

Full Length Research Paper

Transformation of *lacZ* using different promoters in the commercially important red alga, *Gracilaria gracilis*

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This paper reports the first successful transformation of *Gracilaria gracilis* (Stackhouse) M. Steentoft, L.M. Irvine and W.F. Farnham with *lacZ*-containing plasmids by microparticle bombardment. Transient expression of the *lacZ* reporter gene was compared under the control of three different viral promoters including the Simian virus 40 (SV40) promoter, the Cytomegalovirus (CMV) promoter and the Cauliflower mosaic virus 35S (CaMV 35S) promoter. In thalli transformed with vectors containing either the SV40 or CMV promoter, *lacZ* presence was confirmed by histological staining 2 and 3 days post-bombardment. In thalli transformed with the vector containing the CaMV 35S promoter, *lacZ* presence was confirmed by histological staining 1, 2, 3 and 5 days post-bombardment. Sectioning and histological staining of bombarded thalli showed that recombinant bombarded DNA penetrated cells to below the epidermal layer of the thallus. PCR analysis verified the presence of the *lacZ* gene in plasmid-bombarded thalli from the first day post-bombardment onwards. β -Galactosidase activity varied depending on the type of promoter used. These results form an important foundation for the development of a successful transformation protocol for *G. gracilis*.

Key words: *Gracilaria*, *lacZ*, microparticle bombardment, viral promoter.

INTRODUCTION

Gracilaria gracilis (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham 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). The South African *Gracilaria* industry depends solely on the natural *G. gracilis* resource growing in Saldanha Bay. However, the industry has experienced a number of setbacks over the past few years due to major collapses in the *G. gracilis* population (Schroeder et al., 2003). In 1989, the natural *G. gracilis* population experienced one of these die-offs and as a result no commercial harvesting was possible for the next three years (Jaffray et al., 1997). The die-offs are thought to be caused by bacterial epiphytes becoming pathogenic to their host in response to environmental stress (Jaffray and Coyne, 1998). 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 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. A possible approach to overcoming the effects of pathogenic bacteria is to select and/or engineer *G. gracilis* strains that are either more tolerant or resistant to these pathogens. The success of this approach in higher plants and the progress in molecular transformation tools suggests this will soon be possible for macroalgae.

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

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of reporter genes under the control of promoters that had been used for similar purposes 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., 2010), *Porphyra miniata* protoplasts (Kübler et al., 1994), *Porphyra yezoensis* protoplasts and explants (Kuang et al., 1998; Mizukami et al., 2004), *Ulva lactuca* protoplasts (Huang et al., 1996) and *Gracilaria changii* explants (Gan et al., 2003). In many of these cases, transient gene expression was achieved using the Cauliflower mosaic virus (CaMV) 35S promoter. However, CaMV 35S-mediated expression of the GUS reporter gene in *Laminaria* and *Undaria* indicated that this promoter might be tissue specific (Qin et al., 1994). 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). Recently, the use of endogenous promoters, targeted homologous recombination and matrix attachment regions (MARs) to increase reporter gene expression in *P. yezoensis* has received much attention (Fukuda et al., 2008; Gong et al., 2007; Liu et al., 2003; Mizukami et al., 2004; Zuo et al., 2007).

Few groups have, however, reported stable transformation in macroalgae since this requires clonal seaweed culture and techniques for algal 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), chloramphenicol acetyltransferase (CAT) (Jiang et al., 2002), a recombinant tissue-type plasminogen activator (rtPA) (Gao et al., 2005; Zhang et al., 2008) and *uidA*, encoding β -glucuronidase (GUS) (Li et al., 2009). There are reports of stable expression of *lacZ* in *Undaria pinnatifida* (Qin et al., 2003), and although full publications are not available, many have reported stable transformation of *Porphyra* in conference abstracts (Bernasconi et al., 2004; Cheney et al., 2001; He et al., 2001; Lin et al., 2001). Reporter genes that have been stably expressed in *Porphyra yezoensis* include the *gluc*, *cat*, GUS and GFP genes (Cheney et al., 2001; He et al., 2001) and the bacterial nitroreductase gene *nsfl* (Bernasconi et al., 2004).

A genetic transformation system for *G. gracilis* is needed to characterise algal genes expressed in response to environmental stress and disease. Manipulation of endogenous genes and the introduction of foreign genes will also become possible with the development of this tool (Walker et al., 2005).

