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Agrobacterium-mediated gene delivery and transient expression in the red macroalga *Chondrus crispus*

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Abstract: Molecular resources and transgenic studies in red algae are lagging behind those for green algae. The *Agrobacterium*-mediated gene-transfer method routinely used in plant transformation has not been fully utilised in the red algae, which, as an important source of phycocolloids, warrant more studies. In this regard, a stepwise methodology was developed for *Agrobacterium*-mediated transformation of the carrageenophyte *Chondrus crispus* using pCAMBIA 1301 and a construct featuring a codon-optimized beta-glucuronidase (*GUS*) reporter gene driven by the endogenous *Chondrus* actin promoter. The effects of several factors on transformation efficiency were investigated. An intimate association of *Chondrus* and bacterial cells was observed using scanning electron microscopy. *GUS* transient expression within *Chondrus* cortical and medullary cells with both expression cassettes testified to the amenability of *Chondrus* to *Agrobacterium*-mediated transformation. Darker staining, indicative of higher GUS activity, was observed with the *Chondrus*-specific construct, suggesting its superiority over the pCAMBIA 1301. Presence of acetosyringone, the wounding method and the type of co-cultivation medium significantly affected the transformation outcome and efficiency. The *Agrobacterium*-mediated transient expression presented here constitutes a first step towards tailoring a transformation strategy for *Chondrus*, which can serve to facilitate further transgenic studies in this important red alga.

Keywords: algal biotechnology; gene engineering; genetic transformation; transformed algae; transgenic algae.

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Introduction

The red macroalga, *Chondrus crispus* Stackhouse, found along the rocky shores of the North Western and North Eastern Atlantic, is an important commercial source of the phycocolloid carrageenan, which is used as a gelling, thickening and stabilizing agent in the food, cosmetic and phamaceutical industries (Guiseley 1989, Necas and Bartosikova 2013). According to the Food and Agricultural Organisation, 2000 tonnes of *Chondrus* were harvested in the year 2005. *Chondrus* was historically the only source of carrageenan and contributed to the approximately US$417 million per annum carrageenan market (Kraan 2012), highlighting its importance.

The greatest diversity in algae can be observed within the red algae or Rhodophyta (Yoon et al. 2004, Maggs et al. 2008, Verbruggen et al. 2010), many of which have not yet been adequately studied. The red algae deserve more attention for their potential – by virtue of their unique biosynthetic pathways, cell wall components and unique pigments absent in land plants (Collén et al. 2013, Brawley et al. 2017). They are a mine of novel bioactive compounds (Holdt and Kraan 2011) that can be harnessed by the biotechnological industry. Studying the red algae, an ancient eukaryote lineage and a potential sister group to green algae, can additionally increase our understanding of the evolution of other algae and land plants (Collén et al. 2013).

In order to tap into the above-mentioned economic potential of the red macroalgae, a molecular toolkit for red algae is desirable, but lacking. It is crucial to develop reliable transgene delivery techniques for analysing the expression, function and regulation of red algal endogenous genes which will, in time, allow the engineering of economically important traits in algae such as

phycocolloid quality and yield. Currently, several species of red algae have been transformed: the unicellular *Cyanidioschyzon merolae* (Minoda et al. 2004) and *Porphyridium* sp. (Lapidot et al. 2002), and the macroalgae *Pyropia yezoensis* (Mizukami et al. 2004, Hirata et al. 2014), *Pyropia miniata* (Kübler et al. 1994), *Pyropia tenera* (Son et al. 2011), *Pyropia leucosticta* (Lin et al. 2004), *Porphyra haitanensis* (Wang et al. 2010), *Kappaphycus alvarezii* (Kurtzman and Cheney 1991), *Gracilaria changii* (Gan et al. 2003) and *Gracilaria gracilis* (Huddy et al. 2012). So far, algal transgenic research has heavily focused on microalgae, hence more studies on macroalgae are warranted.

