp HR PCR product. When primer pair 18S-R and TSS-R were employed in PCR amplification, a 1383 bp product was amplified when using either p18S-SV-egfp (lane 3) or the 1469 bp HR PCR product (lane 1) as template (Fig. 4.11). However, when the primer pair E18R and TSS-R was used, a 1450 bp product was amplified only from the HR product (lane 2), and not from the plasmid control (lane 4) (Fig. 4.11). The size difference in the fragments resulting from the substitution of E18R for 18S-R was 67 bp. These results confirmed the identity of the 1469 bp HR PCR product and further suggested that HR had indeed taken place between the transfected plasmid DNA and the 3' homologous portion of the *G. gracilis* 18S rRNA gene.



**Figure 4.11** PCR confirmation of HR PCR product sequence identity. Lanes 1 and 2: DNA fragments amplified from HR PCR product with 18S-R and TSS-R (1) or E18R and TSS-R (2); lanes 3 and 4: Plasmid positive (3) and negative (4) control amplified from p18S-SV-egfp with 18S-R and TSS-R (3) or E18R and TSS-R (4), respectively; lane M:  $\lambda$ PstI MW marker with sizes indicated on the left. The sizes of the amplified products, in base pairs (bp), are indicated on the right hand side.

#### 4.4.4 *G. gracilis* protoplast sensitivity to antibiotics and BASTA®

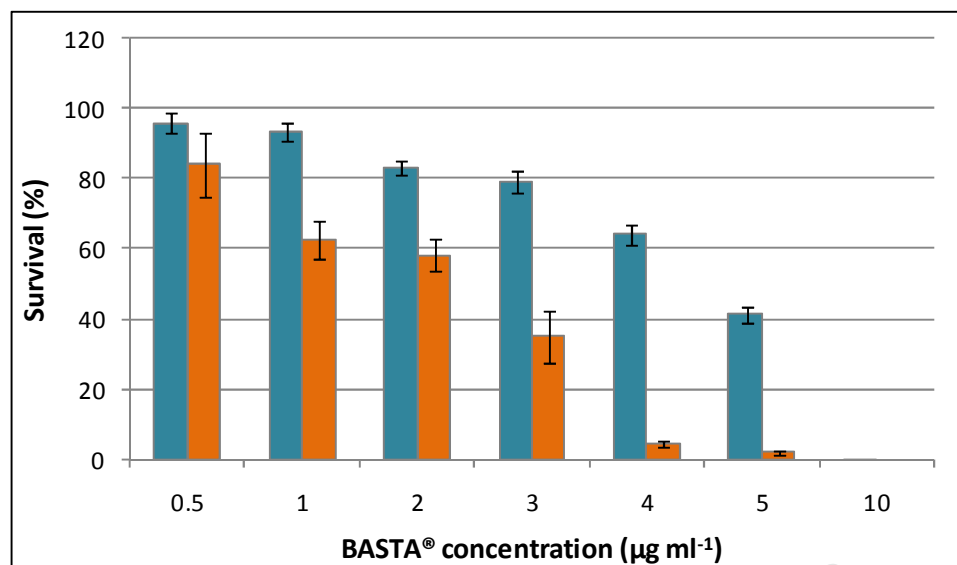
*G. gracilis* protoplasts were tested for their sensitivity to chloramphenicol and kanamycin in order to test whether these commonly employed selective agents would be applicable for use in *G. gracilis* protoplast selection. *G. gracilis* protoplasts however, demonstrated a very high level of resistance to both antibiotics (Fig. 4.12). Concentrations of up to 500  $\mu\text{g ml}^{-1}$  of chloramphenicol (Fig. 4.12A) and kanamycin (Fig. 4.12B) had no significant effect on protoplast viability over a one week period and whole plants could be regenerated in the presence of both antibiotics. Kanamycin supplementation of 20-500  $\mu\text{g ml}^{-1}$  even resulted in increased protoplast survival (Fig. 4.12B) and aided in inhibiting microalgal contamination in later stages of culture.



**Figure 4.12** Effect of chloramphenicol (A) and kanamycin (B) concentrations on the survival of *G. gracilis* protoplasts over a one week period. Data represents the mean  $\pm$  standard error.

Since *G. gracilis* protoplasts displayed no sensitivity to chloramphenicol or kanamycin, neither of these antibiotics would be suitable as a selective agent. Therefore, protoplasts were tested for their sensitivity to the herbicide BASTA<sup>®</sup> at concentrations ranging from 0.5-10  $\mu\text{g ml}^{-1}$  (Fig. 4.13). Protoplasts were supplemented with BASTA<sup>®</sup> two days after isolation (day 0) and again 3 days later. *G. gracilis* protoplast survival was determined 2 and 7 days following the initial BASTA<sup>®</sup> supplementation.

*G. gracilis* protoplasts were sensitive to BASTA<sup>®</sup> supplementation (Fig. 4.13). Protoplasts exhibited only  $41.3 \pm 2.2\%$  survival 2 days after supplementation with 5  $\mu\text{g ml}^{-1}$  BASTA<sup>®</sup> (Fig. 4.13). The addition of 10  $\mu\text{g ml}^{-1}$  BASTA<sup>®</sup> resulted in 0.06% protoplast survival 2 days after BASTA<sup>®</sup> supplementation (Fig. 4.13). The addition of 4 and 5  $\mu\text{g ml}^{-1}$  BASTA<sup>®</sup> limited protoplast survival to  $4.6 \pm 1.0\%$  and  $2.0 \pm 0.6\%$ , respectively, 7 days after BASTA<sup>®</sup> supplementation (Fig. 4.13). In addition, the surviving protoplasts appeared bleached and were no longer round in shape (data not shown). BASTA<sup>®</sup> concentrations of 0.5-3  $\mu\text{g ml}^{-1}$  resulted in less significant decreases in protoplast survival (Fig. 4.13).

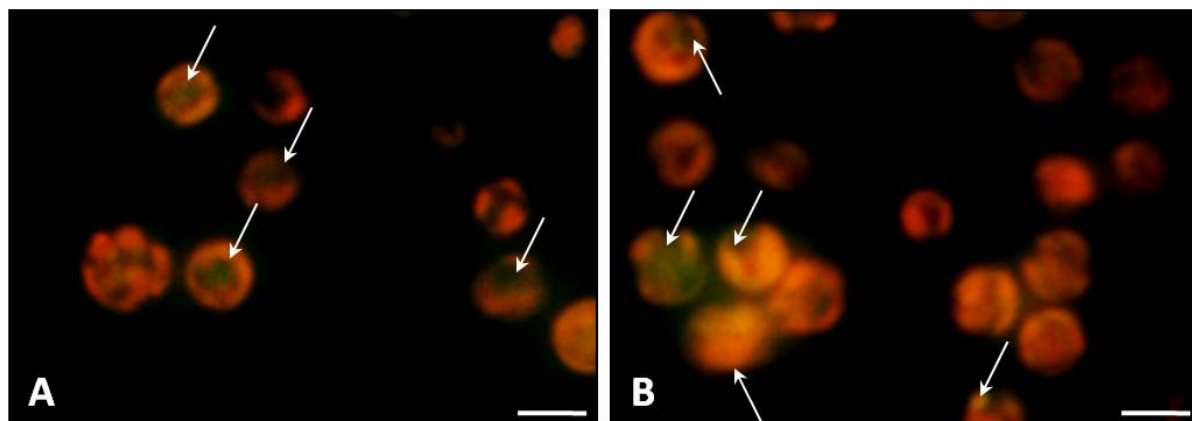


**Figure 4.13** Effect of different BASTA<sup>®</sup> concentrations on the survival of *G. gracilis* protoplasts 2 (■) and 7 (■) days after supplementation. Data represents the mean  $\pm$  standard error.

#### 4.4.5 BASTA<sup>®</sup> selection of transfected protoplasts

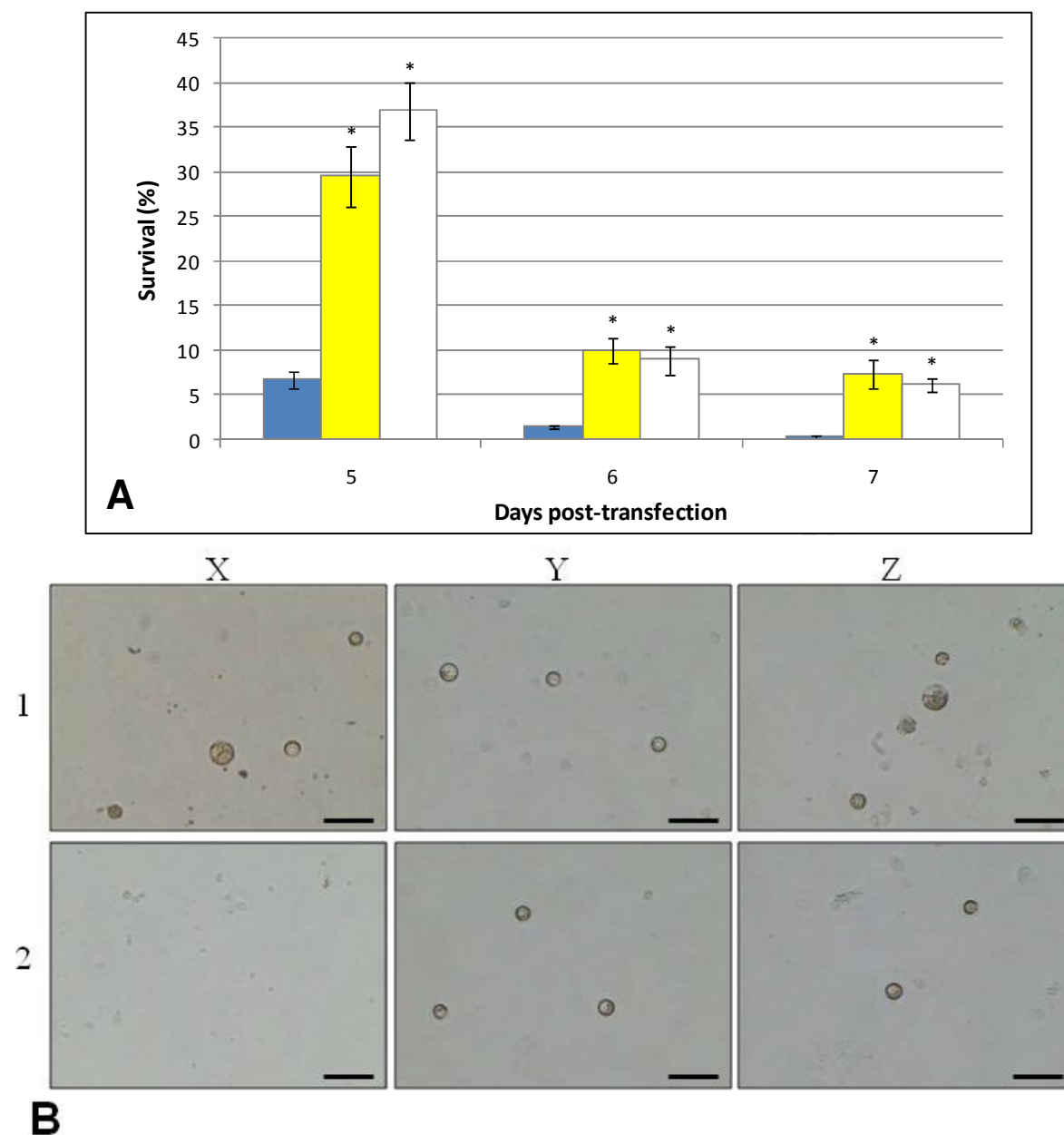
In order to assess whether the herbicide BASTA<sup>®</sup> could be employed as a possible selective agent for transformed *G. gracilis*, protoplasts were transfected with expression plasmids encoding the *bar* gene under the influence of the SV 40 promoter/enhancer. Transfected protoplasts were then supplemented with 5 µg ml<sup>-1</sup> BASTA<sup>®</sup> (a concentration already determined to be detrimental to protoplast survival (4.4.4)) 3 days post-transfection and their survival monitored and compared to untransfected *G. gracilis* protoplasts. The plasmid p18S-SV-*bar*/egfpMARS was constructed and employed in the transfections since 18S rDNA HR regions and MARS had been shown to result in increased transgene expression (4.4.3). Prior to BASTA<sup>®</sup> supplementation, *G. gracilis* protoplast cultures were examined 3 days post-transfection for visible EGFP expression in order to confirm that the plasmids, pSV-*bar*/egfp and p18S-SV-*bar*/egfpMARS, had been successfully introduced into the protoplast with similar efficiencies to facilitate EGFP production. Protoplasts were supplemented with BASTA<sup>®</sup> 3 days post-transfection since EGFP expression under the influence of the SV 40 promoter/enhancer had previously been shown to be maximal at this time point (4.4.3).

Protoplasts transfected with either pSV-bar/egfp or p18S-SV-bar/egfpMARs displayed green fluorescence 3 days post-transfection (Fig. 4.14A and B). Protoplasts appeared yellow in colour where EGFP expression and chloroplast fluorescence overlapped (Fig. 4.14A and B). There was no significant difference ( $P>0.05$ ) in transfection efficiencies between cultures transfected with pSV-bar/egfp or p18S-SV-bar/egfpMARs.



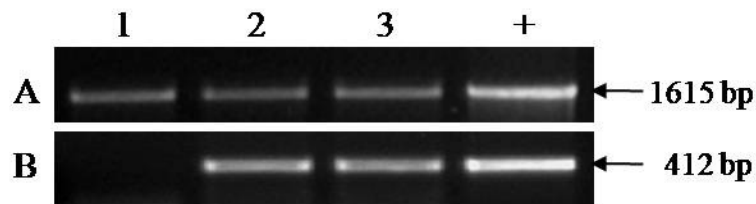
**Figure 4.14** *G. gracilis* protoplasts demonstrating EGFP expression (→, arrow) 3 days post-transfection with pSV-bar/egfp (A) or p18S-SV-bar/egfpMARs (B). Images were taken with a Nikon Diaphot-TMD inverted microscope (fitted with a Nikon epifluorescence attachment) equipped with a 510 nm emission filter. Scale bar = 10  $\mu$ m.

*G. gracilis* protoplasts transfected with either pSV-bar/egfp or p18S-SV-bar/egfpMARs exhibited significantly ( $P<0.01$ ) increased survival 5-7 days post-transfection (2-4 days post-BASTA<sup>®</sup> supplementation) in comparison to the negative control protoplasts (Fig. 4.15A). Protoplasts transfected with pSV-bar/egfp displayed  $7.4 \pm 1.7\%$  survival 7 days post-transfection, while protoplasts transfected with p18S-SV-bar/egfpMARs showed  $6.2 \pm 0.7\%$  survival at the same sampling point. At this stage of *G. gracilis* protoplast culture, untransfected protoplasts exhibited only  $0.4 \pm 0.1\%$  survival, which was significantly ( $P<0.01$ ) less than the transfected protoplasts (Fig. 4.15A). There was no significant difference ( $P>0.05$ ) between survival percentages of protoplasts transfected with the different constructs (Fig. 4.15A). Furthermore, the surviving protoplasts in the plasmid-transfected samples appeared healthy 5 days after BASTA<sup>®</sup> supplementation (8 days post-transfection), while there were no surviving negative control protoplasts at this time (Fig. 4.15B). Approximately 4-5% of the protoplasts survived a second round of selection 21 days post-transfection. However, surviving cells had only undergone limited cell division and had not continued to produce calli after a further 2 month period.



**Figure 4.15** The effect of BASTA<sup>®</sup> supplementation on the survival of *G. gracilis* protoplasts transfected with no plasmid (negative control; ■), pSV-bar/egfp (■) or p18S-SV-bar/egfpMARs (□) (A). Data represents the mean  $\pm$  standard error. Different postscripts (\*) indicate a significant difference ( $P < 0.01$ ) between sample means within time points (one-way ANOVA). Protoplasts 8 days post-transfection (B). Panel 1: survival controls (protoplasts not supplemented with BASTA<sup>®</sup>) for protoplasts transfected with no plasmid DNA (negative control) (X), pSV-bar/egfp (Y) or p18S-SV-bar/egfpMARs (Z). Panel 2: BASTA<sup>®</sup> supplemented protoplasts transfected with no plasmid (X), pSV-bar/egfp (Y) or p18S-SV-bar/egfpMARs (Z). Scale bar = 25  $\mu$ m.

PCR analysis of genomic DNA extracted from protoplasts sampled 3 days post-transfection was carried out to confirm the presence of the *bar* gene in the host cells at the time of initial BASTA<sup>®</sup> supplementation. A 412 bp *bar* PCR product was amplified from DNA samples from *G. gracilis* protoplasts transfected with either pSV-*bar*/egfp or p18S-SV-*bar*/egfpMARs, but was not amplified from untransfected protoplast samples (Fig. 4.16). PCR amplification of a *G. gracilis* 18S rRNA PCR product confirmed the presence of intact *G. gracilis* DNA in all the samples tested (Fig. 4.16).



**Figure 4.16** PCR analysis of total DNA isolated from transfected protoplasts 3 days post-transfection. Lanes 1-3: untransfected protoplasts (1), protoplasts transfected with pSV-*bar*/egfp (2), or p18S-SV-*bar*/egfpMARs (3); +, positive control. Panel A: DNA fragments amplified with *G. gracilis* 18S rRNA gene specific primers; Panel B: DNA fragments amplified with *bar*-specific primers.

## 4.5 Discussion

Protoplasts offer particular advantages as transformation targets, as they are devoid of cell walls and therefore there is one less obstacle to successful transformation. The fluid mosaic characteristics of the plasma membrane means that DNA uptake can be induced chemically and/or by physical procedures (Davey *et al.*, 2005a). PEG-mediated transfection has been employed for the transfection of protoplasts of higher plants (Davey *et al.*, 2005a), microalgae (Coll, 2006; Hallmann, 2007) and the macroalga *P. yezoensis* (Kuang *et al.*, 1998). PEG-mediated transformation is a simple and efficient technique which allows the processing of many samples simultaneously. It does not rely on expensive, specialised equipment, as is the case with electroporation, and it results in cell populations with high survival and division rates (Nicolaisen and Poulsen, 1993; Mathur and Koncz, 1998). We therefore tested whether this technique was applicable for use in the establishment of a transformation protocol for *G. gracilis* protoplasts.

The reporter gene, *gfp*, was first cloned and sequenced from the cnidarian *Aequorea victoria* in the early nineties (Prasher *et al.*, 1992). It has since been widely employed as a reporter gene in a variety of organisms, ranging from bacteria to vertebrates (Leffel *et al.*, 1997). *Gfp* has an important advantage as a reporter gene in that its fluorescence is not dependent on the addition of any external substrates or cofactors (Chalfie *et al.*, 1994). This negates the need for harmful staining techniques. *Gfp* has been successfully used as a reporter gene in *P. yezoensis* (Cheney *et al.*, 2001; Mizukami *et al.*, 2004; Mikami *et al.*, 2011), while *egfp* has been employed as a reporter gene in *P. haitanensis* conchospores (Wang *et al.*, 2010b). EGFP is a red-shifted variation of wild-type GFP and is reported to have a five-fold greater fluorescence than that of the native protein and so may offer an added advantage by being detectable at very low levels (Falk *et al.*, 2001). The *egfp* reporter gene was therefore employed in this study to determine whether *G. gracilis* protoplasts could be successfully transformed through PEG-mediated transfection. The SV40 promoter/enhancer was employed to drive expression of *egfp* since it was already proven to be functional in *G. gracilis* (Chapter 2).

PEG-mediated transfection of *G. gracilis* protoplasts was successful and demonstrated that the *egfp* reporter gene could also be transcribed and expressed by the transformed protoplasts 2 days post-transfection. This was not surprising as the *egfp* reporter gene, under the

influence of the SV40 promoter, has been shown to be functional in *P. haitanensis* (Wang *et al.*, 2010b). However, entire protoplasts did not appear green as was reported for *P. haitanensis* conchospores (Wang *et al.*, 2010b), and instead EGFP fluorescence was seen within the *G. gracilis* protoplasts along with the natural orange-red fluorescence of the chloroplasts. In cases where the green EGFP fluorescence and the orange-red fluorescence of the chloroplasts overlapped, a yellow-orange signal was seen. This appearance of EGFP/GFP-expressing cells is common in higher plant cells (Plautz *et al.*, 1996; Mathur and Koncz, 1998) and has also been reported for *P. yezoensis* protoplasts (Mizukami *et al.*, 2004).

The appearance of the EGFP-expressing cells under the microscope meant that close inspection of individual cells was necessary which made it difficult to screen large numbers of cells. Fluorometer-based quantification of both transiently and stably expressed GFP has been widely reported for higher plants (Remans *et al.*, 1999; Cheng *et al.*, 2001; Mohamed *et al.*, 2006; Robić *et al.*, 2009; Wu *et al.*, 2011). GFP is quantified based on fluorescence units per  $\mu\text{g}$  protein, while untransformed tissue is used as an estimate of natural autofluorescence of the tissue (Cheng *et al.*, 2001). Therefore, a similar approach for detecting and measuring expressed EGFP in transfected *G. gracilis* protoplasts was employed in this study. EGFP values were quantified based on fluorescence units per cell using a fluorometer after excitation at 490 nm and emission maximum at 510-570 nm. This value was then normalized to cell number in order to obtain an accurate measure of fluorescence in each protoplast sample. Since there was a variation in the natural fluorescence of protoplasts between batches and with culture time, data was normalized to the negative control and presented as fold changes in fluorescence per cell (relative fluorescence) to allow for comparison between samples examined at various times. This approach proved effective as plasmid-transfected protoplasts expressing EGFP, confirmed through microscopy, displayed significantly ( $P < 0.05$ ) higher fluorescence in comparison to untransfected protoplasts.

Several parameters have been shown to affect transformation efficiency during PEG-mediated transfection (Nicolaisen and Poulsen, 1993). The parameters influencing transformation efficiency were varied during transfection with pSV-egfp DNA to optimize the production of maximal numbers of transformed *G. gracilis* protoplasts.

In order to assess the effect of different concentrations of DNA on transfection efficiency, the amount of plasmid added to the protoplast suspension during transfection was varied. While there was no significant difference ( $P>0.05$ ) in relative fluorescence between samples transfected with the various amounts of plasmid, maximal fluorescence was observed when  $1 \times 10^5$  protoplasts were transfected with 20  $\mu\text{g}$  of pSV-egfp. Optimal DNA:protoplast ratios vary between reports (Nicolaisen and Poulsen, 1993; Kuang *et al.*, 1998; Bart *et al.*, 2006; Jeon *et al.*, 2007) and therefore should be experimentally determined for each system.

Significantly, the presence of PEG during the transfection process was necessary for successful transformation of *G. gracilis* protoplasts. PEG is responsible for the precipitation of DNA in the presence of different salts in the transfection medium (Maas and Werr, 1989). The precipitation protects the DNA from nucleolytic digestion and this DNA-precipitate is also what is taken up by the cells (Maas and Werr, 1989). While transfection protocols for higher plant protoplasts employ higher concentrations of PEG (20% and above) (Nicolaisen and Poulsen, 1993; Bart *et al.*, 2006; Zelazny *et al.*, 2007), maximal relative fluorescence was observed when *G. gracilis* protoplasts were transfected with 13% PEG. Similarly, 13.3% was reported to be most effective in the transfection of *P. yezoensis* protoplasts (Kuang *et al.*, 1998). This concentration of PEG has also been employed for the successful transfection of the microalga *Chlorella ellipsoidea* (Jarvis and Brown, 1991; Kim *et al.*, 2002).