In this paper, we show that *G. gracilis* can be successfully transformed using the microparticle bombardment method to transiently express *lacZ*. Using histological

staining, expression of the *lacZ* gene was demonstrated under the control of three different viral promoters: The Simian virus 40 (SV40), the Cauliflower mosaic virus 35S (CaMV 35S) and the Cytomegalovirus (CMV) promoters. β -Galactosidase activity in transiently expressing tissue varied, indicating that transient expression of recombinant protein may be promoter-dependent.

MATERIALS AND METHODS

G. gracilis culture and sterilization

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 under a 16/8 h (day/night) photoperiod with a light intensity of 45 $\mu\text{mol photons/m}^2/\text{s}^{-1}$ at 14 to 15°C. One day prior to microparticle bombardment, thalli were rinsed and visible epiphytes removed before sterilising the thalli by placing them in sterile distilled water for 3 h, followed by 5 min incubation in 0.1% (v/v) bleach and a final incubation in 1% (w/v) KI for 1 min. Thalli were cultured overnight in sterile artificial seawater (ASW) [0.42 M NaCl, 23 mM MgCl₂, 8.9 mM KCl, 12.9 mM CaCl₂, 25.6 mM MgSO₄, 2 mM NaHCO₃] enriched according to Provasoli (1968) with PES (1/3 strength) and supplemented with 0.5 mg/ml each of penicillin G and kanamycin sulphate at 15°C.

Plasmid constructs

The plasmid vector pSV- β -Gal which contains the *lacZ* gene under the control of the SV40 promoter/enhancer was obtained commercially (Promega, USA). Recombinant plasmids pCMV- β -Gal and pCaMV- β -Gal were constructed by replacing the SV40 promoter in pSV- β -Gal with promoter sequences CMV from pcDNA3.1/Zeo/CAT (Invitrogen, USA) and CaMV 35S from pEarleyGate201 Δ ccdB (Smart 2010, unpublished), a modified version pEarleyGate201 (Earley et al., 2006), respectively. The bacterial *lacZ* gene encodes the β -galactosidase enzyme which is capable of cleaving 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), a colorless substrate, to produce a stable and insoluble blue compound which is easily detected.

Transformation by microparticle bombardment

Approximately 70 0.5 cm thallus pieces were placed on a solid agar (0.8%) surface in a Petri dish prior to particle bombardment. The vectors were precipitated onto gold particles (1 μm in diameter, Bio-Rad, USA) as described by Dunder et al. (1995). Each bombardment delivered 0.48 mg of gold particles and 1.0 μg plasmid DNA according to Teng et al. (2002), Gan et al. (2003) and Jiang et al. (2003). A rupture disc pressure of 650 psi and a particle travel distance of 6 cm were employed, based on bombardment optimisation conditions carried out previously (results not shown). Negative controls were bombarded with non-DNA-attached gold particles. Each plate was bombarded twice using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, USA) for particle delivery. After bombardment, thalli were thoroughly rinsed in ASW and maintained in ASW supplemented with PES (1/3 strength) under culture conditions described previously, prior to sampling at different time periods post-bombardment to assay for *lacZ* and perform genomic DNA extraction.

Histological *lacZ* assay

The presence of the β -galactosidase enzyme was assayed at 1, 2, 3, 5 and 7 days post-bombardment by employing an *in situ* histochemical stain. The histochemical stain uses X-gal, a chromogenic substrate that turns blue following cleavage by the β -galactosidase enzyme. Thalli were rinsed twice in ASW, followed by a rinse in 1x phosphate buffered saline (PBS, pH 7.0), fixed for 0.5 h in PBS (pH 7.0) containing 1 mM $MgCl_2$ and 1.25% glutaraldehyde and finally placed in stain solution [0.25% X-gal, 10 mM phosphate buffer, pH 7.0, 1.0 mM $MgCl_2$, 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$] and incubated at 37°C for 4 h. Thalli were viewed and photographed under a Nikon Stereoscopic Zoom Microscope SMZ1500.

In addition, a thallus showing blue staining was transversely sectioned through the blue stained region, using a scalpel blade. The section was viewed and photographed under a light microscope.