For transformation in macroalgal systems, methods based on temporary cell membrane permeabilization e.g. electroporation and biolistics are mostly used (Gan and Maggs 2017). There are limited reports on the use of crown gall-causing *Agrobacterium* to transform macroalgae: *Pyropia yezoensis* (Cheney et al. 2001), *Gracilaria changii* (Gan et al. 2005) and *Kappaphycus alvarezii* (Handayani et al. 2014, Triana et al. 2016). Several microalgae such as *Chlamydomonas reinhardtii* (Kumar et al. 2004, Pratheesh et al. 2014), *Chlorella vulgaris* (Cha et al. 2012), *Haematococcus pluvialis* (Kathiresan et al. 2009), *Nannochloropsis* sp. (Cha et al. 2011), *Schizochytrium* (Cheng et al. 2012), *Dunaliella bardawil* (Anila et al. 2011) and *Dunaliella salina* (Srinivasan and Gothandam 2016) have been transformed with *Agrobacterium*. *Agrobacterium* is routinely used in plant transformation because it allows heritable integration of genes, mostly in single copies and with little rearrangement in the host's genome (Gelvin 2003, Tzfira and Citovsky 2006, Bourras et al. 2015, Hwang et al. 2015, 2017). The basic mechanism used by the bacterium has long been known: phenolic exudates released from injured plant tissue attract the bacteria, which move to the tissue by chemotaxis, attach to it and infect the wounded tissue via the transfer of a segment of the Ti plasmid into the cells (Simoh et al. 2007, Bhattacharya et al. 2010, Subramoni et al. 2014). Once inside the cell, the T-DNA will translocate to the nucleus and integrate into the host genome (Zupan et al. 2000, Tzfira and Citovsky 2002, Bourras et al. 2015, Hwang et al. 2017). This infection is mediated by the collective action of virulence genes encoded by the bacterial chromosome (chv) and Ti-plasmid virulence (Vir) genes. With the aid of an exogenous supply of phenolics for virulence induction, the host range of *Agrobacterium* has successfully been expanded, under laboratory conditions, to yeast, fungi, human cells and sea urchins (Kunik et al. 2001, Bulgakov et al. 2006, Zheng et al. 2011, Zhang et al. 2014, Rolloos et al. 2015, Lin et al. 2017).

One way of improving transformation and gene expression efficiency in algae has been through the use of endogenous promoters of genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fukuda et al. 2008), actin1 (Takahashi et al. 2010) and the heat shock protein HSP70 (Son et al. 2011). Proteins, especially those from species using non-canonical codes or containing regulatory elements which limit expression, are difficult to express in their non-native host since their codons might be rarely used in the host (Gustafsson et al. 2004). Hence, optimization of transgene codons to match the native host codon preference has resulted in improvements in transcription and translation efficiencies in various organisms including algae (Fukuda et al. 2008, Mikami et al. 2009, Kucho et al. 2013, Hirata et al. 2014, Šnajder et al. 2015, Barahimipour et al. 2016, Zhou et al. 2016).

The aim of this study was to develop and test a working *Agrobacterium*-mediated transformation protocol tailored to the complex red alga *Chondrus crispus*. The factors known to influence the efficiency of *Agrobacterium*-mediated transformation were investigated: (1) co-cultivation in induction medium or seawater (salinity 20), (2) inclusion (100 μM) or exclusion of the phenolic acetosyringone during co-cultivation and (3) wounding using microparticle bombardment versus mechanical injury administered with a sharp, sterile sewing pin, and (4) using an endogenous *Chondrus* expression cassette. *Chondrus crispus* was considered a good candidate, being an economically valuable carrageenophyte with developed transcriptomic tools and a full genome sequence (Collén et al. 2013) which greatly facilitated adaptation of the expression cassette to *Chondrus*.

Materials and methods

Algal collection, sterilization and culture

Chondrus crispus female gametophytic fronds were collected from rock pools and the lower intertidal zone at various sites near Fanad Head, Co. Donegal, Ireland, and transported to Belfast for processing. Fronds were cut into about 4 cm segments and thoroughly washed with pasteurized seawater to remove associated debris. Epiphytic material and surface contaminants were brushed away under a stereomicroscope (Kyowa Optical SDZ-PL, Singapore). The thallus segments were surface-sterilized by immersion in 1.5% potassium iodide for 10 min followed by thorough rinsing in pasteurized seawater. They were cultured in seawater or one-tenth strength von

Stosch medium (Guiry and Cunningham 1984; chemicals obtained from Sigma-Aldrich, UK) at 10°C under a 16:8 light/dark regime with aeration.

Agrobacterium strains and binary vector constructs

Agrobacterium strain LBA4404 was kindly provided by Andy Bailey, Bristol University, England. A *Chondrus*-specific *GUS* expression cassette was constructed (Figure 1A). The codons of a 1.812 kbp bacterial *GUS* sequence from pBI 221 (GenBank accession AF502128.1) were modified manually by silent mutations to match the favoured codon usage of native *Chondrus* (Collén et al. 2013); this increased the G/C content of the *GUS* coding sequence from 52 to 66% (Supplementary Figure S1). The 3.052 kbp sequence upstream of the start codon of the *Chondrus* actin gene (CHC_T00008840001, NCBI Accession NW_005179192) on scaffold 307 (positions 29997–26946) was fused upstream of the *Chondrus*-specific *GUS* sequence while a 0.253 kbp *NOS* terminator (GenBank accession AF502128.1) was fused downstream of the *GUS* sequence. This construct was cloned into the multiple cloning site of the 9.168 kbp binary vector, pRI 910 (Clontech). For *Agrobacterium* transformation, pRI 910 (Ac-GUS) and the binary vector pCAMBIA 1301 (Figure 1) were introduced separately into competent cells of the *Agrobacterium* strain LBA 4404 using the freeze/thaw method (Wise et al. 2006). For each expression cassette, two independent experiments, each with two replicates of 10 thalli, were conducted.