The divalent cations in the transfection buffers play an important role in the precipitation of DNA during the transfection process (Maas and Werr, 1989).  $\text{Ca}^{2+}/\text{Mg}^{2+}$  are commonly employed in plant protoplast transfections (Maas and Werr, 1989). A concentration of 15 mM of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  proved optimal for transfection of *G. gracilis* protoplasts in this study. The optimal concentrations of these cations are likely to be dependent on other constituents in the transfection buffers and PEG solutions, since collectively they are responsible for DNA precipitation during protoplast transfection.

The transfection period, while having no significant effect on the relative fluorescence of the transfected protoplasts, had an effect on protoplast survival. Longer transfection periods resulted in decreased protoplast survival. PEG is known to be cytotoxic to cells (Ohnuma *et al.*, 2008) and therefore it is possible that shorter transfection periods are likely to limit the toxic effect of PEG on *G. gracilis* protoplasts. Transfection periods of between 2 and 30 min

are commonly reported for PEG-mediated transfection of plant protoplasts (Maas and Werr, 1989; Jarvis and Brown, 1991; Nicolaisen and Poulsen, 1993; Kim *et al.*, 2002; Jeon *et al.*, 2007; Zelazny *et al.*, 2007).

Variations in protoplast concentration during transfection had no significant effect ( $P>0.05$ ) on the transfection efficiency. Varied protoplast concentrations are reported throughout the literature (Maas and Werr, 1989; Jarvis and Brown, 1991; Nicolaisen and Poulsen, 1993; Kim *et al.*, 2002; Jeon *et al.*, 2007; Zelazny *et al.*, 2007) and therefore ideal protoplast concentrations should be determined experimentally for each system.

The transformation/transfection efficiency obtained for PEG-mediated transfection of *G. gracilis* protoplasts prior to protocol optimization was approximately ten in  $10^5$  protoplasts. This was similar to the maximal transformation efficiency of four in  $10^5$  protoplasts reported for PEG-mediated transfection of *P. yezoensis* when assayed 2 days post-transfection (Kuang *et al.*, 1998). Optimization of the parameters in the PEG-mediated transfection method of *G. gracilis* protoplasts however resulted in an increased transformation efficiency of 89 in  $10^5$  protoplasts (0.09%). This is increased compared to those originally obtained and to those reported for PEG-mediated transfection of *P. yezoensis* protoplasts (Kuang *et al.*, 1998). Mizukami *et al.* (2004) also reported expression frequencies of less than 0.3% after 2 and 4 days of culture when employing *gfp* as a reporter gene in the electroporation of *P. yezoensis* protoplasts. The expression frequency could however be dramatically increased to just less than 1% after 2 days culture, and more than 3% after 4 days culture when *uidA* was employed as the reporter gene instead of *gfp* (Mizukami *et al.*, 2004). Huang *et al.* (1996) also report a transformation frequency after 2 days culture of less than 1% for electroporation of *Ulva lactuca* protoplasts when employing the *uidA* reporter gene. However, it is difficult to draw comparisons between these studies and the current study, since they employ different reporter genes in combination with various promoters introduced in various ways, and as previously discussed (Chapter 2.5), these factors have a bearing on foreign gene expression. The transformation efficiency obtained by Wang *et al.* (2010b) through glass bead agitation of *P. haitanensis* conchospores was 6.02 in  $10^6$  conchospores when employing a similar construct as employed in this study, i.e. the *egfp* reporter gene under the influence of the SV40 promoter. While this does represent a much lower efficiency of transformation, the regeneration capacity of the target tissue should also

be taken into account. Lower transformation efficiencies will have less effect on systems with high regenerative capacities, such as algal spores. Transformation efficiencies amongst the microalgae are said to be strongly species dependent (Hallmann, 2007) and this may also be the case with macroalgae. However, it is difficult to come to any conclusion when there are so few published reports.

A silkworm MAR and 18S rDNA-targeted HR was shown to increase the transient expression of chloramphenicol acetyl transferase (CAT) in *P. haitanensis* protoplasts (Zuo *et al.*, 2007). Similarly in this study, a suite of expression constructs were designed and constructed to investigate whether the tobacco Rb7 MAR and 18S rDNA-targeted HR could have a positive effect on transient gene expression in *G. gracilis*. In order to assess this, protoplasts were transfected with one of four plasmids and the relative fluorescence of the protoplasts was monitored over a period of 9 days as a measure of EGFP expression. The results are summarised in Table 4.6.

In all cases increased relative fluorescence, above that of the control sample, was correlated with the presence of plasmid DNA (Table 4.6). Data showed that the presence of pSV-egfp or pSV-egfpMARs resulted in a significant increase ( $P < 0.01$ ) in relative fluorescence of the protoplasts when compared to the negative control and to protoplasts transfected with either 18S-pSV-egfpMARs or 18S-pSV-egfp 2 days post-transfection. While the presence of 18S-pSV-egfpMARs and 18S-pSV-egfp was confirmed through PCR analysis in transfected protoplasts, neither construct resulted in significant increases in relative fluorescence when compared to the negative control sample (Table 4.6). Furthermore, the presence of all four constructs tested resulted in maximal relative fluorescence ( $P < 0.01$ ) 3 days post-transfection. Transfected samples also showed no significant difference in the number of EGFP-expressing cells at this time. The trend of increasing transgene expression over time has also been noted in the transformation of *P. yezoensis* and *P. haitanensis* (Mizukami *et al.*, 2004; Zuo *et al.*, 2007). Mizukami *et al.* (2004) and Zuo *et al.* (2007) however, both report maximal transgene expression of *P. yezoensis* and *P. haitanensis* 4 days post-transfection. In a study by Gong *et al.* (2007), maximal transgene expression was noted in *P. yezoensis* protoplasts 2 days post-transfection. All these studies employed different reporter genes in combination with various promoters which is likely to be the reason for the variation observed in transient expression patterns.

**Table 4.6** Summary of relative fluorescence data and *egfp* PCR results over a sampling time period following transfection of *G. gracilis* protoplasts with 4 different constructs.

Sample time point <sup>a</sup> (Day)	Construct	Relative fluorescence <sup>b</sup>	PCR <sup>c</sup>
2	pSV-egfp	+	+
	pSV-egfpMARs	+	+
	p18S-SV-egfpMARs	-	+
	p18S-SV-egfp	-	+
3	pSV-egfp	+	+
	pSV-egfpMARs	+	+
	p18S-SV-egfpMARs	+	+
	p18S-SV-egfp	+	+
4	pSV-egfp	+	+
	pSV-egfpMARs	+	+
	p18S-SV-egfpMARs	+	+
	p18S-SV-egfp	+	+
5	pSV-egfp	+	+
	pSV-egfpMARs	+	+
	p18S-SV-egfpMARs	+	+
	p18S-SV-egfp	+	+
6	pSV-egfp	-	-
	pSV-egfpMARs	+	+
	p18S-SV-egfpMARs	+	+
	p18S-SV-egfp	+	+
9	pSV-egfp	-	-
	pSV-egfpMARs	-	+
	p18S-SV-egfpMARs	-	+
	p18S-SV-egfp	-	+

<sup>a</sup> Sampling time point given as days post-transfection.

<sup>b</sup> Relative fluorescence is given as present (+) if protoplasts showed a statistically higher fluorescence ( $P < 0.05$ ) than the negative control sample; - indicates no statistical difference ( $P > 0.05$ ) in relative fluorescence in comparison to the negative control sample.

<sup>c</sup> Presence (+) or absence (-) of a *egfp* PCR product following amplification using specific primers.

The presence of p18S-SV-egfpMARs in transfected protoplasts resulted in the highest relative fluorescence at 3 and 4 days post-transfection. In comparison, neither the presence of MARs nor HR regions on their own had any significant effect on EGFP expression when compared to the control plasmid, pSV-egfp, over the first 5 days post-transfection. Thus, it is the presence of both these regions in p18S-SV-egfpMARs that is responsible for the

increased EGFP expression seen 3 and 4 days post-transfection. Zuo *et al.* (2007) also report the highest CAT expression for *P. haitanensis* protoplasts transfected with a plasmid carrying both MARs and HR regions. This is interesting to note, since integration of foreign DNA into a chromosome is required for MARs to greatly influence transgene expression (Cheng *et al.*, 2001).

Six days post-transfection the presence of pSV-egfp was no longer detected in transfected protoplasts and as a consequence the relative fluorescence of these protoplasts had returned to basal levels (Table 4.6). Similarly, Zuo *et al.* (2007) reported that CAT levels returned to basal levels in *P. haitanensis* protoplasts transfected with a vector carrying only the reporter gene and promoter by 6 days post-transfection. Protoplasts transfected with either pSV-egfpMARs, p18S-SVegfpMARs or p18S-SV-egfp still displayed significantly higher ( $P<0.05$ ) relative fluorescence levels when compared to the control protoplasts (Table 4.6). This suggests that both MARs and HR regions may be positively influencing transgene expression. In addition, a slightly higher proportion of EGFP-expressing cells (0.09-0.11%) were observed for the p18S-SVegfpMARs and p18S-SV-egfp transfected samples when compared to the pSV-egfpMARs transfected samples (0.05-0.07%). The Rb7 MAR has been reported to function as an enhancer for transient gene expression in rice plants resulting in increased GFP expression of up to 70% (Cheng *et al.*, 2001). This may explain why a smaller proportion of EGFP-expressing cells in pSV-egfpMARs transfected samples could still result in similar levels of expression when compared to HR transfected samples. Furthermore, the variation noted in EGFP-expressing cell numbers suggests that HR regions may have aided in foreign gene retention. While Zuo *et al.* (2007) report elevated CAT expression levels in protoplast samples transfected with an HR construct 6 days post-transfection, there was no assessment of whether chromosomal integration had taken place. Liu *et al.* (2003) also report increased GUS levels in *P. yezoensis* protoplasts transfected with a construct carrying 18S rDNA HR regions when compared to protoplasts transfected with the control plasmid 2 days post-transfection. The authors do state that a higher transformation efficiency was obtained for the construct carrying 18S rDNA HR regions when compared to the control plasmid, however no further monitoring of GUS expression was carried out (Liu *et al.*, 2003). Liu *et al.* (2003) employed a linearised plasmid during transfection which is known to be more efficient for recombination (Primrose and Twyman, 2006). This may explain why an earlier effect on transgene levels was observed by Liu *et al.*

(2003) when compared to this study and that of Zuo *et al.* (2007), in which circular supercoiled plasmids were employed for transfection. Circular supercoiled plasmids are advantageous when studying transient expression since they offer greater resistance to intracellular DNAses when compared to linear plasmids and they are also known to result in higher levels of transfected plasmid DNA (Coll, 2006).

When considering these results, one should also bear in mind that the protoplast population as a whole was assessed and the detected EGFP expression is an average of many transformants. Therefore, the conclusions drawn from this analysis may not apply to every transformant. It is only through the regeneration of stably transformed plants that the actual effect of MARs and HR regions could be assessed at an individual level. However, the transient system established in this study does allow a preliminary evaluation of the MAR and HR expression constructs.

There was no significant difference ( $P>0.05$ ) between the relative fluorescence of the control *G. gracilis* protoplasts and the protoplasts transfected with any of the plasmids 9 days post-transfection. EGFP fluorescence could also not be detected by fluorescence microscopy. However, the presence of *egfp* was still detected in the DNA of *G. gracilis* protoplast samples transfected with pSV-*egfp*MARs, p18S-SV-*egfp*MARs or p18S-SV-*egfp* by PCR analysis at this stage. This meant that either the transfected plasmids were still present in these protoplasts as episomal DNA or chromosomal integration had occurred.

In order to assess whether targeted HR may have occurred in *G. gracilis* host cells, PCR analysis of genomic DNA extracted from protoplasts transfected with either p18S-SV-*egfp* or pSV-*egfp* 9 days post-transfection was carried out. The strategy employed made use of a pair of primers, one which was located outside the region of homology of the 18S rDNA included in p18S-SV-*egfp*, and one which was located within the transfected DNA and would not be present in the chromosomal DNA unless integration had taken place. A PCR product of the correct size, 1469 bp, was amplified from *G. gracilis* protoplasts transfected with p18S-SV-*egfp*, but not from protoplasts transfected with pSV-*egfp*. In addition, an expected 67 bp size difference was seen between the HR product and the PCR product amplified from the plasmid positive control. Sequencing and further PCR analysis confirmed the identity of the 1469 bp HR PCR product. These results suggested that HR had taken place between the

transfected DNA and the 3' homologous portion of the *G. gracilis* 18S rRNA gene. Further Southern hybridization analysis would however be needed to confirm this but since a selection method and regeneration protocol of stably transformed *G. gracilis* was not available, individual transformants could not be assessed.

It should be emphasized that the putative chromosomal arrangement detected is not likely to be the only product to result from possible HR events occurring between the transfected plasmid DNA and the host chromosome. Since the plasmid was transfected into protoplasts in a circular and not a linear form, HR could have taken place between either the 5' or the 3' homologous portion of the *G. gracilis* 18S rRNA gene carried on the vector and resulted in insertion of the entire construct. The construct was however designed so that when linearised outside of the regions of homology, it could act as a replacement vector where homologous pairing with both sides of the gene target would result in substitution of the chromosomal copy with the exogenous DNA sequence. Such integration products are more stable (Primrose and Twyman, 2006) and would therefore be more desirable when regenerating whole plants. Our efforts in the current study, however, were focused on the detection of possible targeted HR in *G. gracilis*, and hence it was not necessary to be concerned about resulting chromosomal arrangements.

Since the rate of stable integration is so low, recovery of transformed organisms generally requires the use of selectable markers (Hallmann, 2007). Very little research has however been conducted on macroalgal susceptibility to commonly employed antibiotics and herbicides (Qin *et al.*, 1998 referenced in Reddy *et al.*, 2008a). The sensitivity of *G. gracilis* protoplasts to kanamycin, chloramphenicol and BASTA<sup>®</sup> was assessed in order to identify a possible selective agent for successfully transformed *G. gracilis*. *G. gracilis* protoplasts however proved resistant to both chloramphenicol and kanamycin since concentrations of up to 500 µg ml<sup>-1</sup> had no significant effect on protoplast viability. Whole plants were even regenerated from protoplasts cultured in the presence of these antibiotics. It is perhaps not surprising that kanamycin had no effect on protoplast viability since other species of macroalgae have also been reported to be resistant to this antibiotic (Qin *et al.*, 1998 referenced in Reddy *et al.*, 2008a; Reddy *et al.*, 2008a). In fact, kanamycin supplementation of protoplast cultures even had a positive effect on protoplast survival. This same trend was noted for *Ulva lactuca* protoplasts cultured in the presence of kanamycin (Huang *et al.*, 1996)

and it is likely to be due to the inhibition of microorganisms which may still be present in protoplast cultures. *L. japonica* has been reported to be sensitive to chloramphenicol supplementation and the *cat* gene has also been successfully employed to select *L. japonica* transformants (Jiang *et al.*, 2002). *G. gracilis* protoplasts were, however, resistant to high concentrations of this antibiotic. It is likely that different species of macroalgae will vary in their sensitivity to various selective agents, but it should be noted that macroalgae are said to possess significant inherent antibiotic resistance (Kübler *et al.*, 1994).

As an alternative, the herbicide BASTA<sup>®</sup> was assessed as a possible selective agent for use in *G. gracilis*. Glufosinate, also called phosphinothricin, is a potent inhibitor of glutamine synthetase and is the active agent in BASTA<sup>®</sup> (Altenburger *et al.*, 1995). Glufosinate therefore interferes with glutamine biosynthesis and ammonium detoxification. The *bar* gene is commonly employed in terrestrial plant tissue culture as a selectable marker (Altenburger *et al.*, 1995). *G. gracilis* protoplasts proved sensitive to BASTA<sup>®</sup>, at a concentration of 5 µg ml<sup>-1</sup>, resulting in only 2.0 ± 0.6% survival 7 days after BASTA<sup>®</sup> supplementation and surviving protoplasts appeared bleached and unhealthy. BASTA<sup>®</sup> has already been successfully employed as a selective agent in *L. japonica* (Zhang *et al.*, 2006; Zhang *et al.*, 2008) and in the unicellular green alga *Dunaliella salina* (Tan *et al.*, 2005). A concentration of 40 µg ml<sup>-1</sup> was used for *L. japonica* selection (Zhang *et al.*, 2006; Zhang *et al.*, 2008), while 20 µg ml<sup>-1</sup> was used for *D. salina* selection (Tan *et al.*, 2005). The increased sensitivity of *G. gracilis* in comparison to *L. japonica* and *D. salina* may be due to the fact that it is newly regenerated protoplasts that have been targeted for selection and these cells may be more sensitive than the gametophytes of *L. japonica* or the whole cells of *D. salina*.

The *bar* gene encodes phosphinothricin acetyltransferase (PAT) which confers resistance to phosphinothricin/glufosinate and therefore BASTA<sup>®</sup> (Tan *et al.*, 2005). During the course of this investigation the *bar* gene was tested as a selective marker for genetic transformation of *G. gracilis* protoplasts. Protoplasts were transfected with either pSV-*bar*/*egfp*, a vector encoding the *bar* and *egfp* genes both under the influence of a SV 40 promoter, p18S-SV-*bar*/*egfp*MARs, which contained Rb7 MARs as well as 18S rDNA homologous regions flanking the promoter-reporter gene cassette described for pSV-*egfp*. Plasmid p18S-SV-*bar*/*egfp*MARs was constructed and employed since the combination of MARs and HR regions had been shown to positively influence *egfp* expression and by extension, may

offer a similar advantage to *bar* expression, and therefore, BASTA<sup>®</sup> resistance in *G. gracilis* protoplasts. Protoplasts were supplemented with BASTA<sup>®</sup> 3 days post-transfection after confirmation of plasmid presence through both PCR analysis and fluorescence microscopy to confirm EGFP expression. Equal transfection efficiency was also confirmed 3 days post-transfection.

Plasmid transfected *G. gracilis* protoplast samples showed significantly ( $P < 0.01$ ) improved survival rates 5-7 days post-transfection when compared to the negative control protoplast samples. Protoplasts transfected with the different constructs however showed no significant difference ( $P > 0.01$ ) in survival rates at any time. There was also no difference between the survival of the untreated control groups (survival controls). By 7 days post-transfection, protoplasts transfected with pSV-*bar*/egfp showed  $7.4 \pm 1.7\%$  survival, while protoplasts transfected with p18S-SV-*bar*/egfpMARS showed  $6.2 \pm 0.7\%$  survival. At this stage of culture, negative control protoplasts showed only  $0.4 \pm 0.1\%$  survival and by 8 days post-transfection there were no surviving negative control protoplasts. Therefore, increased protoplast survival correlated with plasmid transfection and it is likely that the *bar* gene was successfully transcribed and conferred BASTA<sup>®</sup> resistance to the surviving protoplasts. It is unfortunate that EGFP was no longer visible in transfected protoplasts 7 days post-transfection as this could have served as a confirmation of foreign DNA presence. This indicates that stable, high level expression of EGFP did not occur under the influence of the SV40 promoter. However, when considering the results previously obtained for EGFP expression, it is not surprising that EGFP could no longer be detected in transfected protoplasts.

Approximately 4-5% of *G. gracilis* protoplasts survived a second round of selection 21 days post-transfection, indicating prolonged presence of the *bar* gene. However, after a further 2 month period, surviving cells had only undergone limited cell division and had not continued to produce calli. This may indicate that while the protoplasts do survive the selection, BASTA<sup>®</sup> supplementation may have a negative effect on the protoplasts. While similar problems were not reported for *Laminaria* selection, this is the first time that BASTA<sup>®</sup> selection has been attempted for macroalgal protoplasts. The survival of protoplasts transfected with the *bar* gene does however suggest that BASTA<sup>®</sup> selection may well prove a

viable option for the selection of transformed *G. gracilis* protoplasts. However, further optimization of this system is required.

When considering the survival rate of transfected protoplasts, it appears as if 6-7% of the *G. gracilis* protoplast population successfully expressed the *bar* gene. However, EGFP expression efficiencies were routinely less than 0.2% of the transfected population. The discrepancy between these values brings into question the suitability of *egfp* as a reporter gene in *G. gracilis* protoplasts. Either *egfp* is not being expressed in the same proportion of cells as the *bar* gene is, or it is not being ubiquitously expressed throughout the protoplast population and its expression level is too low to be detected in the majority of cells. Both of these possibilities however suggest that *egfp* is not the optimal reporter gene for *G. gracilis* protoplasts. Varied expression frequency has also previously been reported in *P. yezoensis* when employing *uidA* and *gfp* as reporter genes under the influence of the same promoter (Mizukami *et al.*, 2004). Different reporter genes therefore appear to function with varying efficiency even in the same macroalgal species and until more supporting literature becomes available, reporter gene functionality should be assessed for each new species of macroalgae.

In conclusion, we established a protocol for PEG-mediated transfection of protoplasts by optimizing the various parameters which are known to affect transfection efficiencies. Furthermore, the effects of targeted HR and MARs on EGFP were assessed and both these regions were shown to have positive effects on transient transgene expression. *G. gracilis* protoplasts were assessed for their sensitivity to various selective agents which allowed the herbicide BASTA<sup>®</sup> to be identified as a potential selective agent. The *bar* gene was then shown to confer resistance to transfected protoplasts.

## General discussion and future research

### 5.1 General discussion

*Gracilaria* species are considered to be the most important algae for use in food grade agar production (De Oliveira *et al.*, 2000). The growing demand for agar from *Gracilaria* species over the years has, however, put an increasing strain on the natural stocks (Santelices and Doty, 1989; De Oliveira *et al.*, 2000). In South Africa, the *Gracilaria* industry depended solely on the natural *G. gracilis* resource growing in Saldanha Bay. This resource has experienced a number of population collapses over the past few years which have rendered it unreliable for commercial collections (Schroeder *et al.*, 2003; Rothman *et al.*, 2009). It has been suggested that the only means of establishing a reliable *Gracilaria* industry in South Africa is through suspended (open-water) cultivation (Rothman *et al.*, 2009). However, intensive farming often leads to an increased disease burden as a result of forced growth under unnatural conditions. In order to ensure a regular and healthy supply of seaweed from these cultivated populations, the issue of maintaining a disease- and stress-free *G. gracilis* resource needs to be addressed. In this respect, a better understanding of how *G. gracilis* responds at a molecular level to stresses associated with seaweed aquaculture is essential for selecting for and/or engineering macroalgal strains that are either more tolerant or resistant to these stresses. In order to achieve this long-term goal, a transformation system is required for *G. gracilis*. Transformation systems allow *in vivo* analysis of gene function and regulation, the manipulation of endogenous genes, and the introduction and expression of foreign genes (Walker *et al.*, 2005), which are all necessary for the production of stress tolerant strains. A transformation model should consist of elements such as an effective method to deliver foreign DNA into a target cell, suitable vectors carrying recognizable promoters, and screening mechanisms to select transformants from a multitude of recipients (Qin *et al.*, 2005). Furthermore, regeneration systems are also necessary for multicellular organisms (Qin *et al.*, 2005). The aims of this project were therefore to develop a transformation and tissue culture system for *G. gracilis*, and in so doing, lay the necessary groundwork for future genetic manipulation studies that are essential for improving our understanding of the role various genes play in stress response and tolerance in *G. gracilis*.