β -Galactosidase enzyme assay

Algal tissue was homogenized two days post-bombardment in Z-buffer (Miller, 1972) supplemented with extra β -mercaptoethanol (100 mM final). The extract was cleared in a microcentrifuge and protein content was measured by the standard Bradford protein assay (Bradford, 1976). β -Galactosidase activity was assayed in the extract by measuring the hydrolysis of o-nitrophenyl- β -D-galactopyranoside (ONPG) at 37°C. In order to control for the colored compounds released from the algal tissue, a blank reaction with no substrate was carried out for each sample. One unit of β -galactosidase is defined as the amount of enzyme that will hydrolyze 1 μ mol ONPG to o-nitrophenol and D-galactose per minute at pH 7.5 and 37°C. The β -galactosidase activity data for the samples was analyzed by one-way ANOVA. When the results of the ANOVA were significant, the Tukey Test was used to determine significant differences in β -galactosidase activity due to the presence of the various promoters, using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at $p < 0.05$.

RESULTS AND DISCUSSION

To test for successful transformation of *lacZ* into *G. gracilis* tissue, macroalgal thalli were bombarded with the recombinant vectors pSV- β -Gal, pCMV- β -Gal and pCaMV- β -Gal. Thalli were sampled at 5 time points post-bombardment (days 1, 2, 3, 5 and 7) and stained for the presence of *lacZ* using an *in situ* histochemical stain. A negative control employing gold particles lacking vector DNA was included. The presence of blue-stained areas within the thalli indicates areas of β -galactosidase activity and therefore *lacZ* expression. *LacZ* as a reporter gene is exceptionally useful due to its amenability to histochemical detection. Its gene product is stable and can be immobilised in tissue by using a cross-linking fixative which does not affect activity. This fixation has the added advantage of inactivating endogenous enzymes which is particularly important for organisms which may have high levels of endogenous β -galactosidase activity as is the case with many plant species (Teeri et al., 1989). In all cases, between 6 and 7 thalli of the 40 thalli that were stained per plate, were

showed areas of blue staining. *LacZ* expression was visible on days 1, 2, 3 and 5 post-bombardment in the samples bombarded with pCaMV- β -Gal (Figure 1A). No *lacZ* expression was seen on day 7 in the pCaMV- β -Gal-bombarded samples. *LacZ* expression was visible on days 2 and 3 post-bombardment in the samples bombarded with pCMV- β -Gal (Figure 1B) and pSV- β -Gal (Figure 1C). No *lacZ* expression was seen on days 1, 5 and 7 in these plasmid-bombarded samples. 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* (Figure 1D). PCR analysis of genomic DNA extracted from bombarded thalli confirmed the presence of the *lacZ* gene in the plasmid-bombarded host cells (data not shown).

The blue stained areas on the thalli do 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, portions of thalli exhibiting blue staining were transversely sectioned to examine which particular cells showed β -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 (Figure 2). This result indicated that the gold particles penetrated the outer thallus surface during the bombardment process and became lodged within the underlying medullary cells, allowing for *lacZ* expression in 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. (2010) 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.

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 detected in all DNA samples from tissue bombarded with all 3 vectors (Figure 3) which was not detected in any of the algal samples bombarded with gold particles lacking vector DNA. The presence of a *G. gracilis* 18S rRNA PCR product confirmed the presence of *G. gracilis* DNA in all the samples tested.

The 624 bp PCR product corroborates the β -galactosidase activity observed in thalli bombarded with pCaMV- β -Gal after 1, 2, 3 and 5 days. It is possible that the *lacZ* gene may have been silenced by the time five days had elapsed, which could explain why β -galactosidase activity was not observed in thalli when stained 7 days after bombardment, but the evidence is inconclusive. 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. Thus while some promoters may