Agrobacterium-mediated transformation of *Chondrus*

In order to induce the bacteria for transformation, the virulence induction protocol outlined by Gelvin (2006) was followed with modifications. *Agrobacterium* was grown at 28°C, with agitation at 225 rpm overnight in 5 ml Luria Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} sodium chloride, pH 7; Merck, Germany) containing 50 μg ml^{-1} kanamycin (Sigma-Aldrich, UK). A volume of 500 μl bacterial culture was diluted into 50 ml Induction Medium (IM) [prepared using minimal medium as described by Ando et al. (2009) with addition of 0.5% (w/v) glycerol and 40 mM 2-(*N*-morpholino) ethanesulphonic acid (MES), pH 5.3] supplemented with kanamycin and rifampicin at 50 μg ml^{-1}, and grown at 30°C for about 24 h until it reached an absorbance reading of $A_{600\,nm} = 1.2$.

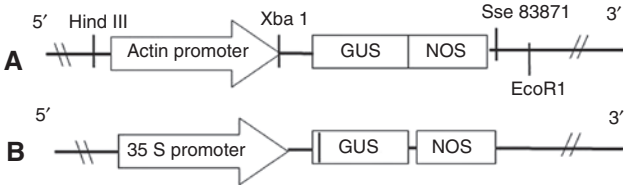

Figure 1: Features of binary vectors for *Agrobacterium*-mediated transformation.
(A) *Chondrus*-specific expression cassette. Salient features: 1.812 kbp *GUS* gene modified to match the favoured codon usage of *Chondrus*, promoter region of the *Chondrus* actin gene, nopaline synthase (*NOS*) gene terminator from Genbank accession AF502128.1; (B) Binary vector pCAMBIA 1301. Salient features: *GUS* gene with castor bean catalase intron driven by a CAMV 35 S promoter and terminated by *NOS*.

The suspension was centrifuged for 15 min at 10,000 *g* to harvest the bacterial cells. The bacterial cells were resuspended in 100 ml IM containing 100 μM acetosyringone (Sigma-Aldrich, UK) and 50 μg ml^{-1} kanamycin and left to shake at 60 rpm (to keep the bacteria suspended) for 24 h at 25°C. The bacterial cells were pelleted by centrifugation and resuspended in IM or seawater (salinity 20) containing 100 μM acetosyringone ready for co-cultivation with *Chondrus* thalli.

Immediately before co-cultivation with *Agrobacterium*, surface-sterilized thallus segments (1 cm in length) were desiccated for 15 min, then pricks and tears were made on the surface using a pointed sterile pin. Biolistic wounding was achieved by bombarding gold microcarriers (0.6 μm) at 1350 psi into thalli using a PDS-1000/He delivery system (Bio-Rad Laboratories, USA). Thallus segments were layered in the centre of a petri dish positioned in the middle of the target shelf at a distance of 3 cm from the microcarrier launch assembly. The bombardment chamber was evacuated to 63.5 cm of mercury and 500 μg of uncoated gold microcarriers (0.6 μm) was bombarded into the tissue with the biolistic machine.

Sterilized thallus segments, both wounded and unwounded (on glass slides in petri dishes), were co-cultivated with a suspension of *Agrobacterium* in seawater with 100 μM acetosyringone. The dishes were incubated at room temperature (18–20°C) for 2 days in the dark. Suitable controls were included: (1) Unwounded and untransformed thalli (thalli not subjected to *Agrobacterium*-mediated transformation), (2) Pin-pricked thalli co-cultivated with untransformed *Agrobacterium* LBA4404 bacteria, (3) Pin-pricked thalli cultivated without bacteria, and (4) Pin-pricked thalli co-cultivated with *Agrobacterium* bearing a construct where the *GUS* gene is absent (pRI 910 binary vector).

Developing a wash protocol for post-transformation

To eliminate residual bacteria after transformation and avoid the occurrence of false GUS positives, a wash protocol was developed and cefotaxime (Sigma-Aldrich, UK) was tested for bacteriostatic effects on *Agrobacterium*. Two cefotaxime concentrations (500 and 800 mg l^{-1}) were tested on thallus segments that had been co-cultivated with *Agrobacterium* LBA4404 cells. Every 2 days, the thallus segments were rinsed for 4 min with sterile seawater six times before incubation for 48 h in fresh seawater with replenished cefotaxime. A thallus segment was streaked on a LBA plate which was incubated at 28°C for up to 4 days to check for the persistence of *Agrobacterium*. This was conducted in triplicate.