In Chapter 2, microparticle bombardment was investigated and optimized as a method to transiently transform *G. gracilis* thalli. This technique allows researchers to study the effects

of mutations, introns, codon use and promoters on transgene expression levels, without the need for the production of genetically transformed plants (Taylor and Fauquet, 2002). To date there has been relatively little research conducted on the functionality of various promoters in macroalgal systems. This optimized bombardment system was therefore used to test the extent of *lacZ* expression under the influence of three different viral promoters: SV40, CaMV 35S and CMV promoters. All three promoters were functional in expressing *lacZ*; however, the SV40 promoter was shown to be the most efficient at expressing *lacZ*, resulting in the highest thallus  $\beta$ -galactosidase activity. This promoter was therefore chosen as the most suitable promoter for further *G. gracilis* transformation studies. While the SV40 and CaMV 35S promoters have been successfully used for foreign gene expression in macroalgae, to the best of our knowledge, this is the first report of successful expression of a reporter gene under the influence of the CMV promoter in macroalgae.

It is all very well to show that transient transformation is possible for *G. gracilis*, but stable transformation is needed for establishing improved macroalgal strains. Stable transformation of macroalgae requires clonal seaweed culture and techniques for plant regeneration from single cells. Chapter 3 therefore sought to establish a cell culture system based on *G. gracilis* protoplasts, with a future view to developing stably transformed macroalgae. In order to do this, a protocol for protoplast isolation and purification was developed and optimized which ensured that large quantities of viable protoplasts could consistently be isolated from *G. gracilis* thalli. Furthermore, under optimized culture conditions, approximately 80% of *G. gracilis* protoplasts underwent cell wall re-synthesis within the first 24 hrs of culturing. This was an important first step towards whole plant regeneration since it demonstrated that protoplasts were in fact viable and being cultured under the correct conditions.

Interestingly, light intensity and incubation temperatures affected protoplast regeneration patterns in *G. gracilis*. While these parameters have been reported to be important factors influencing protoplast regeneration patterns in *Porphyra* (Chen, 1989; Polne-Fuller and Gibor, 1990), this is the first study to investigate and demonstrate their effect on regeneration patterns from *Gracilaria* protoplasts. Under conditions of low light intensity and high incubation temperature, *G. gracilis* protoplasts underwent cell division that resulted in the formation of cell clumps, resembling those produced in cell suspension culture of *Porphyra* (Chen, 1989; Polne-Fuller and Gibor, 1990). These suspension cultures could however not be induced to regenerate through alteration of culture conditions, and suspended *G. gracilis*

cells could only be maintained for a period of 5-6 weeks before they died. It is unfortunate that this is the case, since these suspension cultures would have been an excellent source of “seed stock” for both cultivation and molecular studies of *G. gracilis*. This pattern of protoplast development has not previously been reported for *Gracilaria* and it would be interesting to know if cell suspension cultures can be induced in other species of *Gracilaria*, and under which conditions.

Under conditions of higher light intensity and either high or low culture temperatures, protoplasts divided to produce callus-like cell masses which developed into whole plants. We observed that *G. gracilis* protoplasts either regenerated slowly to produce plants which resembled the parental plants, or they regenerated rapidly to produce plants which remained small with thalli that were thick and unbranched. The reason for the two developmental patterns observed for *G. gracilis* in this study is currently not known and has not previously been reported in other species of *Gracilaria*. We speculate that the developmental patterns may be protoplast-specific and possibly due to an inherited or entrained trait prior to cell treatment for protoplast isolation and which may ultimately be related to origin of the protoplasts themselves. *Porphyra* regeneration patterns have been noted to be related to the area of the thallus from which the protoplasts originated, however the blades of these macroalgae do show a variation in cell morphology (Polne-Fuller and Gibor, 1984; Chen, 1987).

The outcome of this portion of the study resulted in the development of a reliable and efficient method for the production of whole plants from single cells, which is a requirement for any transformation system.

It seemed that protoplasts may be a better option for producing transformed *G. gracilis* plants because whole plants were able to be regenerated from protoplasts. Therefore, Chapter 4 sought to establish a method for PEG-mediated transformation of protoplasts. This optimized transformation protocol was in turn used to investigate the effects of targeted HR and MARs on EGFP expression within *G. gracilis* protoplasts. This was done in an effort to identify a possible means for increasing transgene expression levels. The presence of tobacco Rb7 MARs and 18S rDNA regions within the plasmid vectors used to transform *G. gracilis* protoplasts resulted in significant increases in EGFP levels three and four days post-transfection. Furthermore, PCR amplification of an HR integration product suggested that

targeted HR had taken place in *G. gracilis* protoplasts transfected with an 18S rDNA-containing vector, however Southern blot analysis would be required to confirm this. This is the first report of successful targeted HR in *Gracilaria*.

The recovery of successfully transformed organisms generally requires the use of selectable markers (Hallmann, 2007). In higher plants, resistance to antibiotics or herbicides is often used as a selectable trait. While *G. gracilis* protoplasts proved resistant to both chloramphenicol and kanamycin, they proved sensitive to the herbicide BASTA<sup>®</sup>. Furthermore, BASTA<sup>®</sup> resistance, conferred by a transfected *bar* gene, allowed selection of transformed protoplasts. However, the surviving protoplasts showed no signs of callus development after a period of two months, indicating that BASTA<sup>®</sup> supplementation may have had a negative effect on signals required for protoplast development and regeneration. Further optimization of this system, or even investigation of alternative selection agents, is necessary. Additionally, when considering the low transformation rate along with the rates of whole plant regeneration from protoplasts and the naturally low rate of stable integration, it is perhaps not surprising that whole transformed plants were not regenerated. In other words, this system relies on vector DNA entering and integrating into the chromosome of the “correct” protoplast, one that is able to regenerate, and while MARs and HR regions were employed to promote increased *bar* expression in order to increase the chances of success, further optimization is still required.

## 5.2 Future research

Although we were successful in achieving the primary aims of this study, which were to establish methods for whole plant regeneration from *G. gracilis* protoplasts and for transformation of *G. gracilis*, continued research is required to improve the system.

Algal genetic systems will most certainly profit from the identification of more powerful promoters, either endogenous or from seaweed-associated viruses and bacteria. While the viral promoters utilized in this study were functional, the use of endogenous promoters has been shown to be more efficient in reporter gene expression in the more advanced *P. yezoensis* systems (Mizukami *et al.*, 2004; Gong *et al.*, 2007; Fukuda *et al.*, 2008; Mikami *et al.*, 2011). Therefore, this approach may also be advantageous in the *G. gracilis* system as it is more geared to making use of the natural host system and regulatory mechanisms. The

ongoing *G. gracilis* genomic studies in our research group will aid in identifying possible target promoters for use in transformation studies and in increased understanding of the regulatory mechanisms of gene expression in this red alga. The bombardment system established in this study, will also allow rapid and easy assessment of these promoters in *G. gracilis*.

Very little research has been aimed at improving reporter gene functionality for macroalgal systems. A study by Fukuda *et al.* (2008) demonstrated that codon optimization of *uidA* led to dramatically increased levels of expression in *P. yezoensis*. This approach of employing reporter genes which have a similar codon usage to *P. yezoensis* has been shown to be particularly useful in *Porphyra* (Mikami *et al.*, 2011). It is also well known that codon usage influences foreign gene expression in higher plants (Rybicki, 2010). However, in the case of higher plants, it should be noted that ‘plant codon usage’ does not necessarily result in optimal expression and instead, optimal codon usage for each gene needs to be determined empirically (Rybicki, 2010). Therefore, a similar approach may be required for testing reporter gene expression in *G. gracilis*. In Chapter 4, some issues were raised concerning EGFP levels, the lack of prolonged expression of this reporter gene and the discrepancy between the number of *bar* and *egfp* expressing cells. It may be possible to overcome the problems with *egfp* functionality through codon optimization. Macroalgal researchers should, however, be wary of the assumption that ‘algal codon usage’ would be optimal and should also investigate the effect of various codon optimizations on reporter gene expression.

While we were able to regenerate whole plants from *G. gracilis* protoplasts, the spontaneity of the transition from callus to bud formation should be further investigated in an effort to better understand this stage of algal development. It would also be interesting to investigate the effects that phytohormones may have on this and other regenerative processes. Phytohormones could be added to the protoplast culture medium at various stages of development, and the effects of their presence or absence on development assessed. Studies of this kind in *G. gracilis* and other seaweeds are essential, as this knowledge could potentially lead to increased control over macroalgal tissue culture. Similarly, the role that the presence or absence of epiphytic bacteria play in algal protoplast to plant development should be further investigated. While the effects of the axenic culture conditions on *G. gracilis* plant development are not known at this stage, it is possible that this may in some way have contributed to the aberrant regeneration path of some of the

*G. gracilis* plants and to the spontaneity of bud formation. Plant associated-bacteria are known to contribute to the health, growth and development of seaweeds through the production of plant growth regulators and nitrogen fixation (Sturz *et al.*, 2000). Interestingly, members of *Ulvaceae* lose their foliose thallus morphology when cultured under axenic culture conditions (Provasoli and Pintner, 1980). This morphology can however be successfully restored following the inoculation of particular bacterial isolates to the culture media (Singh *et al.*, 2011b). The role of bacterial isolates in the enhancement of bud induction in *G. dura* was also recently shown (Singh *et al.*, 2011a). Thus, while axenic culture conditions are said to be required for protoplast regeneration (Reddy *et al.*, 1989) and algal tissue culture (Baweja *et al.*, 2009), the effect of the absence of natural algal-associated bacteria on algal development should not be ignored and requires further study.

Perhaps one of the most obvious trends throughout literature concerning macroalgal transformation and tissue culture, is that what “works” for one genus or species will not necessarily be applicable to another. Therefore, successful functionality of promoter and reporter genes, and the efficacy of selective agents, cannot simply be assumed to be such when based on reports in other species, but should be individually assessed for each new species. Especially noticeable is the variation in protoplast regeneration patterns between various genera of macroalgae. Limited literature and the publication of insufficiently detailed studies make comparisons difficult. However, as with promoter and reporter gene functionality, it should be noted that it is likely that protoplast regeneration systems will need to be established for each new species and that the knowledge base of other systems is not necessarily transferable.

In conclusion, to the best of our knowledge, this study was the first to describe microparticle bombardment and successful foreign gene expression in the commercially important macroalga *G. gracilis*. Similarly, this study is the first report of whole plant regeneration from *G. gracilis* protoplasts, PEG-mediated transformation of *Gracilaria* protoplasts, and successful expression of *egfp* and BASTA<sup>®</sup> selection of transformed *Gracilaria* protoplasts. This was also the first study to report successful targeted HR in *Gracilaria*. This research has significantly contributed to the current scientific knowledge concerning general macroalgal and, more specifically, *G. gracilis* transformation systems. Importantly, each of the techniques and findings in this study has laid the groundwork necessary for future genetic manipulation studies in *G. gracilis*.

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## **APPENDIX A**

### **MEDIA AND SOLUTIONS**

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## Contents

A.1	MEDIA .....	183
A.1.1	Artificial Sea Water (ASW).....	183
A.1.2	Fe-solution .....	183
A.1.3	PII metal solution.....	183
A.1.4	PES-enriched seawater medium (1/3 strength) (Provasoli, 1968) .....	184
A.1.5	Luria Broth.....	184
A.1.6	Luria Agar .....	184
A.1.7	Ψ Broth.....	184
A.1.8	Synthetic sea salts .....	184
A.1.9	Marine Broth .....	185
A.1.10	Marine Agar .....	185
A.1.11	ASW agar.....	185
A.2	SOLUTIONS .....	185
A.2.1	General stock solutions .....	185
A.2.2	Antibiotic stock solutions.....	187
A.2.3	Electrophoresis buffers and dye.....	188
A.2.4	Solutions for making Rubidium Chloride (RbCl) competent cells.....	188
A.2.5	Solutions for small scale plasmid isolation.....	189
A.2.6	Solutions for genomic DNA extraction .....	190
A.2.7	Solutions for microparticle bombardment .....	190
A.2.8	Solutions for histological <i>lacZ</i> staining .....	191
A.2.9	Solutions for β-galactosidase assays .....	192
A.2.10	Solutions for protoplasts .....	192
A.2.10.1	1.6 M Mannitol.....	192
A.2.10.2	20 mM Tris-MES (2[N-Morpholino]ethane-sulfonic acid).....	192
A.2.10.3	100 mM CaCl <sub>2</sub> .....	192
A.2.10.4	10% (w/v) cellulase Onozuka R-10.....	193
A.2.10.5	50 mM Potassium phosphate buffer (pH 6.0) .....	193
A.2.10.6	Agarase from <i>Pseudomonas atlantica</i> (200 U ml <sup>-1</sup> ).....	193
A.2.10.7	Enzymatic medium for optimization of cellulase concentration.....	193
A.2.10.8	Enzymatic medium for optimization of agarase concentration.....	193
A.2.10.9	Rinse medium.....	193
A.2.10.10	Cellulase (7%, w/v) and Macerozyme (3.5%, w/v) solution.....	194
A.2.10.11	Enzymatic medium for optimization of Macerozyme R-10 concentration.....	194
A.2.10.12	25x Protease inhibitor (Roche).....	194

A.2.10.13	Plasmolysis medium.....	194
A.2.10.14	1% Papain solution.....	194
A.2.10.15	Enzymatic medium (Optimized).....	195
A.2.10.16	Modified enzymatic medium of (Reddy <i>et al.</i> , 2006).....	195
A.2.10.18	0.4 M Sorbitol-ASW.....	195
A.2.10.19	Solutions for Optiprep <sup>®</sup> density gradient.....	196
A.2.10.20	Solutions for SEM.....	196
A.2.10.21	Transfection medium (TFM).....	196
A.2.10.22	PEG medium.....	197
A.2.10.23	0.125 M NaCl - filter-sterilized natural seawater.....	197
A.2.10.24	BASTA <sup>®</sup> solution.....	197

University of Cape Town

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**APPENDIX A****MEDIA AND SOLUTIONS**

All media were autoclaved at 121°C for 20 min prior to use, unless otherwise stated.

Water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system. Ultrapure water was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system and was used for all protoplast media and solutions.

**A.1 MEDIA****A.1.1 Artificial Sea Water (ASW)**

NaCl (Saarchem)	24.7 g
MgCl <sub>2</sub> .6H <sub>2</sub> O (Saarchem)	4.7 g
KCl (Saarchem)	0.66 g
CaCl <sub>2</sub> .2H <sub>2</sub> O (Saarchem)	1.9 g
MgSO <sub>4</sub> .7H <sub>2</sub> O (Saarchem)	6.3 g
NaHCO <sub>3</sub> (Saarchem)	0.18 g
water to	1 l

**A.1.2 Fe-solution**

Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> .6H <sub>2</sub> O (Saarchem)	702 mg
Na <sub>2</sub> EDTA (Saarchem)	600 mg
water to	1 l

**A.1.3 PII metal solution**

Na <sub>2</sub> EDTA	100 mg
H <sub>3</sub> BO <sub>3</sub>	114 mg
FeCl <sub>3</sub> .6H <sub>2</sub> O	4.9 mg
MnSO <sub>4</sub> .H <sub>2</sub> O	16.4 mg
ZnSO <sub>4</sub> .7H <sub>2</sub> O	2.2 mg
CoSO <sub>4</sub> .7H <sub>2</sub> O	0.48 mg
water to	1 l

**A.1.4 PES-enriched seawater medium (1/3 strength) (Provasoli, 1968)**

NaNO <sub>3</sub>	350 mg
Na <sub>2</sub> glycerophosphate. 5H <sub>2</sub> O	50 mg
Fe solution	25 ml
PII metal solution	25 ml
Vitamin B <sub>12</sub> (Sigma)	10 µg
Thiamine (Sigma)	0.5 mg
Biotin (Sigma)	5 µg
Tris buffer (Sigma)	500 mg
water to	100 ml

Adjust pH to 7.8, autoclave and store at 10 °C.

Add 6.6 ml to 1 L ASW.

The amount of PES solution added (20 ml) to ASW or seawater can be altered to make full strength (2%) PES-enriched seawater medium.

**A.1.5 Luria Broth**

Tryptone (Biolab)	10 g
Yeast extract	5 g
NaCl	5 g
water to	1 l

**A.1.6 Luria Agar**

Tryptone	10g
Yeast extract	5 g
NaCl	5 g
Agar (Biolab)	15 g
water to	1 l

**A.1.7 Ψ Broth**

Tryptone	20 g
Yeast extract	5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	4 g
KCl	0.75 g
water to	1 l

**A.1.8 Synthetic sea salts**

NaCl	30 g
MgCl <sub>2</sub> .6H <sub>2</sub> O	2.3 g
KCl	0.3 g
water to	1 l

<b>A.1.9</b>	<b>Marine Broth</b>	
	glucose (Saarchem)	2 g
	casamino acids (Difco)	5 g
	yeast extract	1 g
	synthetic sea salts to	1 l

<b>A.1.10</b>	<b>Marine Agar</b>	
	glucose	2 g
	casamino acids	5 g
	yeast extract	1 g
	agar	20 g
	synthetic sea salts to	1 l

<b>A.1.11</b>	<b>ASW agar</b>	
	ASW	1 l
	Plant agar (Sigma)	15 g

## A.2 SOLUTIONS

### A.2.1 General stock solutions

- 1 N NaOH**

NaOH (Saarchem)	4 g
water to	100 ml

Store in a plastic bottle.
- 1 M HCl**

37% HCl (Saarchem)	8.4 ml
water to	100 ml

Store in a foil covered glass bottle.
- 0.5 M EDTA**

Na.EDTA	186.1 g
water	800 ml

The pH was adjusted to 8.0 with NaOH pellets prior to making the volume to 1 l with water and autoclaving.
- 1 M Tris-HCl**

Tris base	121.5 g
water	800 ml

The pH was adjusted to 8.0 with HCl prior to making the volume to 1 l with water and autoclaving.

- **Tris-EDTA (TE) buffer (pH 8)**

1 M Tris-HCl (pH 8)	1 ml
0.5 M EDTA (pH 8)	200 µl
water to	100 ml

- **25% (w/v) Sodium dodecyl sulphate (SDS)**

SDS	50 g
sterile water	200 ml

Stir on warm plate to dissolve. Do not autoclave. The concentration of the SDS solution can be altered by altering the amount of SDS added.

- **70% (v/v) Ethanol (EtOH)**

Absolute ethanol	70 ml
water to	100 ml

Do not autoclave.

- **50% (v/v) Glycerol**

glycerol	25 ml
water to	50 ml

- **20% Glucose**

D-Glucose	20 g
water to	100 ml

- **Phosphate-buffered saline (PBS) (10 X stock)**

Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	17.8 g
KH <sub>2</sub> PO <sub>4</sub>	2.4 g
NaCl	80 g
KCl	2 g
water	900 ml

The pH was adjusted to 7.4 prior to making the volume to 1 l with water and autoclaving.

1 X PBS

Dilute 10 X PBS 1:10 in sterile dH<sub>2</sub>O.

- **1 M Sodium chloride (NaCl)**

NaCl	5.84 g
water to	100 ml

- **5 M NaCl**

NaCl	29.22 g
water to	100 ml

- **2 M Sodium phosphate, mono-sodium (NaH<sub>2</sub>PO<sub>4</sub>)**  

NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	55.2 g
water to	200 ml
  
- **2 M Sodium phosphate, di-sodium (Na<sub>2</sub>HPO<sub>4</sub>)**  

Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	71.2 g
water to	200 ml
  
- **1 M Phosphate buffer (pH 7.0)**  

NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O (2 M)	39 ml
Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O (2 M)	61 ml
water to	200 ml
  
- **1 M Potassium phosphate buffer (pH 6.0)**  

KH <sub>2</sub> PO <sub>4</sub>	136.1 g
KOH	17.9 g

Dissolve in 750 ml water and adjust pH to 6.0 using KOH.
  
- **5-Bromo-4-Chloro-3-Indolyl-β-D-galactosidase (X-gal) (20 mg ml<sup>-1</sup>)**  

X-gal (Fermentas)	400 mg
N,N' dimethyl formamide	20 ml

Aliquot and store at -70 °C in the dark.

#### A.2.2 Antibiotic stock solutions

- **Ampicillin (Sigma) (100 mg ml<sup>-1</sup>)**  

Dissolve 2 g in 20 ml water. Filter sterilize through a 0.22 μm syringe filter, and store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 100 μg ml<sup>-1</sup>.
  
- **Chloramphenicol (Sigma) (30 mg ml<sup>-1</sup>)**  

Dissolve 0.6 g in 20 ml ethanol. Store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 30 μg ml<sup>-1</sup>.
  
- **Kanamycin (Sigma) (30 mg ml<sup>-1</sup>)**  

Dissolve 0.6 g in 20 ml water. Filter sterilize through a 0.22 μm syringe filter, and store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 30 μg ml<sup>-1</sup>, unless otherwise indicated.
  
- **Penicillin G (Sigma) (100 mg ml<sup>-1</sup>)**  

Dissolve 2 g in 20 ml water. Filter sterilize through a 0.22 μm syringe filter, and store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 100 μg ml<sup>-1</sup>.

- **Amphotericin B (Sigma) (10 mg ml<sup>-1</sup>)**

Dissolve 1 g in 10 ml DMSO. Filter sterilize through a 0.22 µm syringe filter, and store aliquots at -20 °C. Supplement 2 ml protoplast culture medium with 0.2 µl to reach a final concentration of 0.1 µg ml<sup>-1</sup>.

### A.2.3 Electrophoresis buffers and dye

- **Tris-Acetate-EDTA (TAE) buffer (50 X stock)**

Tris base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8)	100 ml
water to	1 l

1 X TAE

Dilute 50 X TAE 1:50 in dH<sub>2</sub>O.

- **DNA gel tracking dye (6 X)**

bromophenol blue (Saarchem)	0.25 g
sucrose (Saarchem)	40 g
0.5 M EDTA (pH 8)	4 ml
water to	100 ml

- **λ*Pst*I DNA molecular weight marker**

Lambda (λ) phage genomic DNA (Promega)	40 µl
<i>Pst</i> I (Roche)	20 U
10x Buffer R (Roche)	20 µl
sterile water to	200 µl

Perform a standard restriction digest at 37 °C overnight. Add 40 µl tracking dye to stop the reaction, and load 15 - 20 µl per gel lane as a DNA molecular weight marker.

- **Ethidium bromide (10 mg ml<sup>-1</sup>)**

Ethidium Bromide (Sigma)	0.1 g
water to	10 ml

Shake well to dissolve. Do not autoclave. Store in the dark, in a foil covered bottle. Powerful mutagen; wear gloves and clean spills with isopropanol.

