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Heterologous expression of the *Streptococcus pneumoniae yoeB* and *pezT* toxin genes is lethal in *Chlorella vulgaris*☆

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ABSTRACT

Chlorella vulgaris is a eukaryotic microalga with potential for the production of biofuels. However, its thick and rigid cell wall is an impediment to cost-effective, large-scale harvesting of biofuels from these cells. Bacterial toxin-antitoxin (TA) systems, comprising of a stable proteic toxin and its labile cognate antitoxin, have no known homologs in eukaryotic cells. Several bacterial TA toxins have been found to be lethal when expressed in eukaryotes such as yeasts, animal and human cell lines. In this study, the functionality of the $yoeB_{Spn}$ and *pezT* toxin genes from the Gram-positive bacterium *Streptococcus pneumoniae* in *C. vulgaris* was investigated using a two-component inducible expression system. The $yoeB_{Spn}$ and *pezT* toxin genes were each cloned as green fluorescent protein (GFP) fusion constructs and introduced into *C. vulgaris* by *Agrobacterium tumefaciens*-mediated co-transformation with recombinant activator and responder vectors. Following induction for the expression of the toxin-GFP fusion transgenes, GFP fluorescence was observed in the transformed *C. vulgaris* cells which also showed signs of cellular damage and lysis. This is the first report of the lethal expression of bacterial TA toxins in eukaryotic microalgae, which can form the basis of a novel method for harvesting of microalgal cellular contents.

1. Introduction

Chlorella vulgaris is a unicellular eukaryotic microalga that shares many similar production characteristics with plants such as photosynthesis and carbon dioxide fixation with oxygen production [1]. *C. vulgaris* can multiply by asexual reproduction every 24 h, if grown under optimal conditions [2]. This microalga is widely found in natural waters such as ponds or lakes as well as in wastewater ponds. The cultivation of *C. vulgaris* next to combustion power plants with excess carbon dioxide release enabled the microalgae to absorb the unwanted carbon dioxide or secondary products such as nitrates which the microalgae utilizes during the conversion into potential biofuel or feeds [3]. Under nitrogen starvation, *C. vulgaris* is able to accumulate high lipid content that results in favorable fatty acid profiles for biodiesel production [4]. The production of biohydrogen using *C. vulgaris* was also deemed attractive as it requires only the available solar energy and can be used as gas fuel for electricity generation [5]. The production of bio-energies from microalgae are of major importance as they can eventually replace the use of agricultural crops since microalgae-based bioenergy production can be carried out on large scales with higher fuel yield as compared to the former [6]. Besides that, the cost can be effectively decreased as smaller areas are required [7] and the microalgae can be reused for the bioremediation of waste products [8].

Since microalgae are deemed attractive candidates for the generation of a wide range of bioenergy products such as biofuel, biohydrogen and bioethanol [8], a lot of research has been carried out to improve methods to efficiently harvest the cell contents of microalgae. The most used microalgae-biomass harvesting techniques include centrifugation, filtration and flocculation [9] which can provide high biomass recovery from the culture medium [10]. However, these harvesting techniques have limitations which restrict them from being widely used in the microalgal-biomass harvesting industry: the high gravitational force and shear forces during centrifugation was reported to cause cell structural damage and the energy requirement negatively impacted the CO_2 balances in microalgae-based biodiesel production [11] while filtration methods are limited by the differing sizes of the microalgae, which ranged from as small as 1 μM to as large as over 70 μM, leading to high costs for membrane filter replacement used for different microalgae species [11]. Although flocculation can have up to

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80% flocculation efficiency, it is not feasible to sustain large-scale microalgae-biomass harvesting as production plants require large quantities of flocculants which produce excess cationic flocculent that eventually has to be removed, leading to additional operating costs [12]. Furthermore, microalgae have rigid cell walls which impede the harvesting of cell contents, for example, *C. vulgaris* is protected by a rigid trifluoroacetic cell wall that is composed of glucosamine polymer [13]. Although enzymatic degradation of microalgal cell walls prior to biomass harvesting can address this issue, the feasibility of this pretreatment is still questionable in terms of large-scale microalgae-biomass recovery for bio-energy production [13]. Therefore, the need for new approaches for maximizing the harvesting potential from *C. vulgaris* is desirable. One of the potential alternative approaches is through the genetic manipulation of *C. vulgaris* to introduce genes to induce rapid cell death and cell lysis that will release the valuable microalgal cell contents.