Post-transformation elimination of *Agrobacterium*

Following co-cultivation, *Agrobacterium* cells were eliminated by brushing the surface of the thallus segments with an ethanol-sterilized paintbrush followed by rinsing in sterilized seawater. After three consecutive 4-min rinses in sterilized seawater, the transformed thalli were incubated with 500 mg l^{-1} cefotaxime for 48 h in the dark at 18°C. A streak of the thallus was made on a LBA plate (which was then incubated for 2 days at 28°C and checked for the presence of bacteria). The previous step (rinsing and incubating in cefotaxime) was repeated every 2 days, adding fresh seawater with replenished cefotaxime until no bacterial colonies were obtained from the LBA streaking (within approximately 6 days). Antibiotic was washed away, and the tissue was transferred to enriched seawater at 18°C for recovery at a photoperiod of 16 h light: 8 h dark.

Histochemical staining and microscopic analysis of thallus sections

Histochemical staining of thallus segments was conducted after transformation (8 days following co-cultivation). These segments were washed several times in sterile seawater before incubating at 37°C for 2–3 days in GUS staining solution made up in distilled water with 0.1 M $NaPO_4$ at pH 7 (0.12 M Na_2HPO_4 and 0.08 M NaH_2PO_4), 10 mM EDTA, 1 mM $K_3Fe(CN)_6$, 0.1% Triton X-100 and 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). All chemicals were obtained from Sigma-Aldrich, UK. Following the staining, chlorophyll was cleared by incubating the stained tissue for 2–3 days in 70% (v/v) ethanol after which sections made from the thalli were ready for viewing under the microscope.

Longitudinal and transverse sections through the cortical and medullary tissue of thalli were made using a sterile razor blade under a stereomicroscope to investigate the localization pattern of GUS expression within tissues. Sections were mounted on a slide in 50% seawater: 50% glycerol for viewing under a light microscope (Motic B series, USA) and photographed using an attached camera (Leica Microsystems, UK).

To view bacterial attachment to *Chondrus* thalli using scanning electron microscopy (SEM), thalli which had been co-cultivated with *Agrobacterium* (OD 1.2) for 2 days were first rinsed to wash away unattached bacteria, before being fixed. Fixation was carried out for 1 h at room temperature in 4% (w/v) glutaraldehyde (Fisher Scientific, UK) in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose (Sigma-Aldrich, UK). The thalli were subsequently fixed in fresh fixative for a further 3 h at 4°C, then washed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose. After post-fixation treatment in 1% osmium tetroxide (Sigma-Aldrich, UK) for 1 h, the tissues were washed several times in fresh buffer, dehydrated using an ethanol series and dried in hexamethyldisilazane (Sigma-Aldrich, UK). The thalli were then mounted on aluminium stubs, sputter-coated with gold-palladium and viewed with a FEI Quanta 200 SEM operating at 10 kV.

Statistical analysis

The data were tested for normality using the Kolmogorov-Smirnov test. The Mann-Whitney non-parametric test was used to compare between two groups.

Results

Agrobacterium LBA4404 cells successfully attached to *Chondrus* cells

Under the SEM, short, rod-shaped bacterial cells about 0.8 × 2 μm were seen attached in large numbers (an estimated average of one bacterial cell per μm^2) to the *Chondrus* thallus surface at the wound sites. Whole colonies were observed in a fibrillar mesh (Figure 2).

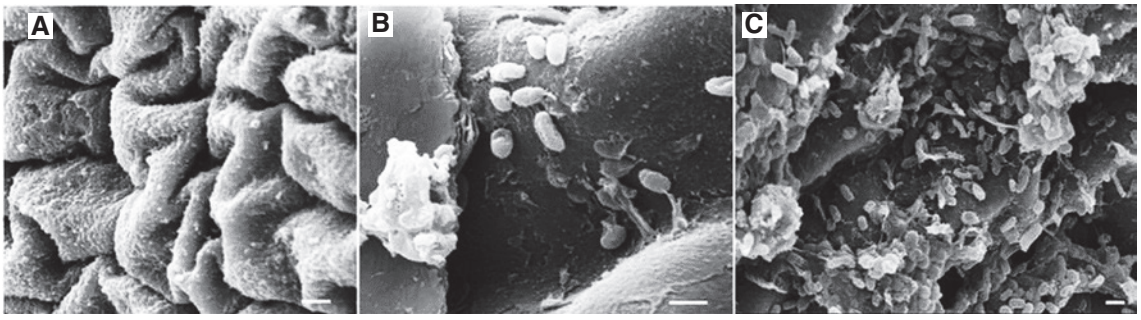

Figure 2: Intimate contact and adhesion of bacteria to wound sites of *Chondrus* thalli.
Thalli co-cultivated for 48 h in induction medium were fixed and viewed under a scanning electron microscope: (A) control thallus incubated without bacteria showing a clean bacterium-free surface; (B) close up view of rod-shaped bacteria attached to thallus surface at wound site; (C) bacterial film at wound sites on thallus surface.