### A.2.4 Solutions for making Rubidium Chloride (RbCl) competent cells

- **1 M Rubidium chloride (RbCl)**

RbCl	6.05 g
water to	50 ml

- **750 mM Calcium chloride (CaCl<sub>2</sub>)**

CaCl <sub>2</sub> .2H <sub>2</sub> O	5.52 g
water to	50 ml
  
- **100 mM 4-Morpholinopropanesulfonic acid (MOPS) (pH 7.0)**

MOPS	1.05 g
water to	50 ml

Adjust pH to 7.0 with NaOH
  
- **TFB 1**

RbCl (1 M)	5 ml
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.495 g
KOAc	0.147 g
CaCl <sub>2</sub> (750 mM)	0.67 ml
glycerol (50%)	15 ml

Adjust pH to 5.8 with glacial acetic acid, make up the volume to 50 ml with water and filter sterilize.
  
- **TFB 2**

MOPS (100 mM, pH 7.0)	5 ml
RbCl (1 M)	0.5 ml
CaCl <sub>2</sub> .2H <sub>2</sub> O (750 mM)	5 ml
glycerol (50%)	15 ml
water to	50 ml

Filter sterilize.

#### A.2.5 Solutions for small scale plasmid isolation

- **Solution 1**

1 M Tris-HCl pH 8	25 ml
0.5 M EDTA	20 ml
20% Glucose	45.5 ml
sterile water to	100 ml
  
- **Solution 2**

10 M NaOH	2 ml
25% SDS	4 ml
water to	100 ml

Make fresh before use.
  
- **Solution 3**

KOAc	147 g
water to	500 ml

Dissolve KOAc in 200 ml water. Adjust pH to 4.8 with glacial acetic acid. Make up to a volume of 500 ml with water.

### A.2.6 Solutions for genomic DNA extraction

- **Proteinase K (20 mg ml<sup>-1</sup>)**

Proteinase K (Sigma)	20 mg
sterile water to	1 ml

Do not autoclave. Store at -20 °C.

- **Stock extraction buffer (SEB)**

Tris-HCl (1 M, pH 8.0)	5 ml
EDTA (500 mM, pH 8.0)	5 ml
NaCl (1 M)	25 ml
water to	50 ml

- **Working extraction buffer (WEB)**

SEB	1.36 ml
SDS (20 %) (w/v)	136 µl
Proteinase K (10 mg/ml)	5 µl

- **RNase A (10 mg ml<sup>-1</sup>)**

RNase A (Sigma)	0.1 g
1 M Tris-HCl (pH 7.5)	100 µl
5 M NaCl	3 ml
water to	10 ml

Heat for 15 minutes at 100 °C and allow to cool slowly to room temperature. Do not autoclave. Aliquot into sterile microfuge tubes and store at -20 °C.

- **Phenol / chloroform / isoamyl alcohol**

Chloroform / isoamyl alcohol

Mix at a ratio of 24:1. Store in a foil covered bottle.

Phenol / chloroform / isoamyl alcohol (25:24:1)

Mix phenol (pH 8) and the chloroform / isoamyl alcohol at a ratio of 1:1.

- **95% (v/v) Ethanol**

Absolute ethanol	95 ml
water to	100 ml

### A.2.7 Solutions for microparticle bombardment

- **50% (v/v) Glycerol**

Glycerol	25 ml
water to	50 ml

- **70% (v/v) Ethanol**

Absolute ethanol	70 ml
water to	100 ml
  
- **2.5 M Calcium chloride**

CaCl <sub>2</sub> ·2H <sub>2</sub> O	36.75 g
water to	100 ml
  
- **0.1 M Spermidine**

Heat bottle containing spermidine to 60 °C.

Spermidine	14 µl
water	96 µl

Filter sterilize  
 Aliquot and store at – 20 °C.  
 Must be freshly prepared once a month.

#### A.2.8 Solutions for histological *lacZ* staining

- **330 mM K<sub>4</sub>Fe(CN)<sub>6</sub>**

K <sub>4</sub> Fe(CN) <sub>6</sub>	139 mg
water to	1 ml

Solution is light sensitive and should be prepared fresh each time.
  
- **330 mM K<sub>3</sub>Fe(CN)<sub>6</sub>**

K <sub>3</sub> Fe(CN) <sub>6</sub>	109 mg
water to	1 ml

Solution is light sensitive and should be prepared fresh each time.
  
- **Stain solution**

X-gal (2%)	0.5 ml
Phosphate buffer (1 M, pH 7.0)	40 µl
MgCl <sub>2</sub> (1 M)	4 µl
NaCl (1 M)	600 µl
K <sub>4</sub> Fe(CN) <sub>6</sub> (330 mM)	40 µl
K <sub>3</sub> Fe(CN) <sub>6</sub> (330 mM)	40 µl
water to	4 ml

Heat solution to 30 °C prior to the addition of X-gal to prevent precipitation.  
 X-gal is light sensitive.

### A.2.9 Solutions for $\beta$ -galactosidase assays

- **Z Buffer**

Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	16.1 g
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	5.5 g
KCl	0.75 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.246 g
water to	1 l

Z Buffer containing  $\beta$ -mercaptoethanol

Prior to using, add 27  $\mu$ l  $\beta$ -mercaptoethanol to 10 ml Z buffer.

- **o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (20 mg ml<sup>-1</sup>)**

ONPG	40 mg
1 M Phosphate buffer (pH 7.0)	2 ml

Heat at 37 °C to help solubilize. Prepare fresh each time. Filter prior to use.

### A.2.10 Solutions for protoplasts

#### A.2.10.1 1.6 M Mannitol

Mannitol (Sigma)	29.152 g
ASW	70 ml

Heat in microwave to dissolve.

ASW to	100 ml
--------	--------

Filter and aliquot out.

Autoclave.

The concentration of the mannitol can be altered by dissolving a different mass of mannitol in the ASW so as to achieve concentrations of 1.2 or 2 M.

#### A.2.10.2 20 mM Tris-MES (2[N-Morpholino]ethane-sulfonic acid)

Tris	2.422 g
MES	3.904 g
water	800 ml

Adjust pH to 6.0 with HCl, make up the volume to 1 l with water and autoclave.

The pH of the medium can be altered through the addition of various amounts of HCl.

#### A.2.10.3 100 mM CaCl<sub>2</sub>

CaCl <sub>2</sub> ·2H <sub>2</sub> O	1.4702 g
Tris-MES (20 mM)	100 ml

**A.2.10.4 10% (w/v) cellulase Onozuka R-10**

cellulase Onozuka R-10	0.6 g
Tris-MES to	6 ml

Filter sterilize.

**A.2.10.5 50 mM Potassium phosphate buffer (pH 6.0)**

Potassium phosphate buffer (1 M, pH 6.0)	1.25 ml
sterile water to	25 ml

**A.2.10.6 Agarase from *Pseudomonas atlantica* (200 U ml<sup>-1</sup>)**

Agarase (Sigma)	5 KU
Potassium phosphate buffer (50 mM)	25 ml

Filter sterilize.  
Aliquot and store – 20 °C.

**A.2.10.7 Enzymatic medium for optimization of cellulase concentration**

Mannitol (1.6 M)	4.5 ml
CaCl <sub>2</sub> (100 mM in Tris-MES)	0.9 ml
Cellulase (10% (w/v) in Tris-MES)	2.7 ml
Tris-MES to	9 ml

This represents a final concentration of 3% (w/v) of cellulase. Volume of cellulase (10% (w/v)) can be varied in order to achieve final concentrations of 0, 1 and 2% (w/v).

**A.2.10.8 Enzymatic medium for optimization of agarase concentration**

Mannitol (1.6 M)	4.5 ml
CaCl <sub>2</sub> (100 mM in Tris-MES)	0.9 ml
Cellulase (10% (w/v) in Tris-MES)	2.7 ml
Agarase	0.45 ml
Tris-MES to	9 ml

This represents a final concentration of 3% (w/v) of cellulase and 10 U ml<sup>-1</sup> of agarase. The volume of cellulase (10% (w/v)) and agarase (200 U ml<sup>-1</sup>) can be varied in order to achieve various final concentrations. Enzymatic medium prior to optimization contained 1% (w/v) cellulase and 10 U ml<sup>-1</sup> agarase.

**A.2.10.9 Rinse medium**

Mannitol (1.6 M)	4.5 ml
CaCl <sub>2</sub> (100 mM in Tris-MES)	0.9 ml
Tris-MES to	9 ml

**A.2.10.10 Cellulase (7%, w/v) and Macerozyme (3.5%, w/v) solution**

Cellulase Onozuka R-10 (Yakult)	495 mg
Macerozyme R-10 (Yakult)	245 mg
Tris-MES (20 mM)	7 ml

Filter sterilize.

This represents a final concentration of 7% (w/v) of cellulase and 3.5% (w/v) Macerozyme. The mass of the Macerozyme can be varied in order to achieve final concentrations of 0 and 7% (w/v).

**A.2.10.11 Enzymatic medium for optimization of Macerozyme R-10 concentration**

Mannitol (1.6 M)	4.5 ml
CaCl <sub>2</sub> (100 mM in Tris-MES)	0.9 ml
Cellulase and Macerozyme solution	2.58 ml
Agarase	0.45 ml
Tris-MES to	9 ml

This represents a final concentration of 2% (w/v) of cellulase, 1% (w/v) of macerozyme and 10 U ml<sup>-1</sup> of agarase. The Macerozyme concentration can be varied by altering the cellulase and Macerozyme solution in A.2.10.10.

**A.2.10.12 25x Protease inhibitor (Roche)**

As per manufacturer's instructions. Made up in 20 mM Tris-MES.

**A.2.10.13 Plasmolysis medium**

Mannitol (1.6 M)	30 ml
sterile water	20 ml

**A.2.10.14 1% Papain solution**

Papain from Papaya latex (Sigma)	180 mg
Plasmolysis medium	18 ml

Filter sterilize.

This represents a final concentration of 1% (w/v) of papain. The papain concentration can be varied by altering the mass of papain in the solution so as to achieve final concentrations of 0, 2 or 5% (w/v).

**A.2.10.15 Enzymatic medium (Optimized)**

Mannitol (1.6 M)	9 ml
Cellulase and Macerozyme solution	5.16 ml
CaCl <sub>2</sub> (100 mM in Tris-MES)	1.8 ml
Agarase	900 µl
Protease inhibitor	180 µl
Tris-MES (20 mM) to	18 ml

The concentration of the mannitol can be altered by using stock solutions of a different concentration (A.2.10.1).

**A.2.10.16 Modified enzymatic medium of (Reddy *et al.*, 2006)**

**NaCl/dextran solution**

NaCl	1 g
Dextran sulphate	0.5 g
MES	0.488 g
Tris	0.3 g
water	90 ml

The pH was adjusted to 6.0 prior to making the volume to 100 ml with water and autoclaving.

This represents a final concentration of 1% (w/v) of NaCl. The NaCl concentration can be varied by altering the mass of NaCl in the solution so as to achieve final concentrations of 3% (w/v).

Cellulase and Macerozyme solution, Mannitol and 100 mM CaCl<sub>2</sub> are all prepared as above with the exchange of Tris-MES for NaCl/dextran solution.

**Enzymatic medium**

Mannitol (1.6 M)	9 ml
Cellulase and Macerozyme solution	5.16 ml
CaCl <sub>2</sub> (100 mM in NaCl/dextran)	1.8 ml
Agarase	900 µl
Protease inhibitor	180 µl
NaCl/dextran solution to	18 ml

This represents a final concentration of 1% (w/v) of NaCl, 0.5% (w/v) dextran sulphate and 0.8 M Mannitol. These can be varied by using various stock solutions prepared as described above.

**A.2.10.18 0.4 M Sorbitol-ASW**

Sorbitol	0.73 g
ASW to	10 ml

**A.2.10.19 Solutions for Optiprep<sup>®</sup> density gradient**

- **Optiprep<sup>®</sup> solution**

NaCl	0.28 g
Optiprep <sup>®</sup>	2 ml
- **58% Optiprep<sup>®</sup> solution (Bottom layer)**

Optiprep <sup>®</sup> solution	290 $\mu$ l
Culture medium containing protoplasts	210 $\mu$ l
- **32% Optiprep<sup>®</sup> solution (2<sup>nd</sup> layer)**

Optiprep <sup>®</sup> solution	160 $\mu$ l
Culture medium	340 $\mu$ l

Culture medium can be either ASW or 0.4 M sorbitol-ASW.

**A.2.10.20 Solutions for SEM****10X Base buffer (bb)**

10 X PBS	5 ml
NaCl	1.17 g

1X bb: Dilute 10 X bb 1:10 in sterile dH<sub>2</sub>O.

**0.4 M Sucrose**

Sucrose	1.369 g
1X bb to	10 ml

Filter sterilize.

**100 mM MgCl<sub>2</sub>**

MgCl <sub>2</sub>	0.203 g
1X bb to	10 ml

Filter sterilize.

**A.2.10.21 Transfection medium (TFM)**

NaCl (0.5 M)	2.922 g
Mannitol (0.5 M)	7.28 g
CaCl <sub>2</sub> (15 mM)	0.22 g
MES	0.2 g
water	80 ml

Heat in microwave to dissolve. Adjust pH to 5.8 with HCl, make up the volume to 100 ml with water and autoclave.

The concentration of the divalent cation (Ca<sup>2+</sup>) in the medium can be altered by changing the amount of CaCl<sub>2</sub> in the medium. The CaCl<sub>2</sub> can also be exchanged for MgCl<sub>2</sub>.

**A.2.10.22 PEG medium**

PEG (4000) 40%	3.2 g
CaCl <sub>2</sub> (0.1 M)	0.116 g
Mannitol (0.4 M)	0.58 g
NaCl (0.5 M)	0.232 g
water	6 ml

Heat to dissolve. Volume will increase to approximately 8 ml. Filter sterilize.  
Prepare fresh weekly.

**A.2.10.23 0.125 M NaCl - filter-sterilized natural seawater**

**0.25 M NaCl – seawater**

NaCl	1.46 g
Natural seawater	100 ml

Filter sterilize.

Refrigerate and prepare fresh weekly.

0.125 M NaCl - filter-sterilized natural seawater

Dilute 0.25 M NaCl - seawater 1:2 in filter-sterilized seawater.

**A.2.10.24 BASTA<sup>®</sup> solution**

BASTA<sup>®</sup> 4 mg ml<sup>-1</sup>

Dilute BASTA<sup>®</sup> 200 SL200 (Glufosinate ammonium 200 mg ml<sup>-1</sup>) 1:50 in sterile water.

**BASTA<sup>®</sup> Working solution 1 mg ml<sup>-1</sup>**

Dilute BASTA<sup>®</sup> (4 mg ml<sup>-1</sup>) 1:4 in sterile water.

## **APPENDIX B**

### **STANDARD METHODS**

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## Contents

B.1	Preparation of <i>E. coli</i> competent cells .....	201
B.1.1	Preparation of <i>E. coli</i> competent cells by CaCl <sub>2</sub> shock treatment .....	201
B.1.2	Preparation of <i>E. coli</i> competent cells by the RbCl method .....	201
B.2	Transformation of competent <i>E. coli</i> cells .....	202
B.3	Small-scale preparation of plasmid DNA .....	202
B.4	Restriction endonuclease digestions .....	202
B.5	Agarose gel electrophoresis .....	203
B.6	Repairing 3' and 5' overhanging ends to generate blunt ends .....	203
B.7	Dephosphorylation of vector DNA .....	204
B.8	Ligations .....	204
B.8.1	Intramolecular ligations .....	204
B.8.2	Intermolecular ligations .....	204
B.9	Genomic DNA isolations .....	205
B.9.1	Modified Wattier method for seaweed tissue .....	205
B.9.2	Modified Wattier method for protoplasts .....	206
B.9.3	Genomic DNA isolation using the DNeasy Plant Mini Kit (Qiagen) with minor alterations .....	206
B.10	Microparticle bombardment .....	207
B.10.1	Microcarrier preparation .....	207
B.10.2	DNA coating of microcarriers .....	207
B.11	Bradford protein quantitation (Microtitre plate procedure).....	208
B.12	β-Galactosidase enzyme assay .....	208
B.13	OptiPrep <sup>®</sup> discontinuous density gradient .....	209
B.14	Plate pool screening method.....	209
B.15	PCR Protocols.....	211
B.15.1	PCR amplification of CMV promoter.....	211
B.15.2	PCR amplification of CaMV promoter.....	212
B.15.3	PCR amplification of <i>lacZ</i> product from bombarded thalli.....	213
B.15.4	PCR amplification of 18S product from bombarded thalli.....	214
B.15.5	PCR amplification of MAReco product .....	215
B.15.6	PCR amplification of MARpstxba product.....	216
B.15.7	PCR amplification of 18S product for cloning .....	217
B.15.8	Plate pool/ colony PCR amplification of 18S product.....	218
B.15.9	Colony PCR amplification of <i>egfp</i> product .....	219
B.15.10	PCR amplification of <i>egfp</i> product from protoplast genomic DNA.....	220
B.15.11	PCR amplification of 18S product from protoplast genomic DNA.....	221

B.15.12 PCR amplification of integration product.....	222
B.15.13 Plate pool/ colony PCR amplification of <i>bar</i> product .....	227
B.15.14 PCR amplification of <i>bar</i> product from protoplast genomic DNA .....	228

## APPENDIX B

### STANDARD METHODS

#### B.1 Preparation of *E. coli* competent cells

##### B.1.1 Preparation of *E. coli* competent cells by CaCl<sub>2</sub> shock treatment

(Dagert and Ehrlich, 1979)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate, into 5 ml  $\Psi$ -broth (Appendix A.1.6) and incubate for 16 hrs at 37 °C with shaking. Inoculate this starter culture into 100 ml pre-warmed  $\Psi$ -broth and incubate as before, until the A<sub>600</sub> reaches between 0.3 - 0.6. Transfer the 100 ml culture to a pre-chilled (-20 °C) GSA centrifuge tube (Beckman) and harvest the cells by centrifugation (3,000x *g* for 5 min at 4 °C). Discard the supernatant fraction, gently resuspend the cells in 100 ml of ice-cold 0.1 M MgCl<sub>2</sub> (Appendix A.2.5) and incubate on ice for one min. Collect the cells as before, gently resuspend them in 50 ml of ice-cold 0.1 M CaCl<sub>2</sub> (Appendix A.2.5) and incubate on ice for approximately 2 hrs. Harvest the cells as before, and gently resuspend them in 10 ml ice cold 0.1 M CaCl<sub>2</sub>. Aliquot (0.1 ml) into sterile microfuge tubes, on ice, and transform with plasmid DNA (Appendix B.2).

##### B.1.2 Preparation of *E. coli* competent cells by the RbCl method

(Draper *et al.*, 1988)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate, into 5 ml  $\Psi$ -broth and incubate for 16 hrs at 37 °C with shaking. Inoculate this starter culture into 100 ml pre-warmed  $\Psi$ -broth and incubate, as before, until the A<sub>600</sub> reaches 0.35 (the culture contains approximately 3.5–4.0 x 10<sup>7</sup> cells ml<sup>-1</sup>). Transfer the 100 ml culture to a pre-chilled (-20 °C) GSA centrifuge tube and chill on ice for 15 min. Collect the cells by centrifugation (3,000x *g* for 5 min at 4 °C) and discard the supernatant fraction. Gently resuspend the cells in 21 ml ice-cold TFB 1 (Appendix A.2.5) and incubate on ice for 90 min. Collect the cells as before and resuspend in 3.5 ml ice-cold TFB 2 solution (Appendix A.2.5). Aliquot (100  $\mu$ l) resuspended competent cells into sterile microfuge tubes, rapidly freeze in liquid nitrogen and store at -70 °C.

## **B.2 Transformation of competent *E. coli* cells**

Thaw the 0.1 ml aliquots of frozen *E. coli* competent cells (Appendix B.1.2) on ice for 10 min until just molten, or incubate freshly prepared 0.1 ml aliquots of *E. coli* competent cells (Appendix B.1.1) on ice for 10 min. Add 10 ng of plasmid DNA to 100  $\mu$ l of thawed competent cells and incubate on ice for 20 min. Heat shock cells at 37 °C for 1 min and immediately incubate on ice for 2 min, before adding 0.8 ml  $\Psi$ -broth and incubating at 37 °C, with shaking, for 30 - 60 min to allow expression. Thereafter, plate 100  $\mu$ l aliquots of the *E. coli* cells onto Luria-Bertani solid media (LA) (Appendix A.1.6) containing an appropriate antibiotic selection and incubate for 16 hrs at 37 °C.

## **B.3 Small-scale preparation of plasmid DNA**

(Ish-Horowicz and Burke, 1981)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate into 5 ml Luria-Bertani broth (LB) (Appendix A.1.4) with antibiotic selection, and incubate for 16 hrs at 37 °C with shaking. Harvest the cells by centrifugation (13,000x *g* for 1 min at 22 °C), resuspend the bacterial pellet in 0.2 ml Solution 1 (Appendix A.2.7) and incubate for 10 min at 22 °C. Lyse the cells by adding 0.4 ml Solution 2 (Appendix A.2.7), gently mix by inverting the microfuge tube several times and incubate at 22 °C for 5 min. To this mixture, add 0.3 ml of ice-cold Solution 3 (Appendix A.2.7) and incubate on ice for 10 min. Pellet the cell debris by centrifugation (13,000x *g* for 5 min at 22 °C), recover the supernatant fraction containing the plasmid DNA and transfer it to a sterile microfuge tube. Precipitate the plasmid DNA by the addition of 0.7 volumes of isopropanol. Mix the solution and centrifuge (13,000x *g* for 15 min at 4 °C) to pellet the plasmid DNA. Gently remove the supernatant, wash the pellet in 70% (v/v) ethanol (Appendix A.2.3) and centrifuge as before. Remove the supernatant and air-dry the pellet before resuspending it in 30  $\mu$ l of TE buffer containing RNase (Appendix A.2.4).

## **B.4 Restriction endonuclease digestions**

(Ausubel *et al.*, 1989 unit 3.1; [www.fermentas.com](http://www.fermentas.com))

All restriction enzymes and their respective buffers were obtained from Fermentas. A total of 0.5 to 10  $\mu$ g of plasmid DNA is pipetted into a sterile microfuge tube. The appropriate

restriction enzyme buffer is added to a final concentration of 1x, or where required 2x (as recommended by the manufacturer) and the volume adjusted to 18  $\mu\text{l}$  with sterile water. Add restriction endonuclease (1 to 5 U  $\mu\text{g}^{-1}$  DNA) and make up to a final volume of 20  $\mu\text{l}$  with sterile water. Pulse tube briefly in a bench-top centrifuge and incubate the reaction mixture for 16 hrs in a water bath at the appropriate temperature ( $^{\circ}\text{C}$ ). Stop the restriction endonuclease reaction by heat inactivation (as recommended by the manufacturer) and/or adding 4  $\mu\text{l}$  tracking dye (Appendix A.2.6).