Toxin-antitoxin (TA) systems are nearly ubiquitous genetic elements that are found in prokaryotic genomes and have been implicated in the regulation of bacterial cell death and dormancy [14]. The mechanism of TA action relies heavily on the differential stability of the two components, namely the stable toxin and the labile cognate antitoxin. Under normal growth conditions, the antitoxin is continuously produced to bind to the toxin, thus neutralizing the toxin's lethal effects. When bacterial cells are under stress conditions, endogenous cellular proteases degrade the antitoxin causing the liberation of the toxin from the TA complex. This will result in the toxin acting on its particular cellular targets, often resulting in cell lysis and death [15]. Bacterial TA systems are currently classified into six types (Types I–VI) with Type II systems being the most prevalent and well-characterized [16–18]. In Type II TA systems, the proteic antitoxin prevents the lethal action of the toxin through tight binding with the toxin, usually at the active site of the toxin [16,18,19]. Most type II toxins are endoribonucleases while other toxins disrupt DNA replication by targeting DNA gyrase and helicase [14], and some inhibits the synthesis of the bacterial cell wall [20].

No eukaryotic homologs of bacterial TA systems have been reported but several bacterial TA toxins have been shown to be functionally lethal in eukaryotic cells, leading to several interesting and novel applications [21]. Recently, it was reported that the expression of the *Streptococcus pneumoniae*-encoded $YoeB_{Spn}$ toxin was lethal in the model plant *Arabidopsis thaliana* [22]. A two-component XVE-based expression system comprising of an activator vector and a responder vector [23] was used to enable strict inducibility with 17-β-estradiol for the cloned $YoeB_{Spn}$ transgene in *A. thaliana* [22]. Until now, there has yet to be any report on the heterologous expression of bacterial TA toxins in eukaryotic microalgae. In this study, the same two-component XVE-based expression system that was used in *A. thaliana* was used to investigate the functionality of the $YoeB_{Spn}$ as well as another *S. pneumoniae*-encoded toxin, PezT [24] in *C. vulgaris*. The functional lethality of the bacterial $YoeB_{Spn}$ and PezT toxins in *C. vulgaris* would thus ultimately pave the way for the development of novel cloning strategies to efficiently harvest valuable cell contents such as biofuels from microalgae.

2. Materials and methodology

2.1. Agrobacterium tumefaciens and Chlorella vulgaris cultures and conditions

Recombinant *A. tumefaciens* LBA4404 cells were cultured in Luria-Bertani (LB) broth and on LB agar solidified with 1.2% (w/v) bacto-agar, supplemented with the appropriate antibiotics. For *A. tumefaciens* harboring the recombinant pMDC150-derived activator vector, the antibiotics used were rifampicin (50 μg mL^{-1}) and spectinomycin (50 μg mL^{-1}); whereas for *A. tumefaciens* harboring recombinant pMDC221-derived responder vector, the antibiotics used were rifampicin (50 μg mL^{-1}) and ampicillin (100 μg mL^{-1}) [23]. Bacterial culture on agar was incubated at 27 °C while those in broth were incubated at 27 °C with shaking at 220 rpm. Both agar and broth cultures of *A. tumefaciens* were incubated in the dark at all times as rifampicin is a light sensitive antibiotic.

C. vulgaris UMT-M1 [25] was cultured in Bold's Basal Medium (BBM) broth and on BBM agar [26] solidified with 1.2% w/v bacto-agar. Microalgae culture on agar was incubated at 27 °C while those in broth were incubated at 27 °C with shaking at 220 rpm. Both agar and broth culture of *C. vulgaris* were exposed to continuous photon flux density of 40 μmol m^{-2} s^{-1}. For selection, the transformed *C. vulgaris* was cultured on selective BBM supplemented with kanamycin (50 μg mL^{-1}) and hygromycin (20 μg mL^{-1}). The agar and broth culture of transformed *C. vulgaris* were incubated in the dark for the first 24 h before exposure to light as hygromycin is a light-sensitive antibiotic.

2.2. Recombinant plasmids

A two-component 17-β-estradiol-inducible expression system comprising of the pMDC150 activator vector and the pMDC221 responder vector [23] was used for the expression of the two prokaryotic toxins genes in this study.

A recombinant activator vector pMDC150_35S, containing the CaMV 35S constitutive promoter as previously described [22] was used for the constitutive expression of the chimeric XVE transcriptional activator in the transformed microalgae, while the responder vector pMDC221_yoeBGFP carries the $yoeB_{Spn}$ toxin gene from *S. pneumoniae* as a translational fusion with a *GFP* gene as described by Abu Bakar et al. [22]. The resulting pMDC221_yoeBGFP (Fig. 1B) recombinant responder places the $yoeB_{Spn}$-*GFP* fusion under the control of the XVE-responsive promoter (OlexA-TATA promoter) thus making its expression dependent on the presence of 17-β-estradiol. A *GFP*-expressing recombinant vector pMDC221_GFP (Fig. 1D) [22] was used a positive control.