Protocol for eliminating *Agrobacterium* using cefotaxime

The results of testing cefotaxime concentrations of 500 and 800 mg l^{-1} confirmed that this cefotaxime-supplemented wash protocol ensured that residual *Agrobacterium* and other culturable bacteria were eliminated on the thalli within 6 days (Table 1).

Agrobacterium successfully delivered pCAMBIA 1301 into *Chondrus* thalli

The methodology for transformation developed using pCAMBIA 1301 followed by the established wash protocol included controls to detect any false positives occurring from indigenous activity of beta-glucoronidase in *Chondrus* (unwounded and untransformed thalli, Figure 3C), indigenous activity from LBA4404 bacterial cells (pin-pricked thalli co-cultivated with untransformed *Agrobacterium* LBA4404 bacteria, Figure 3B), physiological response of algal cells to wounding (pin-pricked thalli cultivated without bacteria, Figure 3A) and vector components (pin-pricked thalli co-cultivated with *Agrobacterium* LBA4404 bearing a construct where the *GUS* gene is absent; pRI 910 binary vector, Figure 3D). None of these controls were stained. However, thalli transformed with pCAMBIA 1301 were stained blue (Figure 3E).

Table 1: Confirmation of the suitability of 500 and 800 mg l^{-1} cefotaxime in eliminating bacteria.

	Mean number of colonies (per plate)		
Day	Concentration of cefotaxime (mg l^{-1})		
	0	500	800
0	>1000	>1000	>1000
2	>300	0.75 ± 0.95	1.25 ± 0.95
4	>300	0.25 ± 1.60	2.00 ± 0.50
6	>300	0	1.00 ± 1.2
8	>300	0	0

Agrobacterium co-cultured with thalli was eliminated by consecutive washes and incubated in fresh antibiotic. Every 2 days, the thallus segments were rinsed for 4 min with sterile seawater six times before incubation for 48 h in fresh seawater with replenished cefotaxime. A thallus segment was streaked on a LBA plate which was incubated at 28°C for up to 4 days to check for the persistence of *Agrobacterium*. This was conducted in triplicates (n = 3). Colonies appearing from periodic streaks of thalli on LBA plates were counted.

Manipulating transformation conditions can enhance transformation efficiency

As shown in Figure 4, among the pin-pricked thalli, a significant increase in transformation efficiency (higher percentage of thallus segments stained blue) was observed when seawater was used in preference to Induction Medium (Mann-Whitney test, $p = 0.029$) in the presence of acetosyringone. However, no transformation was detected in the unwounded thallus segments or those wounded by bombardment when co-cultivation was conducted using seawater. In addition, without the presence of acetosyringone, transformation occurred at a significantly lower efficiency of less than 10% (Mann-Whitney, $p = 0.004$).

Successful expression obtained with the *Chondrus*-specific expression cassette

Using the *Agrobacterium* transformation protocol developed using pCAMBIA 1301, the constructed pRI 910 (Ac-GUS) was delivered into thallus cells using seawater as

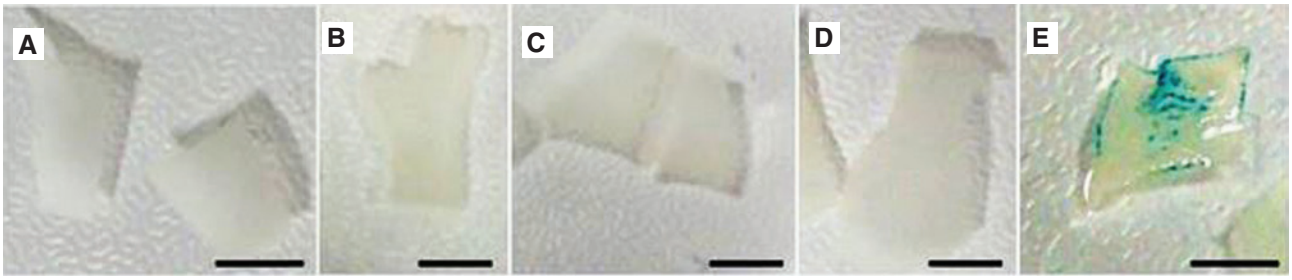

Figure 3: Histochemical staining and low-power microscopic analysis of *Chondrus* thallus segments for transient expression of *GUS* 7 days after co-cultivation.
(A) Pin-pricked thalli co-cultivated without bacteria, (B) pin-pricked thalli co-cultivated with untransformed LBA4404 bacteria, (C) unwounded and untransformed thalli, (D) pin-pricked thalli co-cultivated with LBA4404/pRI 910 (no *GUS* gene), (E) thalli transformed with LBA4404 (pCAMBIA 1301), scale bar: 1 cm.