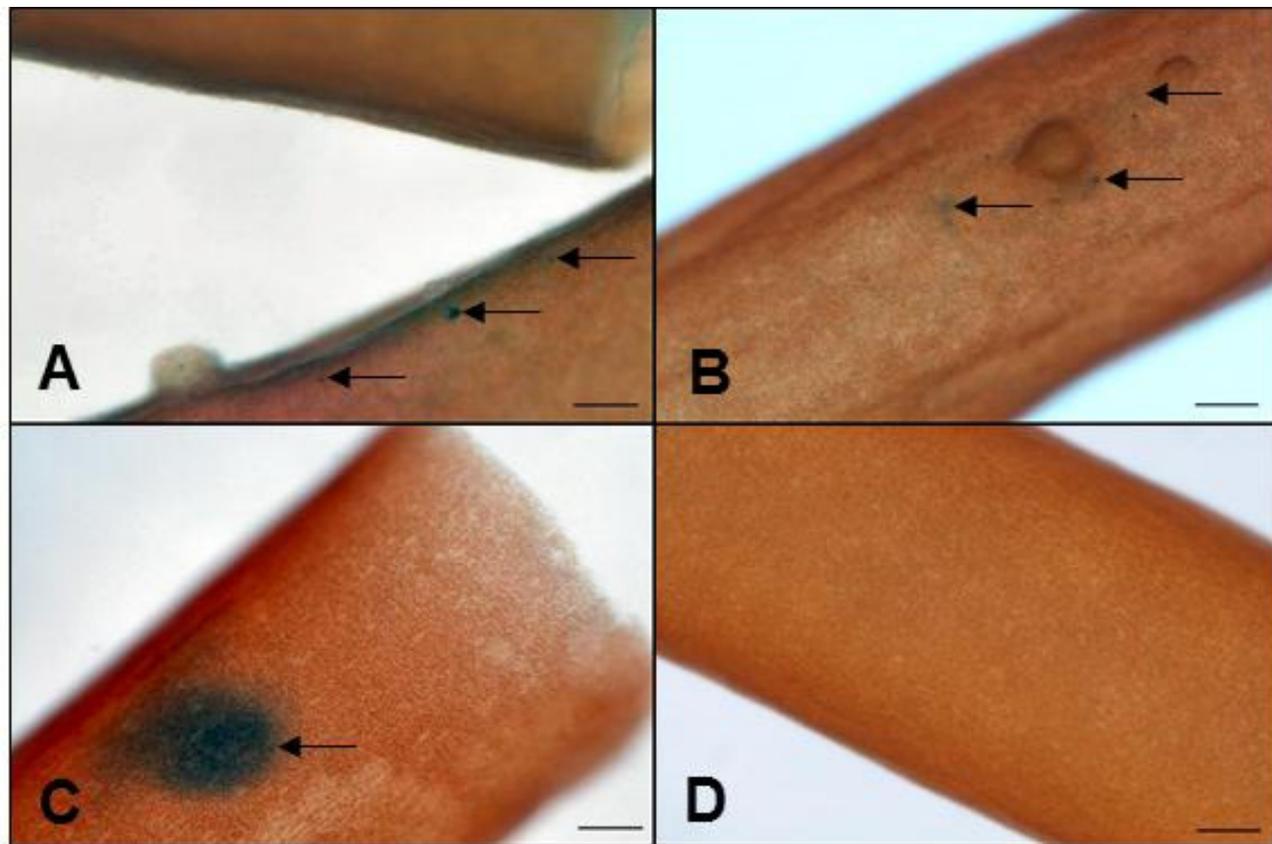


Figure 1. Histological *lacZ* assay. *G. gracilis* thalli (2 days post-bombardment) with blue-stained areas (arrows) indicating β -galactosidase activity in the samples bombarded with pCaMV- β -Gal (A); pCMV- β -Gal (B); pSV- β -Gal (C) bombarded thallus; and, gold particles lacking vector DNA (negative control) (D). Scale bar = 100 μ m (A-D).