### **B.5 Agarose gel electrophoresis**

(Ausubel *et al.*, 1989 unit 2.5)

Melt the agarose (Hispanagar D1 LE) in 1x TAE (Appendix A.2.6) by heating in a microwave. Agarose concentrations can vary from 2% (w/v) for separating small DNA fragments to 0.7% (w/v) for separating larger DNA fragments such as restriction enzyme digested chromosomal DNA. Add ethidium bromide (Appendix A.2.3) solution to the melted agarose to a final concentration of 0.5  $\mu\text{g ml}^{-1}$ . Allow agarose to cool before pouring into a gel-casting platform that has been sealed with masking tape and has a gel comb approximately 1 cm from the top of the gel-casting platform. After the gel has hardened, remove the masking tape from the gel-casting platform and carefully withdraw the comb ensuring that the wells are not damaged in the process. Place the gel-casting platform containing the set gel into an electrophoresis tank and add sufficient 1x TAE buffer to cover the gel. Load DNA samples into the wells of the gel. Attach leads so that DNA migrates into the gel towards the anode. Electrophorese the gel at 1 to 10 V  $\text{cm}^{-1}$  until the dye reaches the end of the gel. Visualize the DNA on a Gel Doc XR (Bio-Rad) system using Quantity One Version 4.5.2 Software.

### **B.6 Repairing 3' and 5' overhanging ends to generate blunt ends**

(Ausubel *et al.*, 1989 unit 3.5.8)

Prepare the reaction mixture by adding the restriction enzyme digested DNA (1 - 4  $\mu\text{g}$ ), 2  $\mu\text{l}$  of 10x Klenow Fragment reaction buffer, 5 U Klenow Fragment and 1  $\mu\text{l}$  of 0.5 mM dNTP mix into a sterile microfuge tube and make up to a total volume of 20  $\mu\text{l}$  with nuclease-free water. Incubate the reaction mixture at 37  $^{\circ}\text{C}$  for 30 min and stop the reaction by incubating the reaction mix at 75  $^{\circ}\text{C}$  for 10 min.

## **B.7 Dephosphorylation of vector DNA**

(Coyne *et al.*, 2002)

Calf intestinal phosphatase (CIP) is commonly used to enzymatically remove the 5'-phosphate groups from linearised DNA. This prevents linearised vector DNA recircularising in an intermolecular ligation.

Heat-inactivate the restriction enzyme digest according to the restriction enzyme manufacturer's instructions. Prepare the reaction mixture by adding the heat inactivated restriction enzyme digested DNA (1 -20 pmol ends), 3 µl of 10x CIP buffer, 0.1 U CIP into a sterile microfuge tube and make up to a total volume of 30µl with sterile water. Incubate the reaction mixture at 37 °C for 30 min and then stop the reaction by incubating the reaction mix at 65 °C for 10 min. Prepare the DNA for ligation by ethanol precipitation (Appendix B.11) or electrophoresis on an agarose gel followed by gel extraction using a commercial kit.

## **B.8 Ligations**

(Coyne *et al.*, 2002)

### **B.8.1 Intramolecular ligations**

In order to recircularize plasmid DNA for the construction of deletion subclones, use approximately 1 pmol of plasmid DNA. Add 2 µl of 10x T4 ligase buffer (Fermentas) to the plasmid DNA in a sterile microfuge tube. Adjust the volume to 18 µl with sterile nuclease-free water, add 2 U of T4 ligase (Fermentas) and incubate the ligation reaction for approximately 16 hrs at 15 °C.

### **B.8.2 Intermolecular ligations**

In order to ligate two different DNA fragments (vector and insert) the total DNA concentration should not exceed 10 pmol and the ratio of vector to insert (V : I) should be in the range of 1:1 to 1:4 pmol. Add 2 µl of 10x T4 ligase buffer (Fermentas) to the DNA fragments in a sterile microfuge tube. Adjust the volume to 18 µl with sterile nuclease-free water, and add 2 U of T4 ligase (Fermentas). For the ligation of DNA fragments with cohesive ends, incubate the reaction overnight at 15 °C. For the ligation of DNA fragments

with blunt ends, use 10x more T4 ligase enzyme and incubate the reaction for 4 to 16 hrs at 22 °C.

## **B.9 Genomic DNA isolations**

### **B.9.1 Modified Wattier method for seaweed tissue**

(Wattier *et al.*, 2000)

Grind a total of 0.1 g of *G. gracilis* thalli in liquid nitrogen using a sterile mortar and pestle. Transfer the ground tissue into a 2 ml sterile eppendorf tube containing 1.5 ml Working Extraction Buffer (WEB) (Appendix A.2.6). Incubate tubes horizontally with shaking at 37°C for 1 hr. Pellet the thallus debris by centrifugation (20,000x g for 15 min at 4 °C) and split the resulting supernatant (approximately 1.4 ml) equally between two fresh tubes. Extract with an equal volume of phenol/chloroform/isoamyl alcohol (Appendix A.2.6) and separate the phases by centrifugation (20,000x g for 5 min at 22 °C). Remove the aqueous phase to a clean eppendorf tube and extract with an equal volume of chloroform/iso-amyl alcohol (Appendix A.2.6). Separate the phases by centrifugation as described before. Pool the aqueous phases of two extractions into one tube and add 5 µl of RNase (10 mg ml<sup>-1</sup>) (Fermentas) (Appendix A.2.6). Incubate at 37 °C for 30 min followed by a 30 min incubation on ice in order to precipitate any polysaccharides that may have carried through the extraction. Centrifuge to pellet any precipitated matter as described before. Transfer the supernatant to a sterile eppendorf tube and precipitate the genomic DNA by the addition of 2.5 volumes of ice cold 95% (v/v) ethanol (Appendix A.2.6) followed by gentle mixing by inverting the tubes. Incubate the tubes at -20 °C over night. Pellet the genomic DNA by centrifugation (20,000x g for 15 min at 4°C). Gently remove the supernatant, wash the pellet in 70% (v/v) ethanol (Appendix A.2.1) and centrifuge as before. Remove the supernatant and air-dry the pellet before resuspending it in 50 µl of TE buffer (Appendix A.2.1). Quantitate the chromosomal DNA using the Nanodrop<sup>®</sup> ND-100 spectrophotometer (Nano Drop Technologies, Inc.) and assess the integrity of the chromosomal DNA by electrophoresis of 500 ng of DNA on a 0.7% (w/v) TAE (Appendix A.2.3) agarose gel (Appendix B.5) containing ethidium bromide (Appendix A.2.3).

### **B.9.2 Modified Wattier method for protoplasts**

Protoplast pellets are thawed on ice. 0.4 ml Working Extraction Buffer (WEB) (Appendix A.2.6) is added to the protoplasts and tubes are incubated at 37°C for 1 hr. Extract with an equal volume of phenol/chloroform/isoamyl alcohol (Appendix A.2.6) and separate the phases by centrifugation (20,000x g for 5 min at 22 °C). Remove the aqueous phase to a clean eppendorf tube and extract with an equal volume of chloroform/iso-amyl alcohol (Appendix A.2.6). Separate the phases by centrifugation as described before. Add 2 µl of RNase (10 mg ml<sup>-1</sup>) (Fermentas) (Appendix A.2.6) and incubate at 37 °C for 30 min. Precipitate the genomic DNA by the addition of 2.5 volumes of ice cold 95% (v/v) ethanol (Appendix A.2.6) followed by gentle mixing by inverting the tubes. Incubate the tubes at -20 °C over night. Pellet the genomic DNA by centrifugation (20,000x g for 15 min at 4°C). Gently remove the supernatant, wash the pellet in 70% (v/v) ethanol (Appendix A.2.1) and centrifuge as before. Remove the supernatant and air-dry the pellet before resuspending it in 30 µl of TE buffer (Appendix A.2.1). Quantitate the chromosomal DNA using the Nanodrop<sup>®</sup> ND-100 spectrophotometer (Nano Drop Technologies, Inc.) and assess the integrity of the chromosomal DNA by electrophoresis of 500 ng of DNA on a 0.7% (w/v) TAE (Appendix A.2.3) agarose gel (Appendix B.5) containing ethidium bromide (Appendix A.2.3).

### **B.9.3 Genomic DNA isolation using the DNeasy Plant Mini Kit (Qiagen) with minor alterations**

The protocol for purification of total DNA from plant tissues (mini protocol) is done according to manufacturer's instructions, outlined in the DNeasy<sup>®</sup> Plant Handbook, with minor alterations. Alterations are as follows: Tissue lysis after the addition of Buffer AP1 is performed at 37 °C for 10 min and not at 65 °C as indicated in the DNeasy<sup>®</sup> Plant Handbook. Prior to pipetting the lysate onto to QIAshredder Mini spin column, the lysate is centrifuged twice for 5 min at 20 000x g, instead of just once. DNA elution was done three times using 50 µl for the first elution and 100 µl each for the second and third elutions. Elutions two and three were pooled and genomic DNA was quantitated using the Nanodrop<sup>®</sup> ND-100 spectrophotometer (Nano Drop Technologies, Inc.). The integrity of the genomic was assessed by electrophoresis of 500 ng of DNA through 0.7% (w/v) TAE (Appendix A.2.3) agarose gel (Appendix B.5) containing ethidium bromide (Appendix A.2.3).

## **B.10 Microparticle bombardment**

### **B.10.1 Microcarrier preparation**

(Adapted from Dunder *et al.*, 1995)

Weigh out 60 mg of gold microparticles. Add 1 ml of absolute ethanol to the tube containing the microparticles and vortex at high speed for 1 min. Pellet the particles by pulsing in a centrifuge. The centrifugation should not exceed 1 min. Discard the resulting supernatant carefully and repeat the absolute ethanol described before a further three times. Following this, add 1 ml sterile dH<sub>2</sub>O and vortex at high speed for 1 min. Pellet the gold particles as previously described and discard the supernatant carefully. Repeat the sterile dH<sub>2</sub>O wash once more. Add 1 ml sterile 50% glycerol (Appendix A.2.7) to the washed gold particles. Vortex the tube to resuspend the gold particles. The gold should be aliquoted into 50 µl aliquots while vortexing to prevent agglomeration of the particles. Each tube will contain 3 mg of gold particles and once these are DNA-coated, will be sufficient for six bombardments. Aliquots are stored at room temperature.

### **B.10.2 DNA coating of microcarriers**

(Dunder *et al.*, 1995)

This procedure describes the precipitation of DNA onto one tube of gold microcarriers. Place the gold on the vortex and allow mixing at high speed for 5 min. Reduce the speed of the vortex and add 6 µg of plasmid DNA and 50 µl 2.5 M CaCl<sub>2</sub> (Appendix A.2.7) to the tube of gold microcarriers. Mix the tube at high speed on the vortex for 30 s. Add 20 µl of 0.1 M Spermidine (Appendix A.2.7) by reducing the speed of the vortex. Vortex the tube at high speed for 3 min. Pellet the particles by centrifugation as described in Appendix B.10.1. Remove the supernatant, being careful to leave the microparticles behind. Add 250 µl absolute ethanol to the pelleted microparticles and mix at high speed on the vortex for 1 min. Pellet the coated microparticles as described before and carefully remove the supernatant. Add 75 µl absolute ethanol and resuspend the DNA-coated microcarriers by vortexing. This precipitation will yield enough DNA-coated microcarriers for six bombardments, delivering 0.48 mg of gold microcarriers coated with 1 µg of plasmid DNA. If fewer bombardments are needed, prepare enough microcarriers for three bombardments by reducing all volumes by one half.

### **B.11 Bradford protein quantitation (Microtitre plate procedure)**

(Bradford, 1976)

The Bio-Rad Protein Assay was used. Protein Assay Dye Reagent Concentrate (Bio-Rad) is diluted 1:5 with sterile dH<sub>2</sub>O. Prepare BSA standards (0.5-0.015 mg ml<sup>-1</sup>) by performing doubling dilutions of a 1 mg ml<sup>-1</sup> stock of BSA (Appendix A.x) in dH<sub>2</sub>O (or the buffer in which the protein is). Aliquot 10 µl of the protein sample or BSA standard into the wells in triplicate. Add 200 µl of diluted Protein Assay Dye Reagent and allow to stand for 5 min. Absorbance is read at 595 nm using a spectrophotometer. An assay blank is prepared by replacing the sample with 10 µl of dH<sub>2</sub>O (or buffer). A standard curve of protein concentration is constructed by plotting the absorbance values against the known concentrations of the BSA standards. The standard curve can then be used to calculate the protein concentration in the samples.

### **B.12 β-Galactosidase enzyme assay**

Grind a total of 0.3 g of *G. gracilis* thalli in liquid nitrogen using a sterile mortar and pestle. Extract briefly in 1 ml Z buffer containing β-mercaptoethanol (Appendix A.2.9). The extract was clarified by microcentrifugation and the protein content was determined using the standard Bradford protein assay (Bradford 1976, Appendix B.11). Add 150 µl of the protein extract to 600 µl of Z buffer containing β-mercaptoethanol and mix before the addition of 75 µl of the ONPG substrate (Appendix A.2.9). Samples are placed at 37 °C for 2 hrs before the addition of 325 µl of 1 M Na<sub>2</sub>CO<sub>3</sub>. Samples are centrifuged before the absorbance is read at 420 nm. Assays are performed in duplicate for each protein extraction. An assay blank is performed by adding only Z buffer and substrate. In order to control for the coloured compounds released from the algal tissue itself, a blank reaction with no substrate is performed for each sample. Samples are quantitated against a standard curve. The standard curve is prepared by employing known concentrations (1, 2, 3, 4, 5 and 6 mU) of β-galactosidase from *E. coli* (Sigma) in the enzyme assay. Activity data is expressed as specific activity, i.e. total activity in mU per mg of total soluble protein (mU mg<sup>-1</sup> protein). One unit of β-galactosidase is defined as the amount of enzyme that will hydrolyze 1 µmol of ONPG to o-nitrophenol and D-galactose per min at pH 7.5 at 37 °C

### **B.13 OptiPrep<sup>®</sup> discontinuous density gradient**

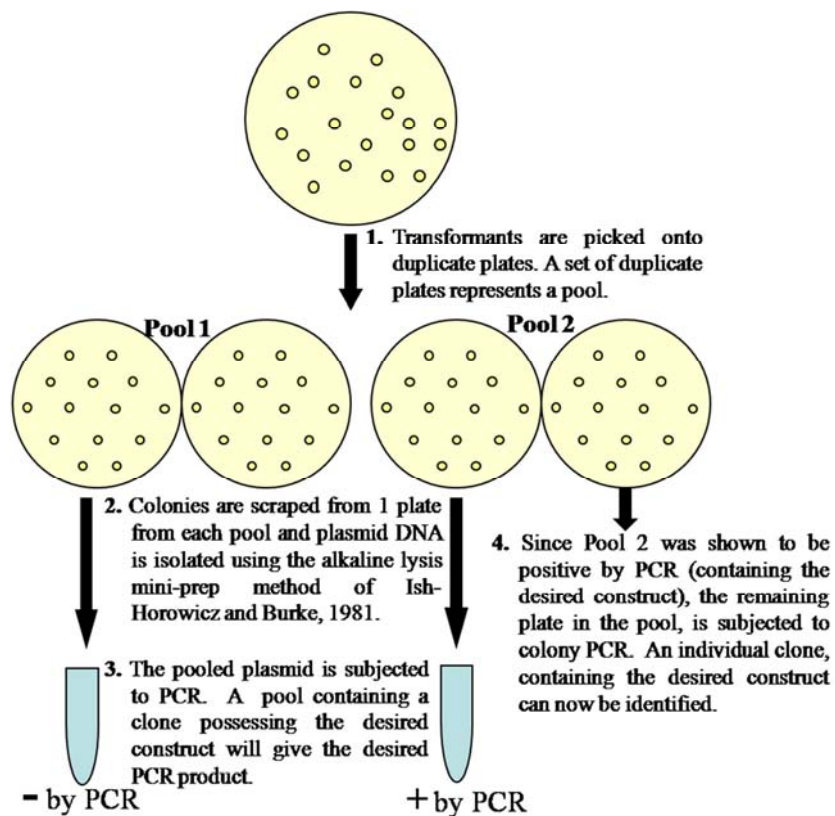
Solutions for the OptiPrep<sup>®</sup> discontinuous density gradient are prepared as described in Appendix A.2.10.19. This protocol describes the preparation of small scale gradients in 2 ml eppendorf tubes. Gradients can also be prepared in 10 ml sterilin tubes and volumes should be adjusted accordingly. A volume of 400  $\mu$ l of culture medium (ASW or 0.4 M sorbitol-ASW) is added into the tube first. The second layer (500  $\mu$ l) of the density gradient is then added by upward displacement. This is done by placing the pipette tip containing the solution at the bottom of the tube, and gently pipetting the solution in to displace the culture medium. A clear boundary between the two solutions can clearly be seen. The third and bottom layer of the density gradient, containing the protoplasts, is added as described above. Again a clear boundary between the various layers will be visible. Gradients are then centrifuged (160x *g* for 10 min at 22 °C). During centrifugation, the protoplasts migrate to the interface between the top two layers of the gradient and are visible as a red-brown band. Protoplasts can then be collected with a pipette. If protoplasts are still visible at the lower interface, the gradient may be centrifuged again and protoplasts collected as described.

### **B.14 Plate pool screening method**

This method can be used to screen large numbers of transformants following a fairly difficult ligation, i.e. blunt-end ligations or ligations with more than one insert, particularly when blue-white screening is not possible

After the overnight incubation on a selective medium, *E. coli* DH5 $\alpha$  capable of growth on ampicillin (or other antibiotics) are picked onto duplicate plates in a set layout, using sterile toothpicks (Fig. B.1). A set of duplicate plates will constitute a pool. The numbers of pools is dependent on the initial number of transformants obtained, or on how many wish to be screened. The pool plates are incubated overnight at 37 °C. One plate from each pool is then used for plasmid isolation as follows. The plate is flooded with 2 ml of LB (Appendix A.1.5) and the colonies are scraped off using a sterile spreader. The cell suspension is pipetted into a sterile microfuge tube and the cells pelleted by centrifugation (20,000x *g* for 1 min). The pool of plasmid DNA can now be isolated from the pool of colonies using the alkaline lysis mini-prep method of Ish-Horowicz and Burke, 1981 (Appendix B.3). The resulting pools of plasmid are screened for the presence the desired fragment by PCR. A positive signal by

PCR would indicate that that specific pool contains one or more recombinant plasmids originally isolated from a positive clone. The remaining plate in a positive pool can then be used for colony PCR in order to identify a positive clone.



**Figure B.1.** Schematic representation of plate pool screening method.

## B.15 PCR Protocols

### B.15.1 PCR amplification of CMV promoter

#### Primers

CMV<sub>pro-F</sub> 5' - TTGACCGAGAATTCCATGAAG - 3'

CMV<sub>pro-R</sub> 5' - CGCTAGCAAGCTTGGGTCT - 3'

#### PCR Protocol

This is an optimized PCR protocol for 25 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume (µl)
pcDNA3.1/Zeo/CAT (25 ng µl <sup>-1</sup> )	0.5
MgSO <sub>4</sub> (25 mM)	4
PCR buffer (10x)	2.5
Primer CMV <sub>pro-F</sub> (10 µM)	1
Primer CMV <sub>pro-R</sub> (10 µM)	1
<i>Pfu</i> Polymerase (0.6 U µl <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 30
55	30	
72	120	
72	300	1

**B.15.2 PCR amplification of CaMV promoter****Primers**

CaMVpro-F 5' - ATCTCAGAATTCCAATCCC - 3'

CaMVpro-R 5' - GTAAAAATAAAGCTTTTATACTCG - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
pEarleyGate201 $\Delta$ <i>ccdB</i> (25 ng $\mu$ l <sup>-1</sup> )	0.5
MgSO <sub>4</sub> (25 mM)	4
PCR buffer (10x)	2.5
Primer CaMVpro-F (10 $\mu$ M)	1
Primer CaMVpro-R (10 $\mu$ M)	1
<i>Pfu</i> Polymerase (0.6 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
TOTAL	25

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 30
50	30	
72	120	
72	300	1

**B.15.3 PCR amplification of *lacZ* product from bombarded thalli****Primers**

lacZ-F 5' - GGTTGAACTGCACACCGCCG - 3'

lacZ-R 5' - GATGGACCATTTCGGCACAG - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (50 ng $\mu$ l <sup>-1</sup> ) or	2 /
pSV- $\beta$ -Galactosidase (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer lacZ-F (10 $\mu$ M)	1
Primer lacZ-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
63	30	
72	60	
72	300	1

**B.15.4 PCR amplification of 18S product from bombarded thalli****Primers**

18S-F 5' - GAGTGAATTGTACAACGAAACTGC - 3'

18S-R 5' - GATCTGAATAATCAGTTCATCTAGC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (50 ng $\mu$ l <sup>-1</sup> ) or	1 /
p18S-21 (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer 18S-F (10 $\mu$ M)	1
Primer 18S-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
60	30	
72	60	
72	300	1

**B.15.5 PCR amplification of MAReco product****Primers**

MAReco-F 5' - ATATCCATTGAATTCCATGATCATTCCGCC - 3'

MAReco-R 5' - CTTTGGACCGAATTCGCCCGCGCC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu\text{l}$  reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu\text{l}$ )
pTRAc (10 ng $\mu\text{l}^{-1}$ )	2
MgSO <sub>4</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer MAReco-F (10 $\mu\text{M}$ )	1
Primer MAReco-R (10 $\mu\text{M}$ )	1
<i>Pfu</i> Polymerase (0.6 U $\mu\text{l}^{-1}$ )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
60	30	
72	180	
72	300	1

**B.15.6 PCR amplification of MARpstxba product****Primers**

MARxba-F 5' - ATATCCATTCTAGAGCAAGTCAATTCCGCC - 3'

MARpst-R 5' - GGTCTTTGGCTGCAGTGATCACCGCGCC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume (µl)
pTRAc (10 ng µl <sup>-1</sup> )	2
MgSO <sub>4</sub> (25 mM)	3.5
PCR buffer (10x)	2.5
Primer MARxba-F (10 µM)	1
Primer MARpst-R (10 µM)	1
<i>Pfu</i> Polymerase (0.6 U µl <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
58	30	
72	180	
72	300	1

**B.15.7 PCR amplification of 18S product for cloning****Primers**

18S-F 5' - GAGTGAATTGTACAACGAAACTGC - 3'

18S-R 5' - GATCTGAATAATCAGTTCATCTAGC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
Genomic DNA (50 ng $\mu$ l <sup>-1</sup> )	2
MgSO <sub>4</sub> (25 mM)	4
PCR buffer (10x)	2.5
Primer 18S-F (10 $\mu$ M)	1
Primer 18S-R (10 $\mu$ M)	1
<i>Pfu</i> Polymerase (0.6 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
TOTAL	25

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	120	1
95	45	} 35
58	30	
72	210	
72	300	1

**B.15.8 Plate pool/ colony PCR amplification of 18S product****Primers**

18S-F 5' - GAGTGAATTGTACAACGAAACTGC - 3'

18S-R 5' - GATCTGAATAATCAGTTCATCTAGC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
Bacterial colony / pooled plasmid DNA	*
p18S-21 (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	1.5
PCR buffer (10x)	2.5
Primer 18S-F (10 $\mu$ M)	1
Primer 18S-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

\* Note that 1 ng of pooled plasmid DNA is added for every colony.