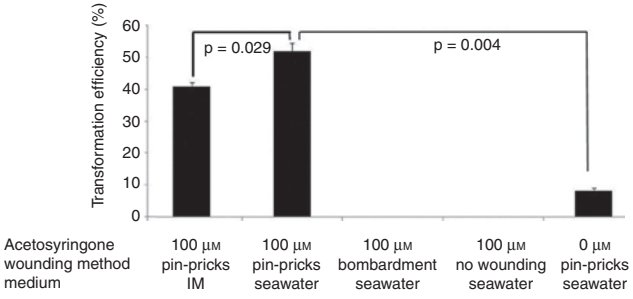

Figure 4: Effect of cultivation medium, wounding method and acetosyringone on *Agrobacterium* transformation efficiency. The transformation efficiencies of *Chondrus* thalli segments co-cultivated with pCAMBIA 1301-transformed *Agrobacterium* in seawater and Induction Medium (IM) were compared. Using seawater as co-cultivation medium, the wound-related treatments, namely biolistics wounding, wounding using pin-pricks and no wounding, were compared. The effect of acetosyringone (100 µM) was also studied. Data are expressed as means ± SE (n = 4). The Mann-Whitney non-parametric test was conducted to compare between two groups.

the co-cultivation medium and with the same controls as for pCAMBIA 1301. Blue colouration was apparent at the sites of tear, cut or wounding on *Agrobacterium*-mediated transformed thalli when compared to controls (Figure 5). Transverse sections through the tissue revealed blue localization of the GUS precipitate within the peripheral cortical cells and central medullary filaments. In general, a darker staining intensity was observed with pRI 910 (Ac-GUS). Furthermore, a higher transformation efficiency of 85% was obtained with this *Chondrus*-specific construct as opposed to 52% with pCAMBIA 1301.

Discussion

In order to induce virulence in *Agrobacterium* and trigger the deployment of a Type IV secretion system (T4S) for T-DNA delivery into the host, intimate contact between the bacterial cells and the host cells is essential (Matthysse and Wagner 1994, Gelvin 2003, Lacroix and Citovsky 2013, Heindl et al. 2014). Hence, the observed close association of the bacteria with the *Chondrus* thalli (Figure 2) constitutes a first line of evidence pointing to the amenability of the algal tissue to agro-infection.

Following *Agrobacterium*-mediated transformation with pCAMBIA 1301, successful T-DNA delivery and transient expression of the reporter *GUS* gene were evidenced by the clear visualization of the blue colour localized within *Chondrus* thallus cells (Figure 3E). This also pointed to the functionality of the Cauliflower Mosaic Virus 35S promoter in *Chondrus* which has also worked well in *Dunaliella bardawil* (Anila et al. 2011), *Gracilaria changii* (Gan et al. 2003), *Kappaphycus alvarezii* (Kurtzman and Cheney 1991) and *Pyropia miniata* (Kübler et al. 1994).

As observed with other red algae (Kübler et al. 1994, Kuang et al. 1998, Gan et al. 2003, Fukuda et al. 2008), the GUS reporter system unambiguously allowed the visualization of cellular GUS expression in *Chondrus* in the absence of endogenous beta-glucuronidase-like activity. In addition, to ensure that residual *Agrobacterium* and other culturable bacteria were eliminated after *Agrobacterium*-mediated transformation, cefotaxime was chosen for its wide spectrum of activity and low toxicity to eukaryotes (Mathias and Boyd 1986, Duan et al. 2013). No bacterial colonies were detected from the LBA streaking of thalli, which were subjected to the robust wash protocol using 500 mg l^{-1} cefotaxime after the *Agrobacterium*-mediated transformation (Table 1). Effective elimination of bacteria, together with the use of suitable transformation controls and the intron-containing *GUS*, helped to ensure the absence of false positives from endogenous GUS activity or non-target organisms.