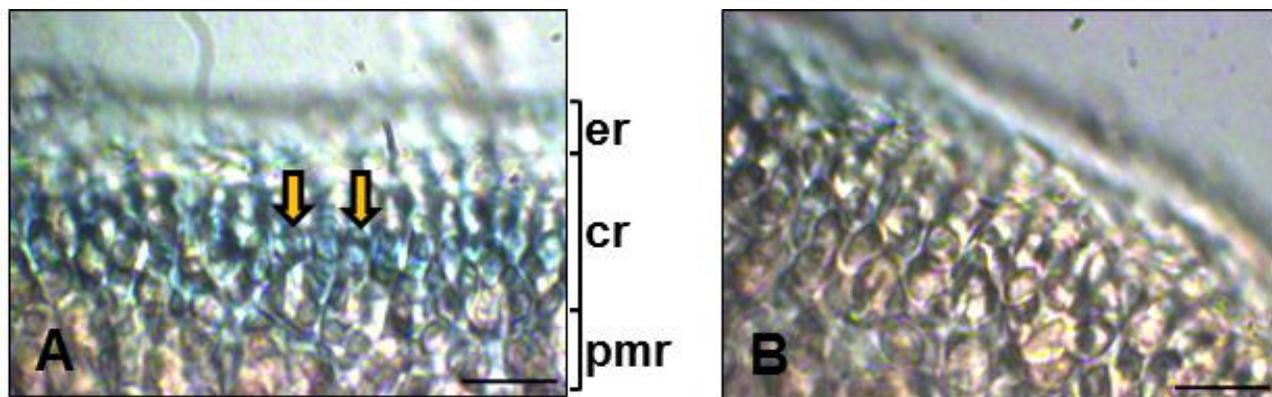


Figure 2. Sectioned *G. gracilis* thalli stained two days post-bombardment. pSV- β -Gal-bombarded thallus exhibited blue staining (arrows) within cells located in the cortical region (cr), which lies between the epidermal (er) and perimedullary (pmr) regions (A), while thalli bombarded with gold particles lacking vector DNA (negative control) showed no blue staining (B). The sections were viewed and photographed under a Nikon Eclipse 50i compound microscope. Scale bar = 40 μ m.

prove efficient in driving high levels of transient gene expression, they may not necessarily continue to drive expression once integration has occurred. *LacZ* expression was only observed in thalli, 2 and 3 days post-bombardment with pSV- β -Gal or pCMV- β -Gal

(Figure 1), despite detection of *lacZ* DNA in thalli sampled 1, 2, 3, 5 and 7 days post-bombardment. During the bombardment process, gold particles coated with plasmid DNA enter many cells within a thallus. Gene expression is however ultimately related to location of the

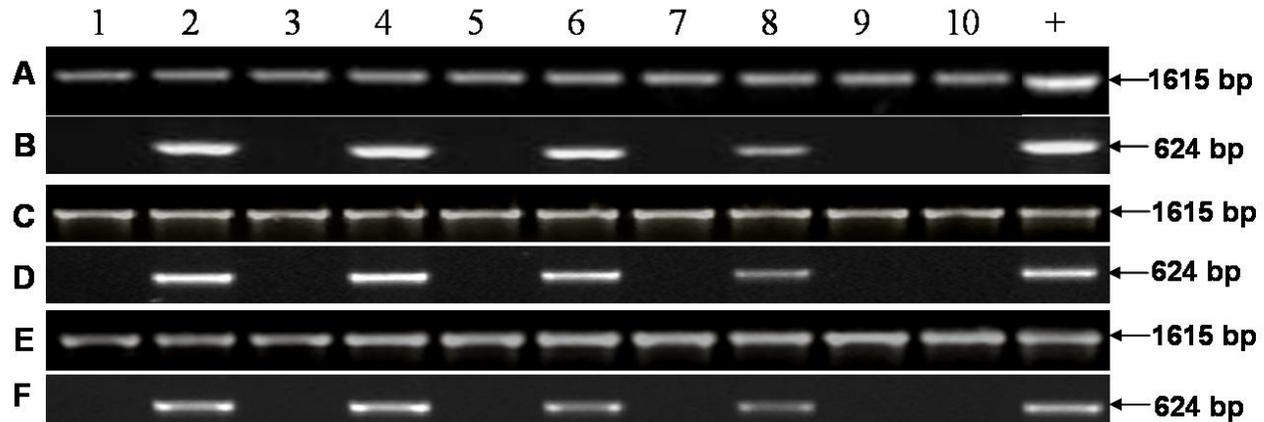


Figure 3. PCR analysis of total DNA isolated from bombarded thalli. Lanes 1, 3, 5, 7 and 9 show seaweed bombarded with non-DNA-attached gold particles (negative control) sampled 1, 2, 3, 5 and 7 post-bombardment, respectively; lanes 2, 4, 6, 8 and 10 show plasmid-bombarded seaweed (test sample) sampled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

Table 1. Effect of viral promoters on transient β -galactosidase activity in microparticle bombarded *G. gracilis* thalli.