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
96	300	1
94	30	} 25
60	30	
72	60	
72	300	1

**B.15.9 Colony PCR amplification of egfp product****Primers**

egfp-F 5' - ATGGTGAGCAAGGGCGAGG - 3'

egfp-R 5' - CACGAACTCCAGCAGGACC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu\text{l}$  reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu\text{l}$ )
Bacterial colony /	
pSV-egfp (5 ng $\mu\text{l}^{-1}$ )	1
MgCl <sub>2</sub> (25 mM)	1.5
PCR buffer (10x)	2.5
Primer egfp-F (10 $\mu\text{M}$ )	1
Primer egfp-R (10 $\mu\text{M}$ )	1
Taq Polymerase (1 U $\mu\text{l}^{-1}$ )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature ( $^{\circ}\text{C}$ )	Time (s)	Cycles
96	300	1
94	30	} 25
57	30	
72	60	
72	300	1

**B.15.10 PCR amplification of egfp product from protoplast genomic DNA****Primers**

egfp-F 5' - ATGGTGAGCAAGGGCGAGG - 3'

egfp-R 5' - CACGAACTCCAGCAGGACC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (50 ng $\mu$ l <sup>-1</sup> ) /	1
pSV-egfp (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	1.5
PCR buffer (10x)	2.5
Primer egfp-F (10 $\mu$ M)	1
Primer egfp-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
57	30	
72	60	
72	300	1

**B.15.11 PCR amplification of 18S product from protoplast genomic DNA****Primers**

18S-F 5' - GAGTGAATTGTACAACGAAACTGC - 3'

18S-R 5' - GATCTGAATAATCAGTTCATCTAGC - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (15 ng $\mu$ l <sup>-1</sup> ) /	1
p18S-21 (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer egfp-F (10 $\mu$ M)	1
Primer egfp-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 40
60	30	
72	60	
72	300	1

**B.15.12 PCR amplification of integration product****Primers**

E18R 5' - CTACGGAAACCTTGTTACGACTTCTCC - 3'

egfp-R 5' - CACGAACTCCAGCAGGACC - 3'

**First round of PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (25 ng $\mu$ l <sup>-1</sup> )*	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer E18R (10 $\mu$ M)	1
Primer egfp-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

\* genomic DNA isolated from protoplasts transfected with pSV-egfp / p18S-SV-egfp

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 40
56	30	
72	90	
72	300	1

**Second round of PCR Protocol (nested)****Primers**

E18R            5' - CTACGGAAACCTTGTTACGACTTCTCC - 3'  
 egfp-2R        5' - CCTCGCCCTTGCTCACCAT - 3'

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
PCR template from previous PCR	2
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer E18R (10 $\mu$ M)	1
Primer egfp-2R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 40
54	30	
72	90	
72	300	1

**Controls****Plasmid positive and negative controls****Primers**

E18R	5' - CTACGGAAACCTTGTTACGACTTCTCC - 3'
egfp-2R	5' - CCTCGCCCTTGCTCACCAT - 3'
18S-R	5' - GATCTGAATAATCAGTTCATCTAGC - 3'

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
p18S-SV-egfp (5 ng $\mu$ l <sup>-1</sup> )	2
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer E18R / 18S-R (10 $\mu$ M)*	1
Primer egfp-2R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

\* Primers E18R and egfp-2R were employed for the plasmid negative control, while primers 18S-R and egfp-2R were employed for the plasmid positive control.

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 25
54	30	
72	90	
72	300	1

**Genomic DNA control****Primers**

E18R 5' - CTACGGAAACCTTGTTACGACTTCTCC - 3'

R18F 5' - CCTGGTTGATCCTGCCAGTGG - 3'

This is an optimized PCR protocol for 25  $\mu\text{l}$  reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu\text{l}$ )
genomic DNA ( $15 \text{ ng } \mu\text{l}^{-1}$ )	2
MgCl <sub>2</sub> (25 mM)	1.5
PCR buffer (10x)	2.5
Primer E18R (10 $\mu\text{M}$ )	1
Primer R18F (10 $\mu\text{M}$ )	1
Taq Polymerase ( $1 \text{ U } \mu\text{l}^{-1}$ )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
58	30	
72	60	
72	300	1

**Confirmation of integration PCR product identity****Primers**

E18R 5' - CTACGGAAACCTTGTTACGACTTCTCC - 3'

18S-R 5' - GATCTGAATAATCAGTTCATCTAGC - 3'

TSS-R 5' - GGTGGCGACCGGTACCAGACC - 3'

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
gel purified integration product (5 ng $\mu$ l <sup>-1</sup> ) / p18S-SV-egfp (20 ng $\mu$ l <sup>-1</sup> )	0.5
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer E18R / 18S-R (10 $\mu$ M)*	1
Primer TSS-R (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

\* Primers E18R and TSS-R were employed for the plasmid negative control, while primers 18S-R and TSS-R were employed for the plasmid positive control.

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 25
56	30	
72	60	
72	300	1

**B.15.13 Plate pool/ colony PCR amplification of *bar* product****Primers**

pat-f 5' - CCAGAAACCCACGTCATGCCAGTT - 3'

pat-r 5' - CTACATCGAGACAAGCACGGTCAACTT - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
Bacterial colony / pooled plasmid DNA	*
pSV- <i>bar</i> /egfp (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer pat-f (10 $\mu$ M)	1
Primer pat-r (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

\* Note that 1 ng of pooled plasmid DNA is added for every colony.

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
96	300	1
94	30	} 25
55	30	
72	60	
72	300	1

**B.15.14 PCR amplification of *bar* product from protoplast genomic DNA****Primers**

pat-f 5' - CCAGAAACCCACGTCATGCCAGTT - 3'

pat-r 5' - CTACATCGAGACAAGCACGGTCAACTT - 3'

**PCR Protocol**

This is an optimized PCR protocol for 25  $\mu$ l reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume ( $\mu$ l)
genomic DNA (50 ng $\mu$ l <sup>-1</sup> ) /	1
pSV- <i>bar</i> / <i>egfp</i> (5 ng $\mu$ l <sup>-1</sup> )	1
MgCl <sub>2</sub> (25 mM)	2
PCR buffer (10x)	2.5
Primer pat-f (10 $\mu$ M)	1
Primer pat-r (10 $\mu$ M)	1
Taq Polymerase (1 U $\mu$ l <sup>-1</sup> )	1
dNTPs (2.5 mM)	2
Sterile dH <sub>2</sub> O to	25
<b>TOTAL</b>	<b>25</b>

The PCR cycle profile:

Temperature (°C)	Time (s)	Cycles
95	60	1
95	30	} 35
55	30	
72	60	
72	300	1

## **APPENDIX C**

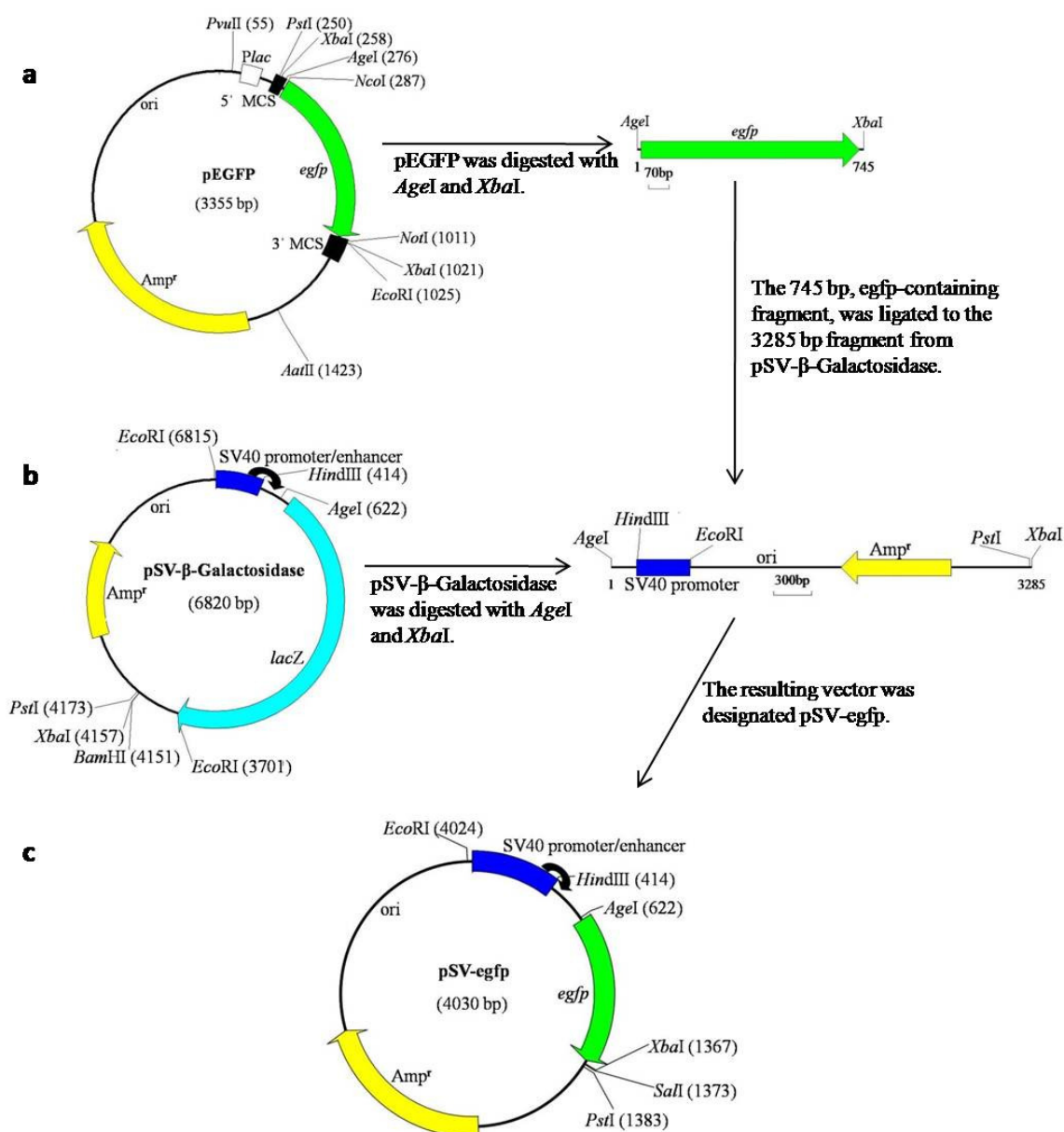
### **CONSTRUCTION OF VECTORS FOR MICROPARTICLE BOMBARDMENT**

University of Cape Town

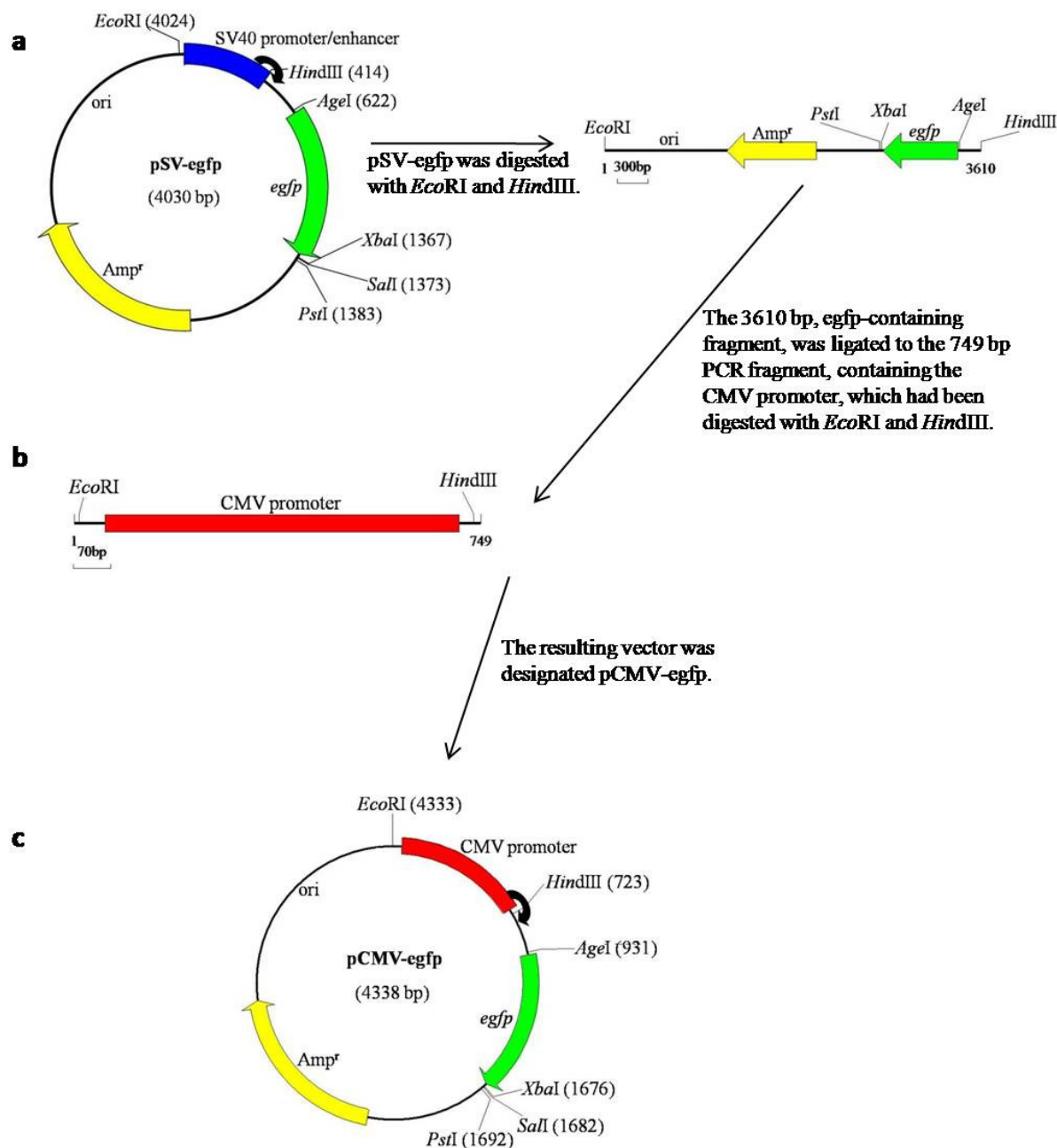
## Contents

C.1	Construction of pSV-egfp .....	231
C.2	Construction of pCMV-egfp .....	232
C.3	Construction of pCMV- $\beta$ -Galactosidase .....	233
C.4	Construction of pCaMV-egfp .....	234
C.5	Construction of pCaMV- $\beta$ -Galactosidase.....	235

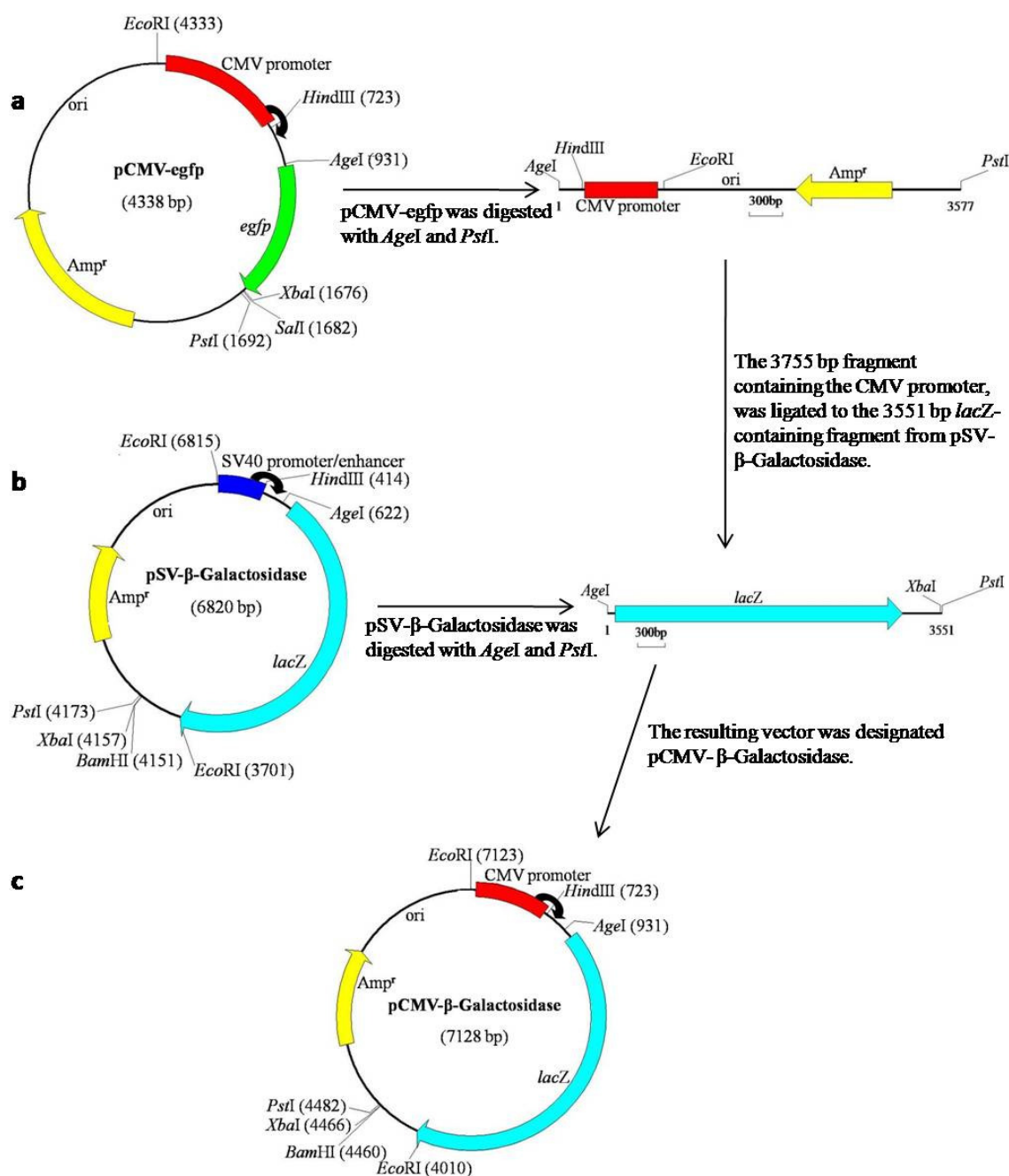
University of Cape Town



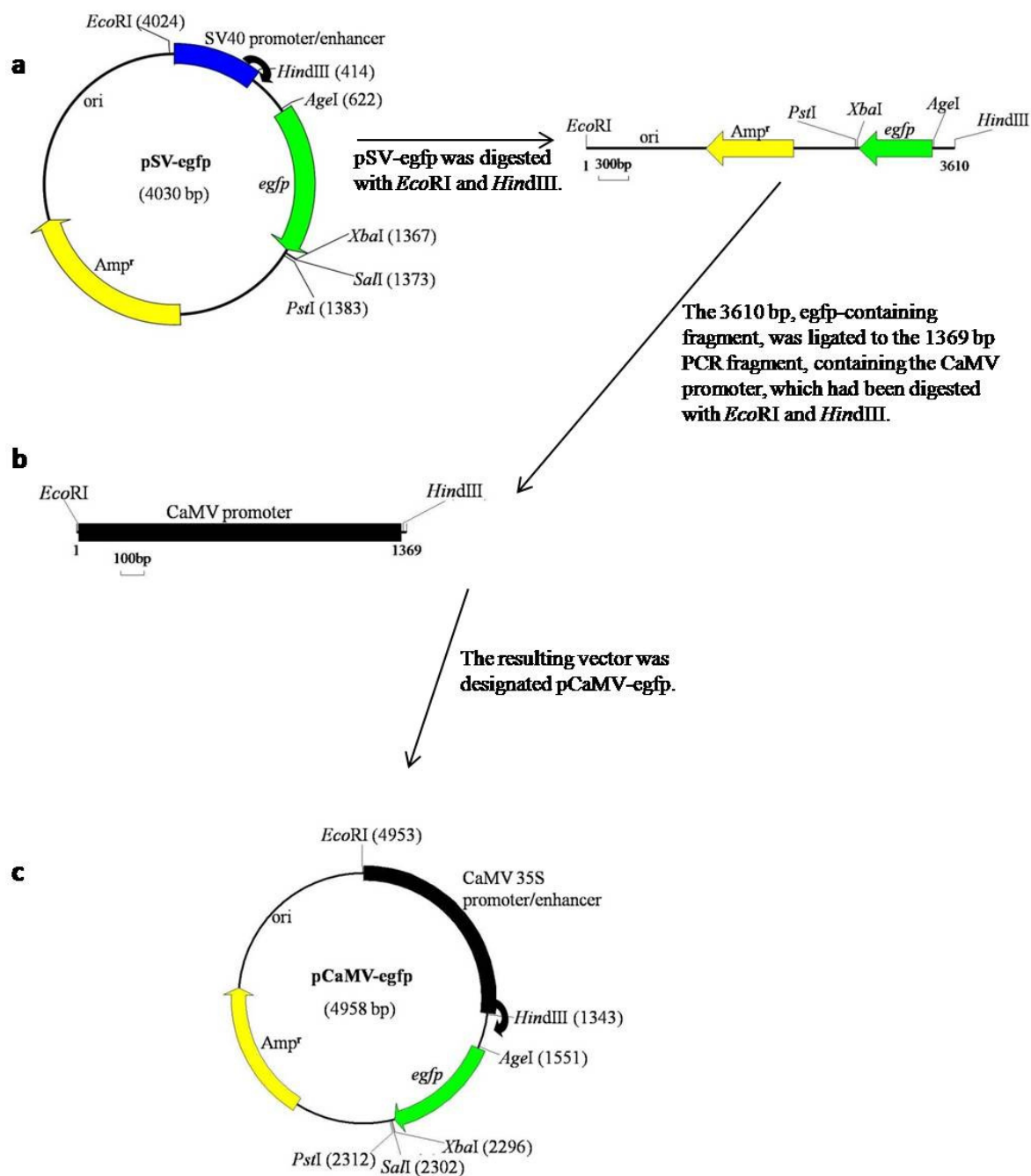
**Figure C1** Schematic representation (a-c) of the construction of the expression vector pSV-egfp (c). A 745 bp *egfp*-containing fragment was excised from pEGFP (BD Biosciences) using the restriction enzymes *AgeI* and *XbaI* (a). The fragment was ligated to a 3285 bp fragment which had been excised from pSV-β-Galactosidase using the restriction enzymes *AgeI* and *XbaI* (b). This sub-cloning strategy resulted in the substitution of *lacZ* with *egfp*. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, *Amp<sup>r</sup>*; ➔, *lacZ*; ➔, indicates direction of transcription from the promoter; —, SV40 promoter/enhancer.