The darker staining pattern of cells with pRI 910 (Ac-GUS) can be explained by a synergistic effect on the level of gene expression resulting from combining a

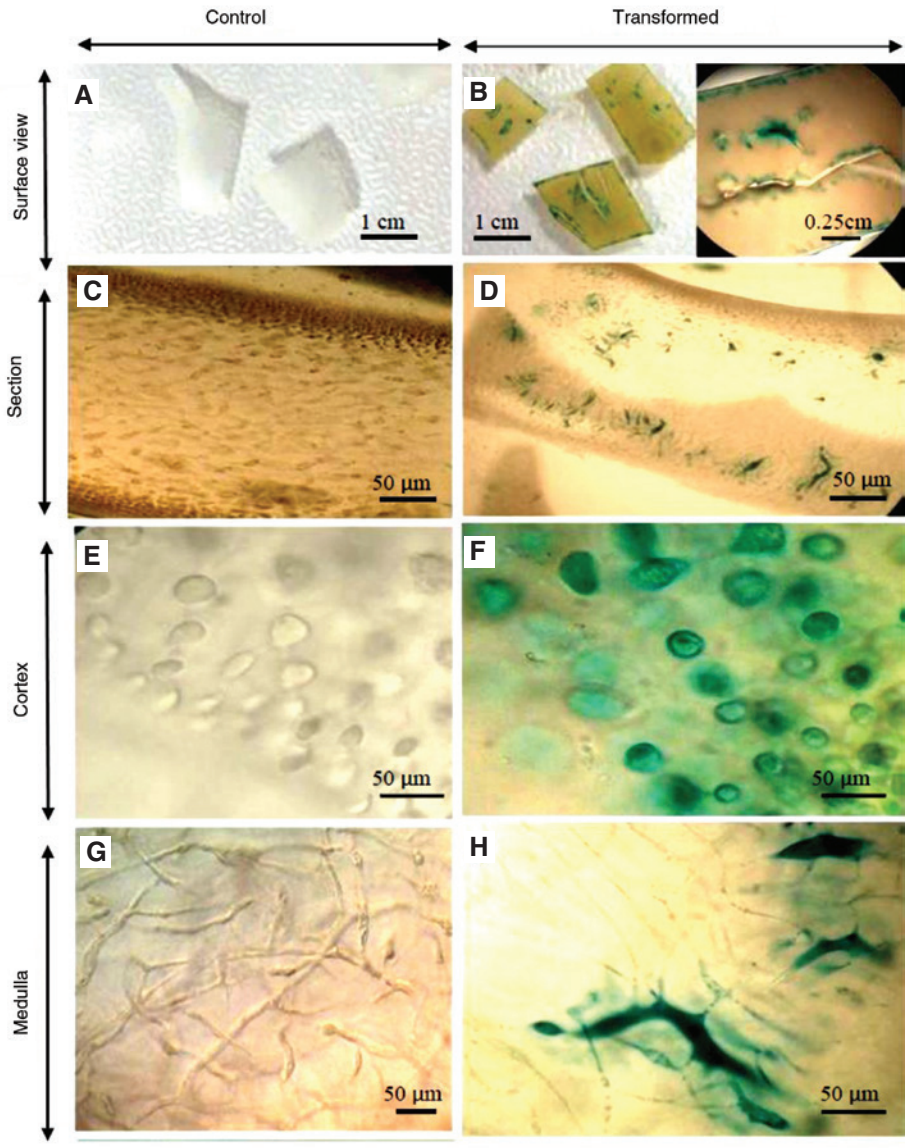

Figure 5: GUS expression in *Chondrus* thallus segments transformed with LBA 4404/pRI 910 (Ac-GUS).
(A) Surface view of control thallus not co-cultivated with *Agrobacterium*, (B) surface view of transformed thallus showing GUS expression at wound sites, (C) section through untransformed thallus, (D) thallus section within wounded area showing blue medullary cells at wound spots, (E) cortical cells from untransformed control thallus, (F) GUS-expressing cortical cells in transformed thallus, (G) medullary filaments from untransformed control thallus, (H) medullary filaments from transformed thallus.

strong native promoter with a codon-optimized reporter gene. This effect has also been reported by Fukuda et al. (2008). Although it was not possible in this study to tease out the separate effects of the actin promoter or the *GUS* gene on the level of gene expression, interesting interpretations can still be made. Adjusting the codon usage to that favoured by *Chondrus*, in conjunction with using its endogenous actin promoter, seemed to have enhanced recognition by the molecular machinery of *Chondrus*. Successful transcription of the *Chondrus*-tailored *GUS* gene indicated that the constitutive actin promoter together with any necessary upstream elements were contained within the 3 kbp sequence upstream of the *Chondrus* actin start codon. These same observations were also made by Takahashi et al. (2010) who isolated a 3.1 kbp region encompassing the promoter of the *actin*1 gene in *Pyropia yezoensis*. This housekeeping gene, expected to be functional in all tissues at all times as an essential component of the cell cytoskeleton (Seagull 1989), proved to be the right choice of promoter for the construct. Moreover, other researchers have also found that the actin promoter works well in driving gene

expression (Dennis et al. 1984, McElroy et al. 1990, Christensen et al. 1992). Unlike the catalase-intron-containing *GUS* of pCAMBIA 1301, no intron was included in the modified *Chondrus GUS* construct given the clear preference of *Chondrus* for monoexonic genes which constitute 88% of all its genes (Collén et al. 2013).