| Promoter | β -Galactosidase activity \pm SEM (mU/mg protein) [†] | Relative β -galactosidase activity (%) |
|----------|--|--|
| Negative | 7.738 \pm 0.154 ^a | - |
| CMV | 9.031 \pm 0.316 ^b | 16.7 |
| CaMV 35S | 7.828 \pm 0.244 ^a | 1.2 |
| SV 40 | 9.367 \pm 0.161 ^b | 21.1 |

[†] Values are means \pm SEM of three independent samples. Activities with different superscripts are significantly different ($p < 0.05$) (one-way ANOVA).

microprojectiles within the cells (Southgate et al., 1995). The *lacZ* DNA detected in the samples by PCR could therefore have originated not only from cells in which transient *lacZ* expression occurred, but also from cells into which recombinant DNA was successfully delivered. Comparison of the PCR results with the *in situ* β -galactosidase staining observations showed that detection of *lacZ* DNA does not necessarily coincide with β -galactosidase activity in the host cells. It is possible that this variation in observable β -galactosidase activity is influenced by differences in promoter activity. In order to compare the three promoter strengths *in vivo*, β -galactosidase activity assays were carried out on macroalgal samples 2 days post-bombardment with the three recombinant vectors. This time point was selected as it was the first day on which thalli bombarded with the recombinant vectors exhibited β -galactosidase activity. Enzyme activity levels detected in thalli samples bombarded with gold particles lacking vector DNA showed that the algal tissue exhibited a β -galactosidase activity of 7.738 ± 0.154 mU/mg protein (Table 1). This is not surprising, as marine macroalgal tissue has

previously been shown to exhibit β -galactosidase activity (Davies et al., 1994). However, this is very low when compared to levels detected in some higher plant species. Using *lacZ* as a reporter gene in plants is limited by the fact that many plants contain endogenous β -galactosidase activity at neutral pH values which complicates the direct measurement of heterologous β -galactosidase activity using enzymatic assays (Teeri et al., 1989). The levels of β -galactosidase in tobacco has been reported to be between 10 and 20 U/mg protein, depending on the tissue sampled (Teeri et al., 1989). The levels of endogenous activity measured for *G. gracilis* are approximately 1000 times less. The fact that *G. gracilis* shows such a low endogenous thallus β -galactosidase activity at a neutral pH means that measuring *lacZ* expression using enzymatic assays should not be skewed by high endogenous β -galactosidase levels. Similarly, *lacZ* use as a reporter gene in macroalgae may not be limited to the same extent as it is in plants and direct assays may well be possible as long as the endogenous β -galactosidase activity is low. β -Galactosidase activity in thalli bombarded with the pSV- β -

Gal and pCMV- β -Gal vectors was significantly ($p < 0.05$) higher, 9.367 ± 0.161 and 9.031 ± 0.316 mU/mg protein, respectively, than endogenous β -galactosidase activity detected in control thalli (Table 1). Thalli bombarded with CaMV- β -Gal did not show significantly higher activity (7.828 ± 0.244 mU/mg protein) when compared to negative control thalli (Table 1). Overall, thalli samples bombarded with pSV- β -Gal, pCMV- β -Gal and pCaMV- β -Gal vectors resulted in a 21.1, 16.7 and 1.2% increase in thallus β -galactosidase activity, respectively (Table 1). These results indicate that while all three promoters are functional in *G. gracilis*, the CMV and SV40 promoters could be of particular use in short term transient expression studies in macroalgae.

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 the macroalgae will continue to rely on using promoters that have been successfully implemented in microalgae and higher plants. While these promoters may not be as effective as native algal promoters, their functionality allows the establishment of a suitable transformation system and for this reason should not be disregarded for future macroalgal expression studies.

This is the first report of successful transformation of *G. gracilis*. This study shows that at least three different promoters including the SV40 promoter/enhancer, the CMV promoter and the CaMV 35S promoter can be used successfully to express the *lacZ* reporter gene in *G. gracilis* transformed by particle bombardment. These results provide an important foundation for the optimisation of a suitable method for stable transformation of the commercially important agarophyte, *G. gracilis*. This could ultimately lead to the introduction and establishment of suspended (open-water) cultivation of disease-resistant macroalgae which would ensure a more economically viable and reliable *Gracilaria* industry not only in South Africa, but in other countries as well.

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