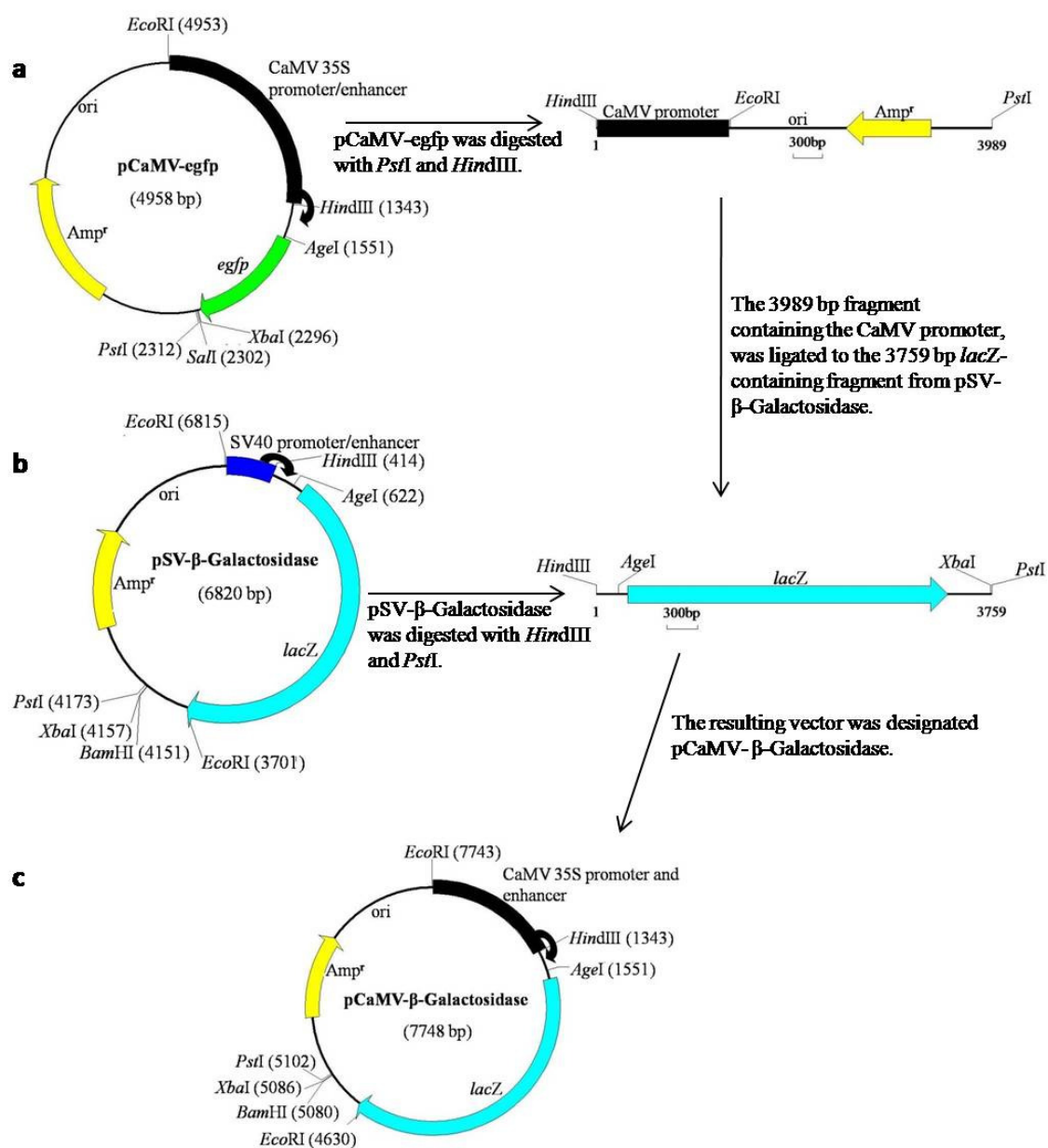
**Figure C2** Schematic representation (a-c) of the construction of the expression vector pCMV-egfp (c). A 3610 bp egfp-containing fragment was excised from pSV-egfp using the restriction enzymes *Eco*RI and *Hind*III (a). The fragment was ligated to a 749 bp CMV promoter-containing fragment which had been PCR amplified from pcDNA3.1/Zeo/CAT (Invitrogen) (not shown) and restricted using the restriction enzymes *Eco*RI and *Hind*III (b). This sub-cloning strategy resulted in the substitution of SV40 promoter/enhancer with the CMV promoter. Relevant restriction sites are shown. Elements of vectors are as follows: ➡, egfp; ➡, Amp<sup>r</sup>; ➡, indicates direction of transcription from the promoter; —, SV40 promoter/enhancer; —, CMV promoter.



**Figure C3** Schematic representation (a-c) of the construction of the expression vector pCMV-β-Galactosidase (c). A 3755 bp fragment was excised from pCMV-egfp using the restriction enzymes *AgeI* and *PstI* (a). The fragment was ligated to a 3551 bp *lacZ*-containing fragment which had been excised from pSV-β-Galactosidase using the restriction enzymes *AgeI* and *PstI* (b). This sub-cloning strategy resulted in the substitution of *egfp* with *lacZ*. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, *Amp<sup>r</sup>*; ➔, *lacZ*; ➔, indicates direction of transcription from the promoter; —, SV40 promoter/enhancer; —, CMV promoter.



**Figure C4** Schematic representation (a-c) of the construction of the expression vector pCaMV-egfp (c). A 3610 bp egfp-containing fragment was excised from pSV-egfp using the restriction enzymes *EcoRI* and *HindIII* (A). The fragment was ligated to a 1369 bp CaMV 35S promoter-containing fragment which had been PCR amplified from pEarleyGate201-ccDB (Earley *et al.*, 2006; Smart unpublished) (not shown) and restricted using the restriction enzymes *EcoRI* and *HindIII* (b). This sub-cloning strategy resulted in the substitution of the SV40 promoter/enhancer with the CaMV 35S promoter. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, egfp; ➔, Amp<sup>r</sup>; ➔, indicates direction of transcription from the promoter; —, SV40 promoter/enhancer; —, CaMV 35S promoter.



**Figure C5** Schematic representation (a-c) of the construction of the expression vector pCaMV-β-Galactosidase (c). A 3989 bp fragment was excised from pCaMV-egfp using the restriction enzymes *HindIII* and *PstI* (A). The fragment was ligated to a 3759 bp *lacZ*-containing fragment which had been excised from pSV-β-Galactosidase using the restriction enzymes *HindIII* and *PstI* (b). This sub-cloning strategy resulted in the substitution of *egfp* with *lacZ*. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, Amp<sup>r</sup>; ➔, *lacZ*; ➔, indicates direction of transcription from the promoter; —, SV40 promoter/enhancer; —, CaMV 35S promoter.

## **APPENDIX D**

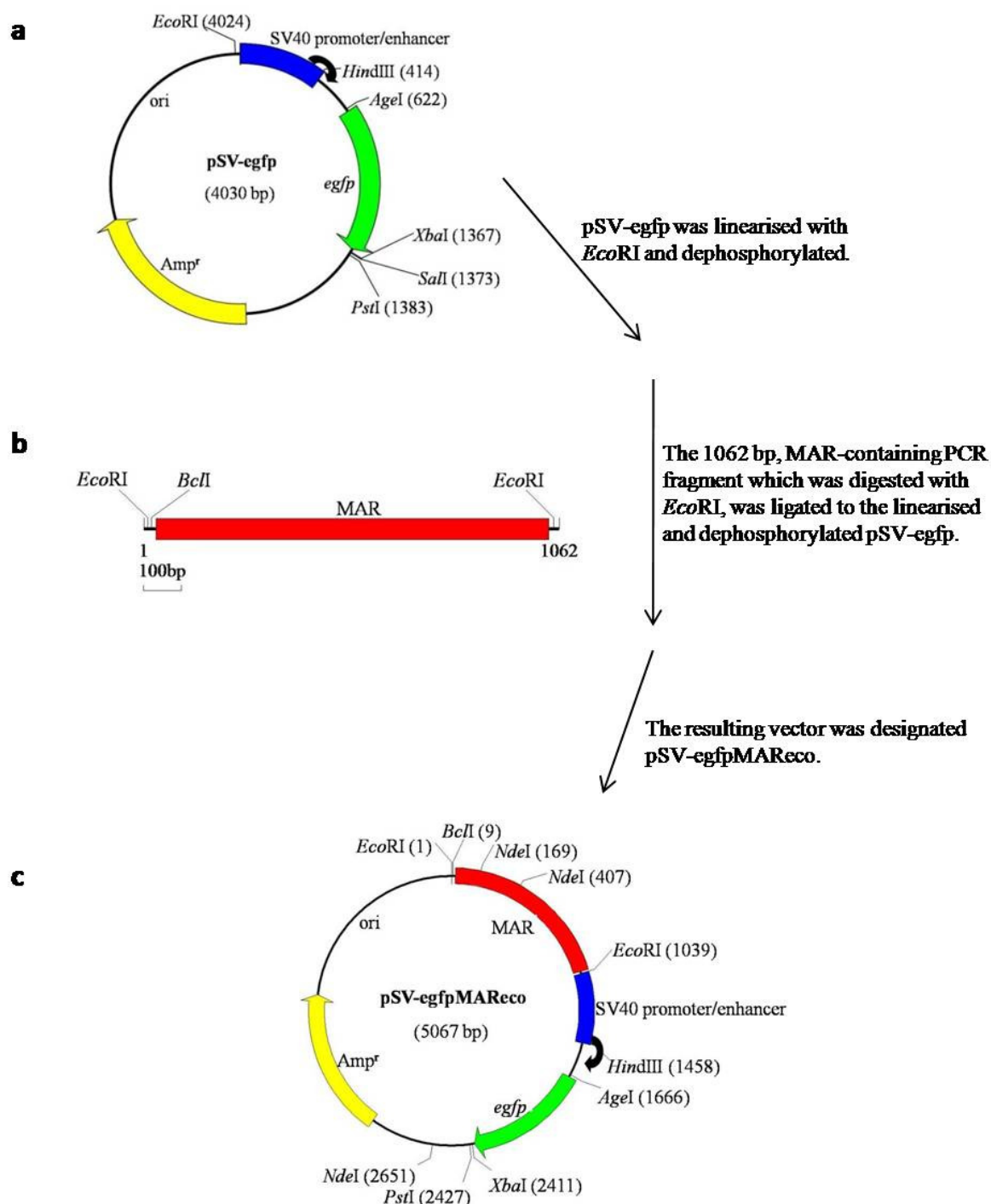
### **CONSTRUCTION OF VECTORS FOR PROTOPLAST TRANSFECTION**

University of Cape Town

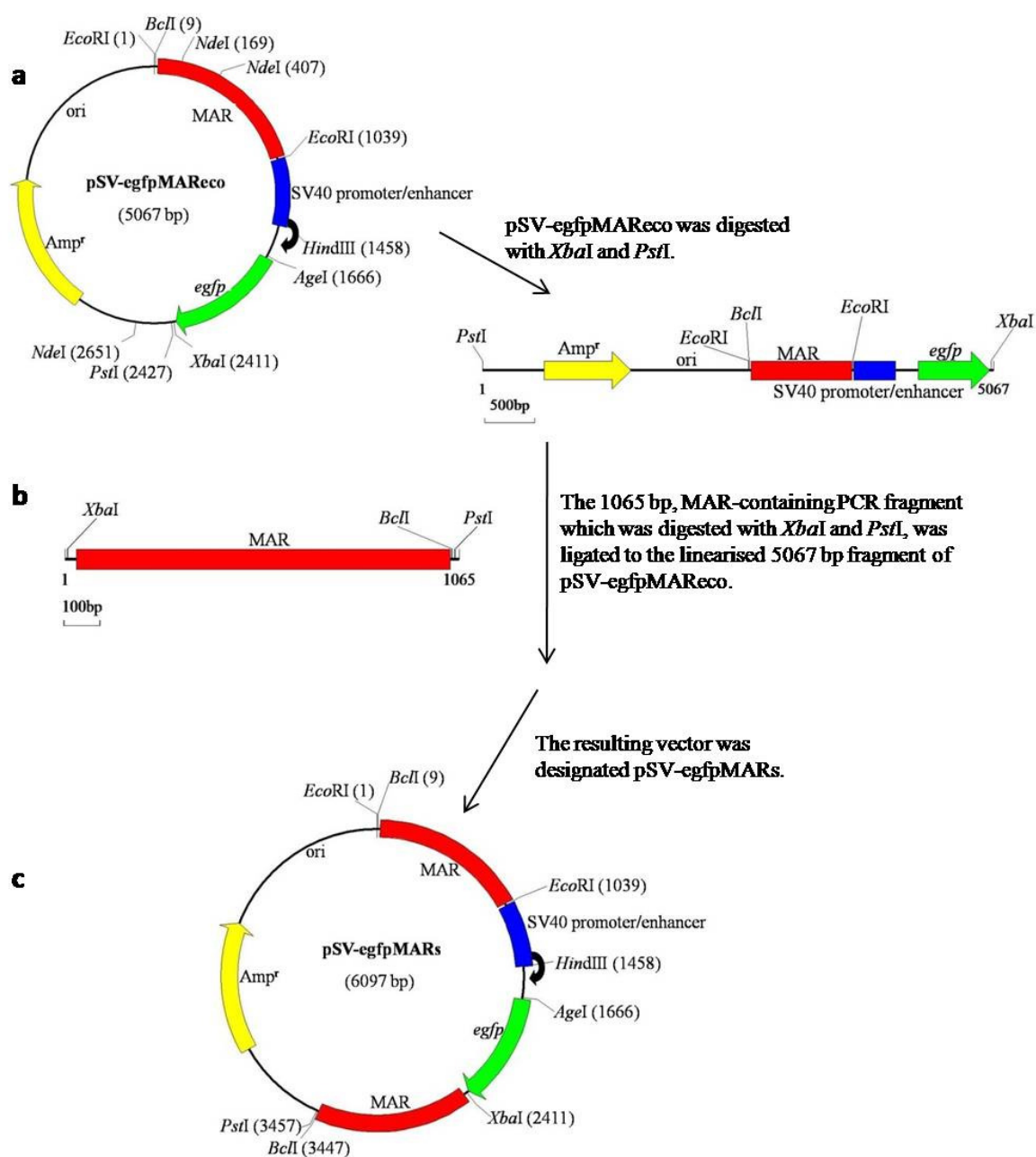
## Contents

D.1	Construction of pSV-egfpMAREco .....	238
D.2	Construction of pSV-egfpMARs .....	239
D.3	Construction of p18S-21 .....	240
D.4	Construction of p18S-SV-egfpMARs.....	241
D.5	Construction of p18S-SV-egfp .....	242
D.6	Construction of pKS-bar .....	243
D.7	Construction of pSV-bar .....	244
D.8	Construction of pSV-bar/egfp.....	245
D.9	Construction of p18S-SV-bar/egfpMARs.....	246

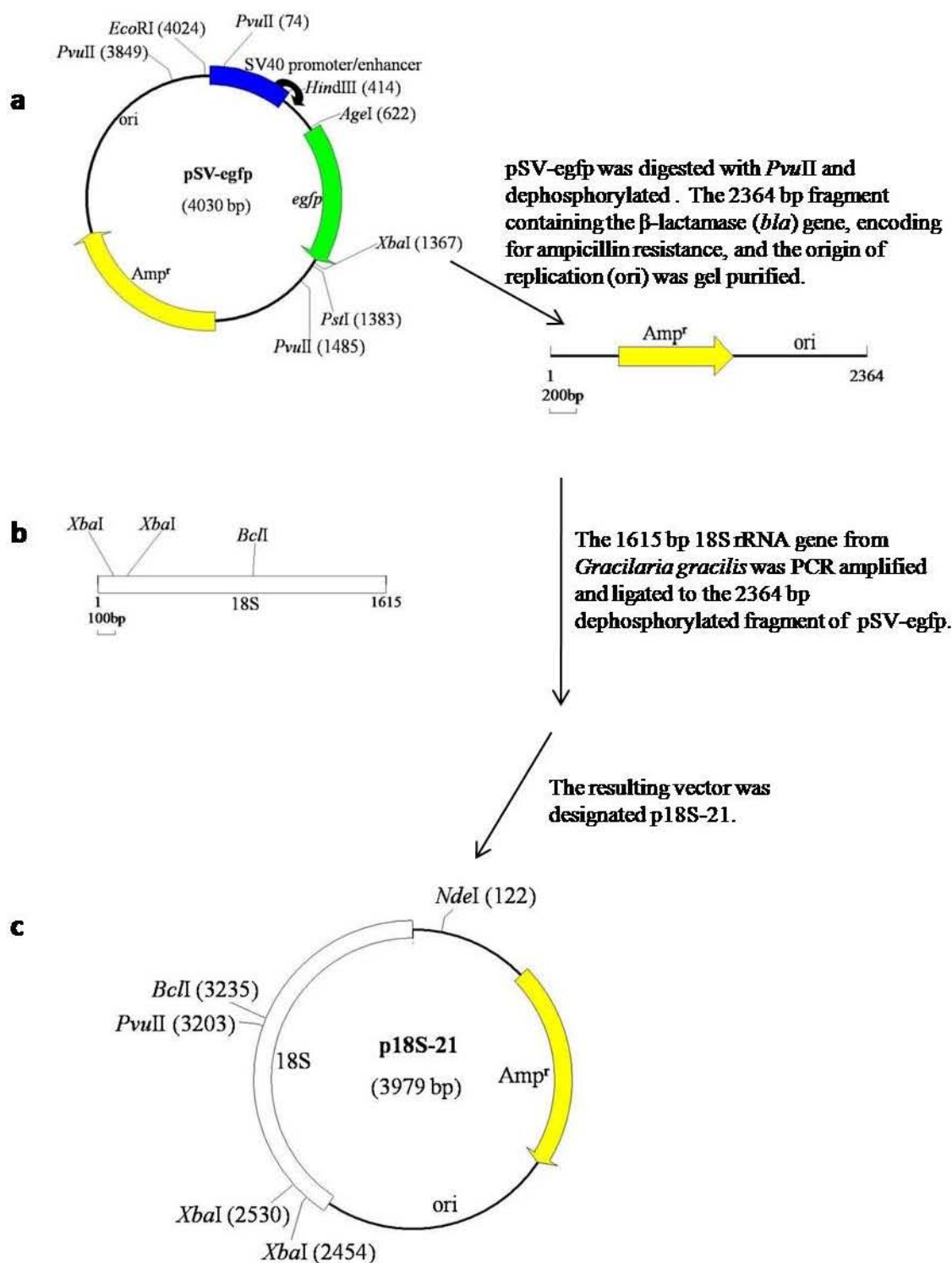
University of Cape Town



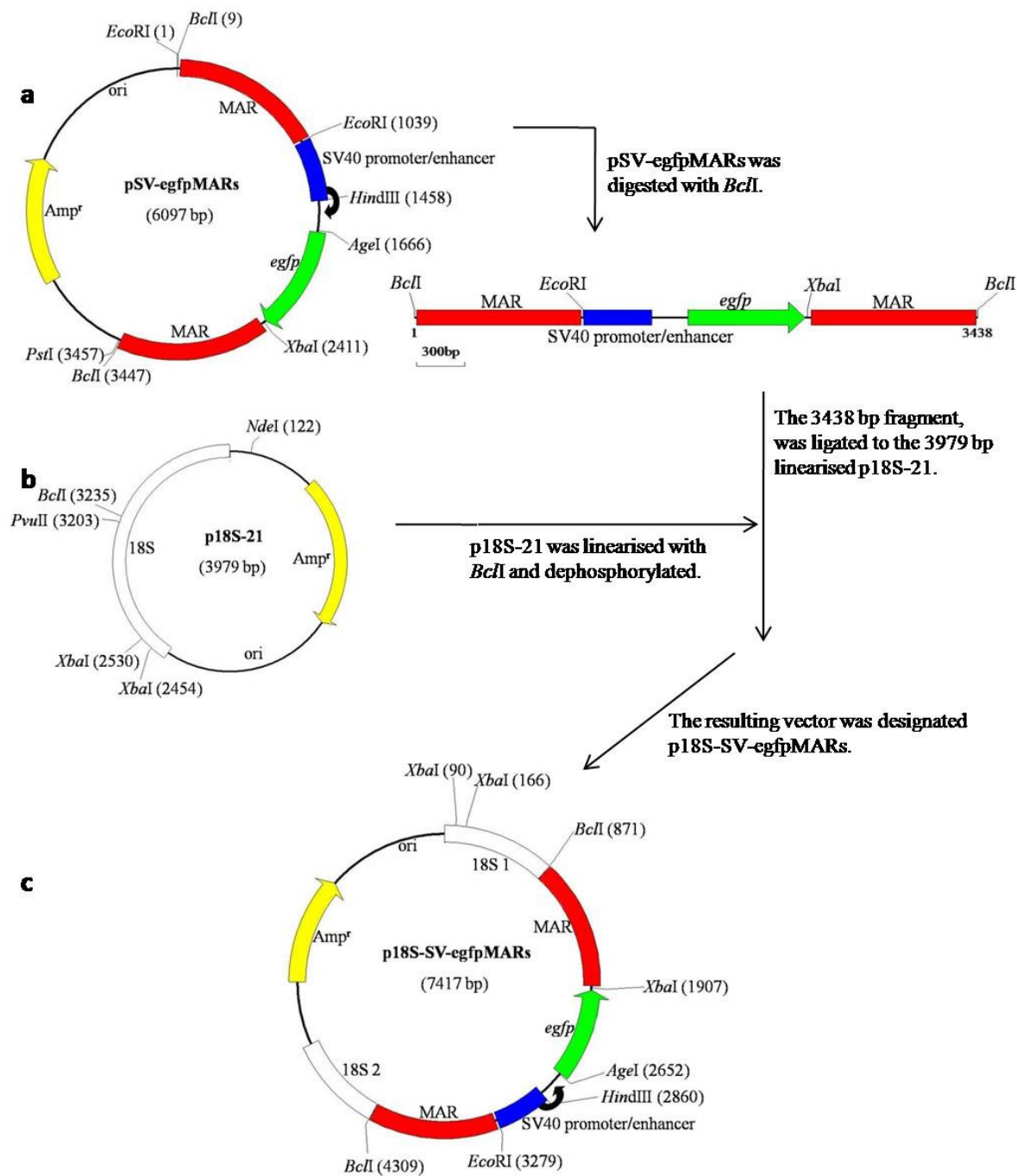
**Figure D1** Schematic representation (a-c) of the construction of pSV-egfpMAREco (c). pSV-egfp was linearised by *EcoRI* restriction (a). The fragment was ligated to a 1062 bp fragment, containing the tobacco Rb7 matrix attachment region (MAR), which had been PCR amplified and subsequently restricted with *EcoRI* (b). This sub-cloning strategy resulted in the insertion of the Rb7 MAR upstream of the SV40 promoter/enhancer. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, *Amp<sup>r</sup>*; ➔, indicates direction of transcription from the promoter; ■ SV40 promoter/enhancer; ■, Rb7 MAR.