It is known that success in transformation also largely depends on the right combination of factors such as the *Agrobacterium* strain, culture medium, tissue wounding, suppression and elimination of the bacterium post transformation (Opabode 2006). Subsequently, the transformation methodology used was tailored to foster optimal *Chondrus-Agrobacterium* interaction. The *Agrobacterium* LBA4404 strain was chosen because it is comparatively salt-tolerant. Furthermore, it has proved efficient in transforming plant species ranging from tobacco (Liang et al. 1997), rice and *Arabidopsis* (Kuriakose et al. 2009) to algae such as *Chlamydomonas* (Kumar et al. 2004), *Chlorella vulgaris* (Cha et al. 2012) and *Schizochytrium* (Cheng et al. 2012). Successful transient expression confirmed that *Agrobacterium* cells were viable in seawater (salinity 20) and could deliver T-DNA at this salinity at an even higher transformation efficiency (Figure 4). This could possibly be ascribed to the *Chondrus* cells being under less physiological stress than in induction medium (salinity 0.15) and better able to synthesize the GUS protein.

In *Chondrus*, as previously reported with *Haematococcus pluvialis* (Kathiresan et al. 2009), transformation occurred, unaided by acetosyringone, pointing to the possible release of a small amount of endogenous phenolic compounds from the wounded cells. Algal extracts are known to be rich in polyphenols particularly phlorotannins in brown algae (O'Sullivan et al. 2011, Stabili et al. 2012). It has been postulated that phenolics are specifically mobilized in *Chondrus* in response to pathogenic attack (Bouarab et al. 2004). However, the significantly higher transformation efficiency obtained with acetosyringone highlighted the importance of using phenolics in transforming *Chondrus* (Figure 4). A significantly higher transformation efficiency was reported in the presence of a phenolic compound (Kimura et al. 2015). However, the effects of phenolic compounds such as acetosyringone on transformation efficiency depend on the host types and bacterial strains (Ainsley et al. 2001, Wang et al. 2002).

While T-DNA transfer to unwounded cells was reported in the microalgae *Haematococcus pluvialis* (Kathiresan et al. 2009) and *Chlamydomonas reinhardtii* (Kumar et al. 2004), wounding was required with *Chondrus* (Figure 4). *Agrobacterium* has a Vir A/Vir G system which is finely tuned to respond to wound site signals (Pitzschke and Hirt 2010). According to Stachel et al. (1985), wounding not only favours the production of signal phenolics but also enhances accessibility of putative cell wall binding factors to the bacterium (Lippincott and Lippincott 1969). The tears, cuts and pinpricks might have also facilitated penetration of the bacteria into the tissue as evidenced by the blue inner medullary filaments (Figure 5). With biolistics, microparticles penetrate the cuticle layer and cell walls, creating numerous potential infection sites simultaneously. Although no visual damage was apparent, it was possible that this micro-wounding method compromised gene expression through cellular damage. A large amount of wounding could cause instant cell lysis while sublethal amounts could temporarily suppress RNA and protein synthesis, and moderate levels could induce cell wall rupture (Joersbo and Brunstedt 1992). On the other hand, insufficient puncture and wounding of cells would fail to attract *Agrobacterium*, possibly accounting for the absence of staining at some wound sites (Figure 4). This confirmed the importance of choosing a suitable wounding method for the organism to be transformed.

Conclusion

This study points to the amenability of *Chondrus* to be transformed by *Agrobacterium in vitro*. This study could serve as a stepping stone to facilitate further transgenic studies of *Chondrus* such as those aimed at deciphering the function of important *Chondrus* red algal genes involved in carrageenan metabolic pathways (Waaland et al. 2004). It would be worthwhile, for example, to enhance expression cassette elements, and test different virulent bacterial strains, other phenolic inducers or wounding methods. To obtain a more comprehensive insight into the superiority of the *Chondrus*-specific cassette, quantitative methods of gene expression such as quantitative real-time PCR would be useful. In particular, other methods or wounding strategies favouring more homogeneous penetration and infection of a larger area of thallus tissue by *Agrobacterium* e.g. vacuum infiltration, would further enhance the transformation protocol. Furthermore, the prior development of robust *Chondrus* regeneration and selection protocols from transformed cells is warranted in order to establish stable *Agrobacterium*-mediated transformation of *Chondrus*.

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