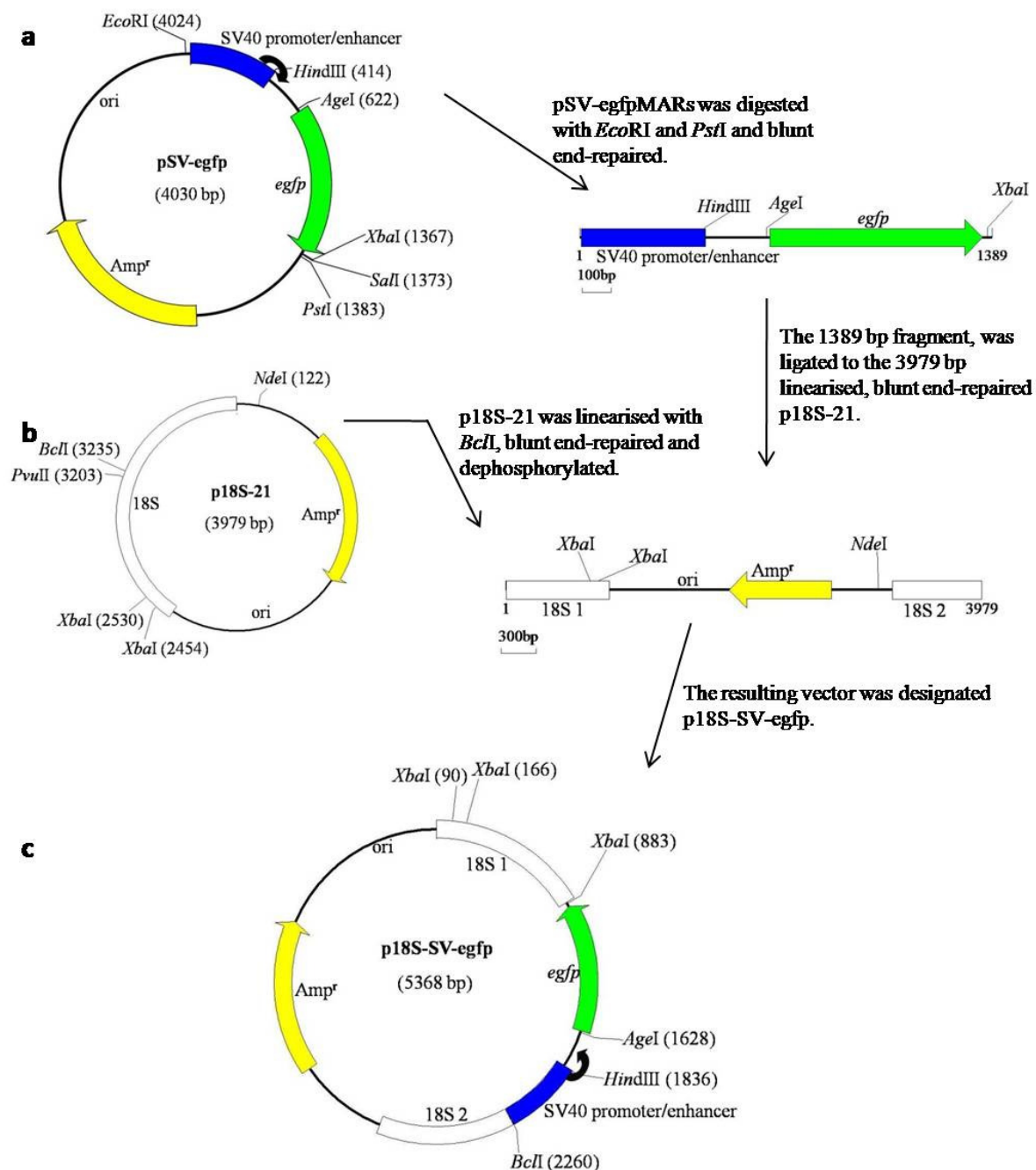
**Figure D2** Schematic representation (a-c) of the construction of pSV-egfpMARs (c). A 5067 bp fragment was excised from pSV-egfpMAREco using the restriction enzymes *XbaI* and *PstI* (a). The fragment was ligated to a 1065 bp fragment, containing the tobacco Rb7 matrix attachment region (MAR), which had been PCR amplified and subsequently restricted with *XbaI* and *PstI* (b). This sub-cloning strategy resulted in the insertion of a second Rb7 MAR downstream of the *egfp* gene. Relevant restriction sites are shown. Elements of vectors are as follows: ➔, *egfp*; ➔, *Amp<sup>r</sup>*; ➔, indicates direction of transcription from the promoter; ■, SV40 promoter/enhancer; ■, Rb7 MAR.



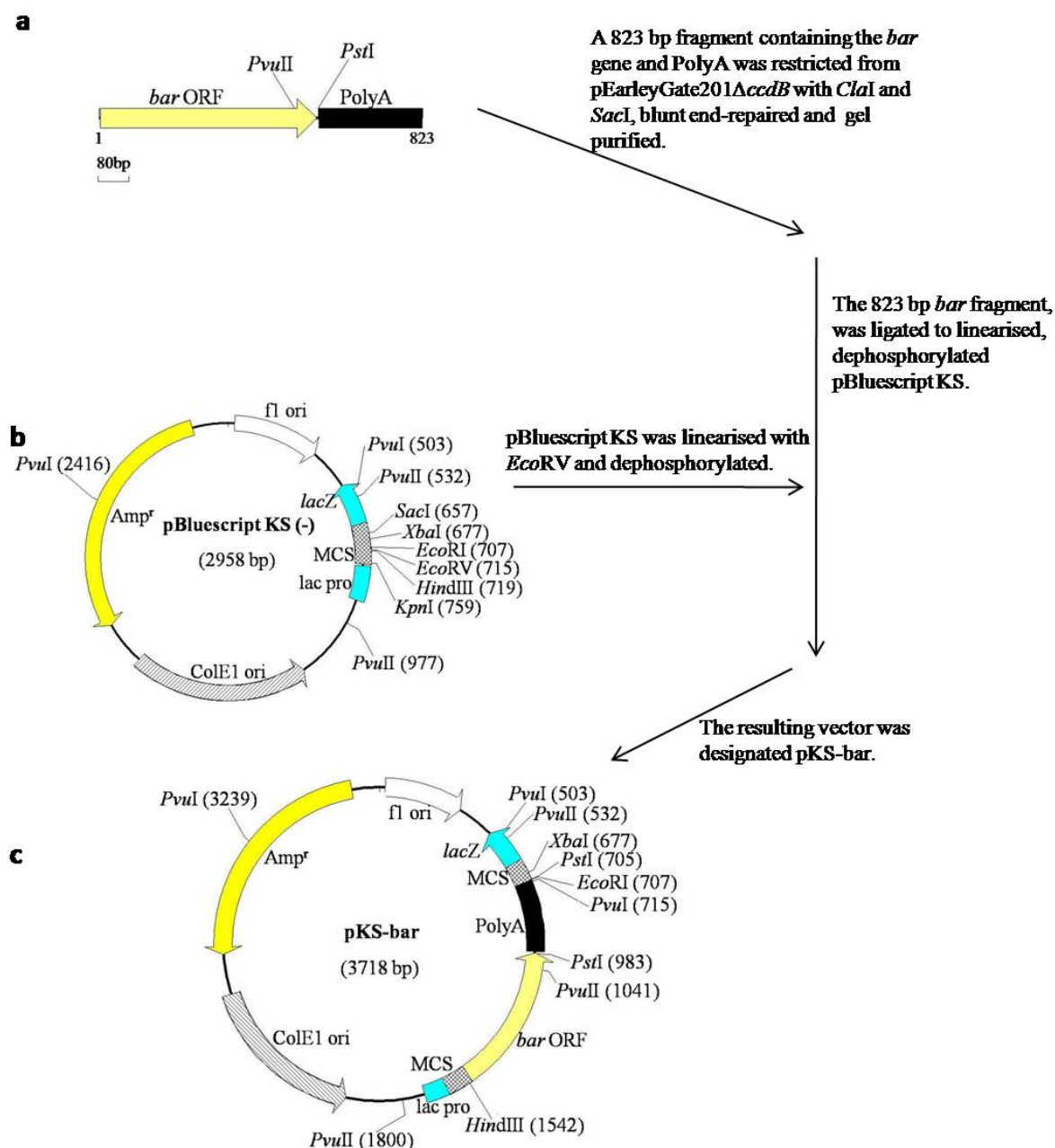
**Figure D3** Schematic representation (a-c) of the construction of p18S-21 (c). A 2364 bp fragment was excised from pSV-egfp using the restriction enzyme *PvuII* (a). The fragment was ligated to a 1615 bp fragment of the *G. gracilis* 18S rRNA gene which had been PCR amplified (b). Relevant restriction sites are shown. Elements of vectors are as follows:  $\rightarrow$ , Amp<sup>r</sup>;  $\square$ , *G. gracilis* 18S rRNA gene.



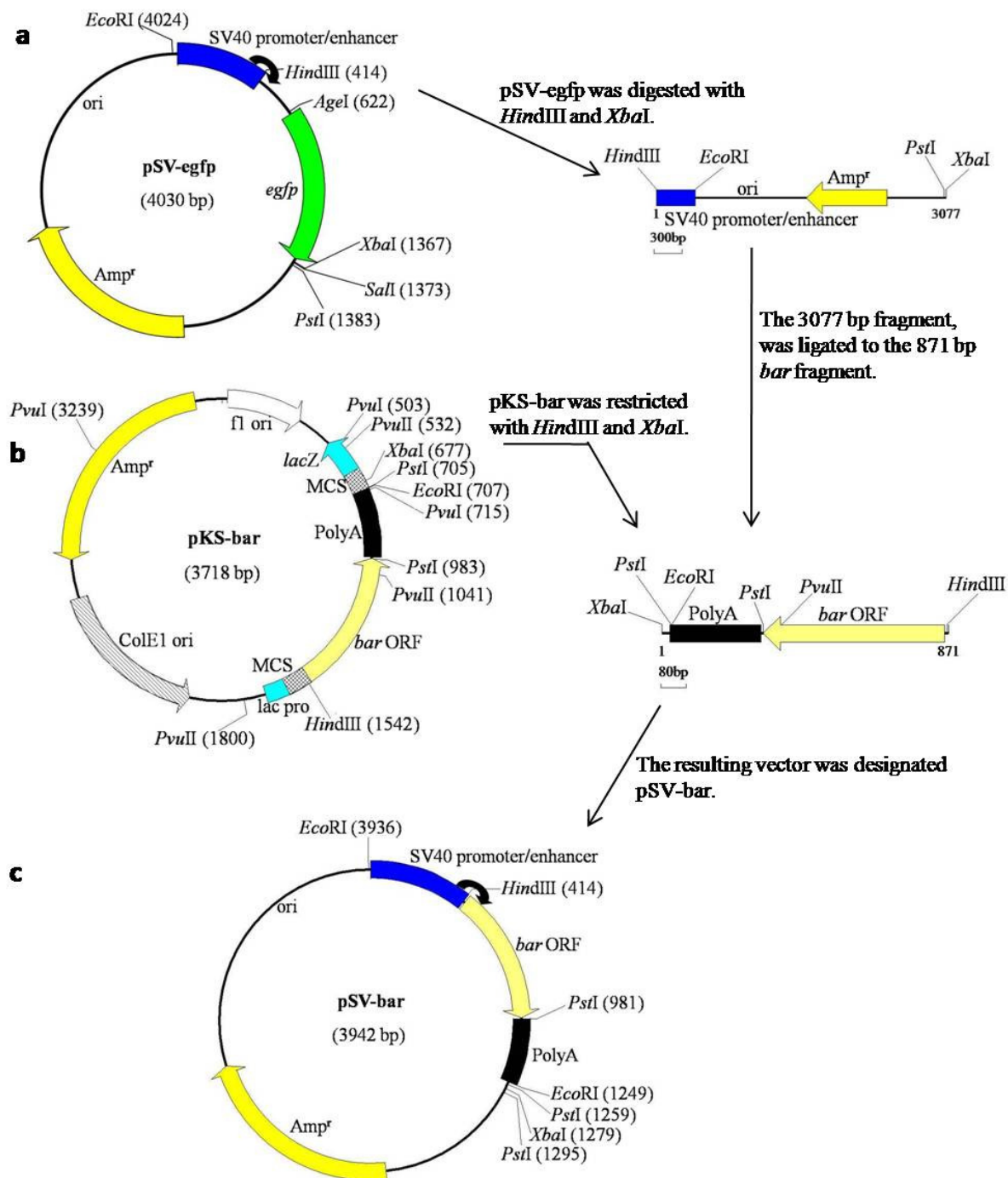
**Figure D4** Schematic representation (a-c) of the construction of p18S-SV-egfpMARs (c). A 3438 bp fragment was excised from pSV-egfpMARs using the restriction enzyme *BclI* (a). The fragment was ligated to p18S-21, which had been linearised by *BclI* restriction and dephosphorylated (b). This sub-cloning strategy resulted in the interruption of the *G. gracilis* 18S rRNA gene by the MAR cassette, containing the *egfp* gene and SV40 promoter. Elements of vectors are as follows: ▶, *egfp*; ▶, *Amp<sup>r</sup>*; ▶, indicates direction of transcription from the promoter; ■, SV40 promoter/enhancer; ■, Rb7 MAR; , 18S 1/2, where the 1 and 2 represent 5' and 3' portions of the gene, respectively.



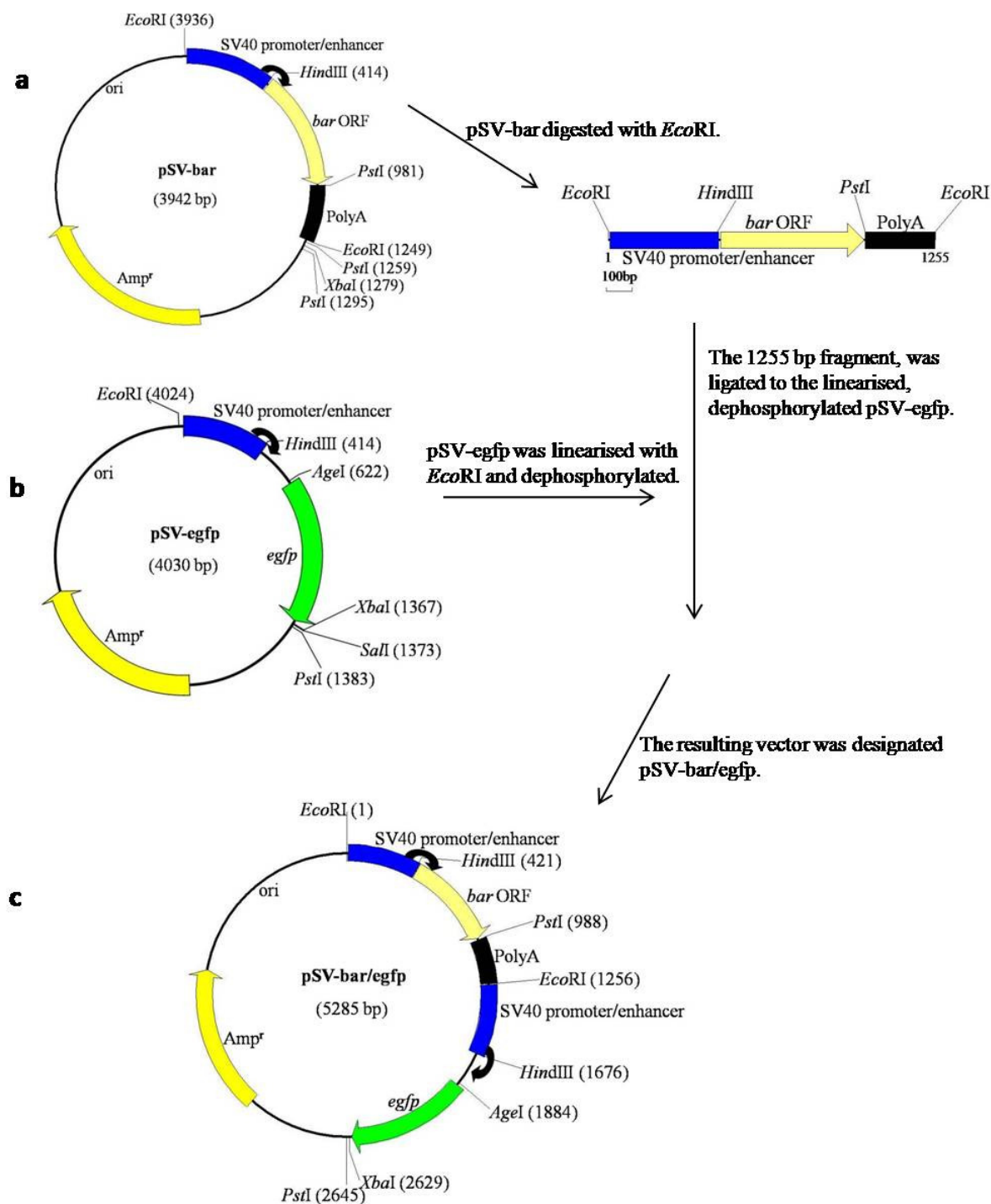
**Figure D5** Schematic representation (a-c) of the construction of p18S-SV-egfp (c). A 1389 bp fragment was excised from pSV-egfp using the restriction enzymes *Eco*RI and *Pst*I, before being blunt end-repaired (a). The resulting fragment was ligated to p18S-21, which had been linearised by *Bcl*I restriction, blunt end-repaired and dephosphorylated (b). This sub-cloning strategy resulted in the interruption of the *G. gracilis* 18S rRNA gene by the SV40 promoter and *egfp* gene. Elements of vectors are as follows: ▶, *egfp*; ▶, *Amp*<sup>r</sup>; ▶, indicates direction of transcription from the promoter; ■, SV40 promoter/enhancer; □, 18S 1/2, where the 1 and 2 represent 5' and 3' portions of the gene, respectively.



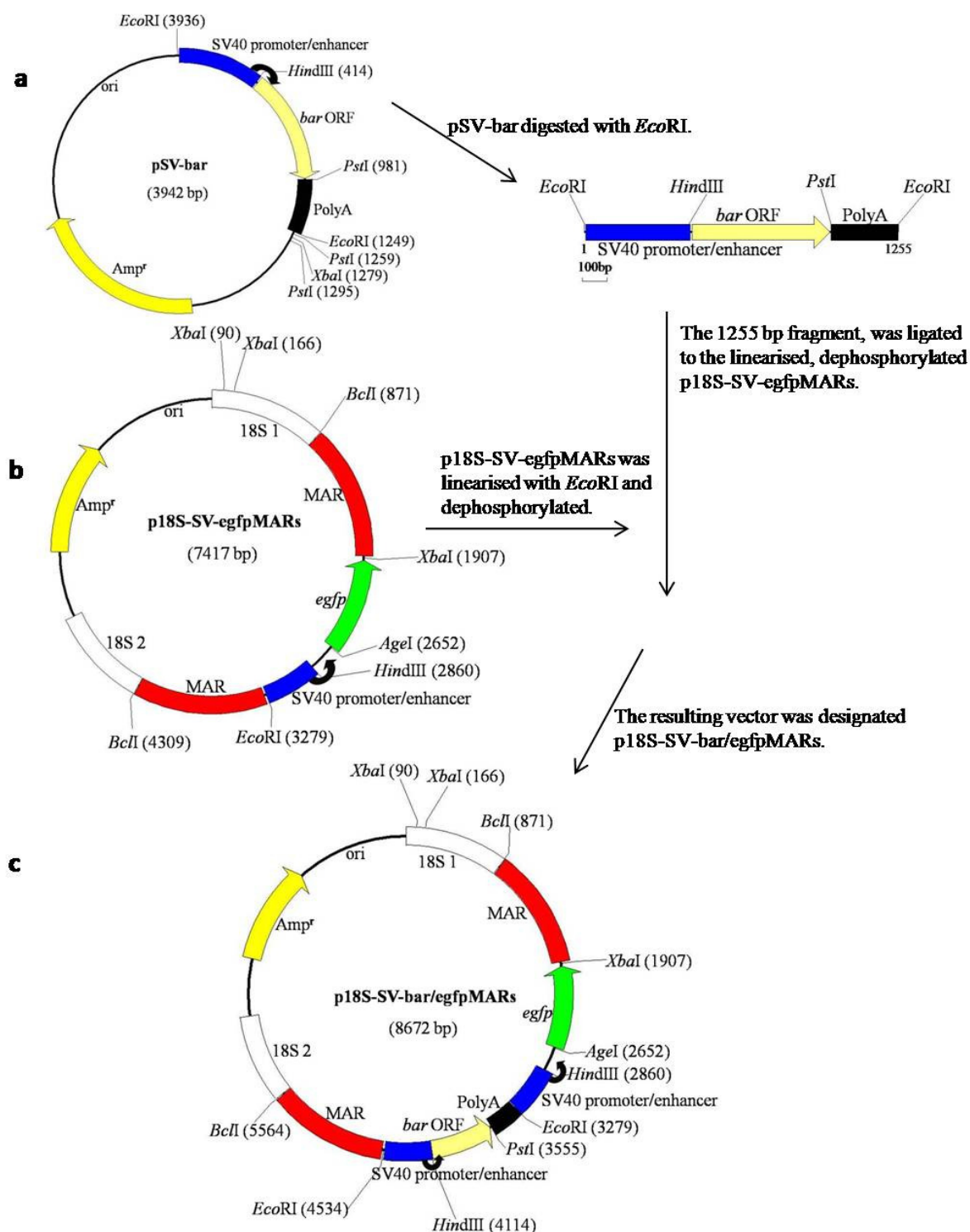
**Figure D6** Schematic representation (a-c) of the construction of pKS-bar (c). A 823 bp fragment was excised from pEarleyGate201-ccDB using the restriction enzymes *Sac*I and *Cla*I, before being blunt end-repaired (a). The fragment was ligated to pBluescript KS, which had been linearised by *Eco*RV restriction and dephosphorylated (b). Elements of vectors are as follows: , *bar*; , PolyA; , multiple cloning site (MCS); , *lacZ*; , f1 origin of replication; , *Amp*<sup>r</sup>; , ColE1 origin of replication; , *lac* promoter.



**Figure D7** Schematic representation (a-c) of the construction of pSV-bar (c). A 3077 bp fragment was excised from pSV-egfp using the restriction enzymes *HindIII* and *XbaI* (a). The fragment was ligated to an 871 bp fragment containing the *bar* gene which had been excised from pKS-bar using the restriction enzymes *HindIII* and *XbaI* (b). This sub-cloning strategy resulted in the substitution of the *egfp* gene with the *bar* gene. Elements of vectors are as follows: ▶, Amp<sup>r</sup>; ▶, *egfp*; ■, SV40 promoter/enhancer; ▶, indicates direction of transcription from the promoter; ▶, *bar*; ■, PolyA; , multiple cloning site (MCS); ▶, *lacZ*; ▶, f1 origin of replication; , ColE1 origin of replication; ▶, *lac* promoter.



**Figure D8** Schematic representation (a-c) of the construction of pSV-bar/egfp (c). A 1255 bp fragment was excised from pSV-bar using the restriction enzyme *EcoRI* (a). The fragment was ligated to pSV-egfp, which had been linearised by *EcoRI* restriction and dephosphorylated (b). Elements of vectors are as follows: ▶, *Amp<sup>r</sup>*; ▶, *egfp*; ■, SV40 promoter/enhancer; ▶, indicates direction of transcription from the promoter; ▶, *bar*; ■, PolyA.



**Figure D9** Schematic representation (a-c) of the construction of p18S-SV-bar/egfpMARs (c). A 1255 bp fragment was excised from pSV-bar using the restriction enzyme *EcoRI* (a). The fragment was ligated to p18S-SV-egfpMARs, which had been linearised by *EcoRI* restriction and dephosphorylated (b). Elements of vectors are as follows: ▶, Amp<sup>r</sup>; ▶, *egfp*; ■, SV40 promoter/enhancer; ▶, indicates direction of transcription from the promoter; ▶, *bar*; ■, PolyA; ■, Rb7 MAR; , 18S 1/2, where the 1 and 2 represent 5' and 3' portions of the gene, respectively.