The *pezT* toxin gene (GI: 446327505; coordinates 1657560–1658321 of accession no. NZ_AKBW01000001) from *S. pneumoniae* was also cloned as a translational fusion with the *GFP* gene into pMDC221. The *pezT* toxin gene was PCR-amplified as a 762 bp fragment from the pET11a-PezT recombinant plasmid [24] using the primers pezT_F and pezT_R (Table 1). A *Bam*HI (5′-GGATCC) restriction site was included at the 5′-end of the pezT_R reverse primer and the 5′-end of the *GFP* forward primer (GFP_F) (Table 1) to enable the synthesis of a *pezT-GFP* fusion. The *GFP* gene was PCR-amplified as a 732 bp fragment as described in [22]. PCR amplifications were carried out under the following conditions: initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s; annealing at 57 °C for 30 s; and extension at 72 °C for 3 min; and a final extension at 72 °C for 7 min. Both the *pezT* and *GFP* amplified products were subjected to *Bam*HI digestion for 2 h at 37 °C prior to overnight ligation using T4 DNA ligase (Promega, WI, USA) at 4 °C. The resulting 1497 bp *pezT-GFP* ligated product was then cloned into the Gateway pENTR_D_TOPO cloning vector (Invitrogen, USA) according to the supplier's instructions. The recombinant *pezT-GFP* Gateway entry clone was validated by conventional Sanger dideoxy sequencing prior to the transfer of the *pezT-GFP* fragment into pMDC221 as the Gateway destination vector using the LR clonase reaction (Invitrogen, USA). The constructs obtained were transformed into *Escherichia coli* TOP10 cells and transformants screened by colony PCR using pezT_F and GFP_R primers. The responder recombinant vector was designated pMDC221_pezTGFP (Fig. 1C) after validation by conventional sequencing.

2.3. Co-transformation of two-component inducible expression vectors into C. vulgaris

A. tumefaciens-mediated transformation of *C. vulgaris* was carried out according to Cha et al. [25] with modifications. A total of 5×10^7

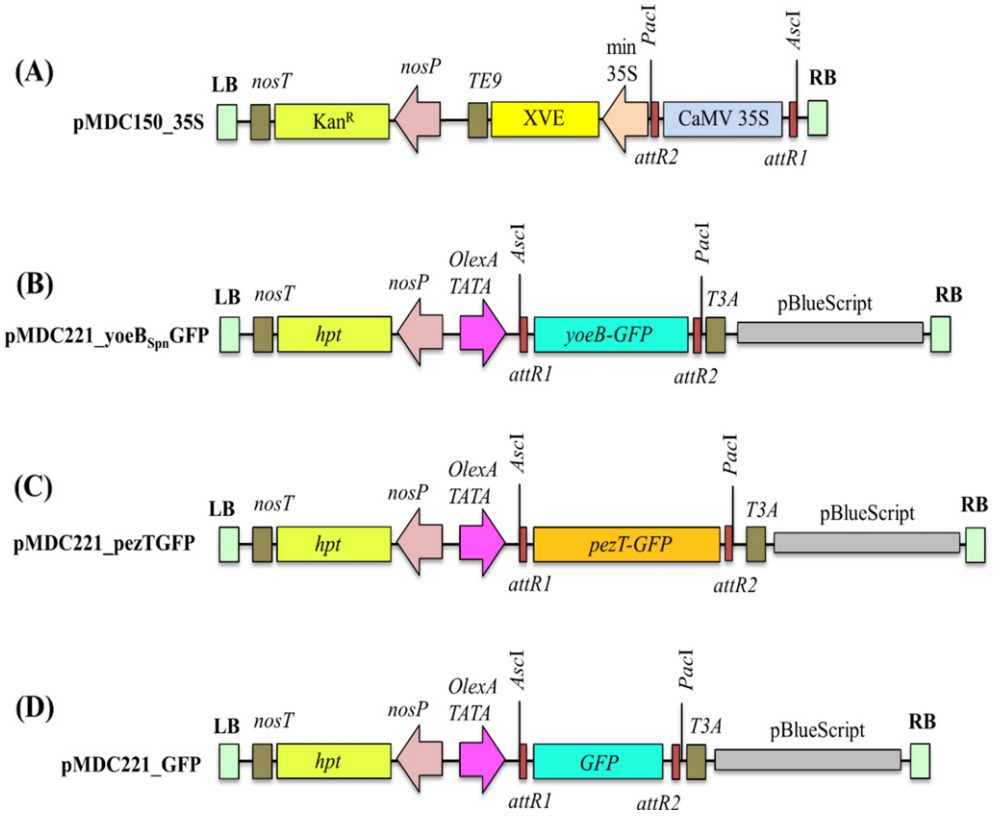


Fig. 1. A schematic illustration of the T-DNA region of the recombinant vectors used in this study. The activator vector (A) pMDC150_35S with an inserted CaMV 35S promoter and *XVE* gene. The responder vectors (B) pMDC221_yoeBGFP, (C) pMDC221_pezTGFP and (D) the positive control, pMDC221_GFP. Each of the responder vectors has the XVE-responsive promoter which is designated as "*OlexA-TATA*". LB and RB denotes the left and right borders of the T-DNA, respectively. The *attR1* and *attR2* are the Gateway recombination sites used to clone in the transgene of interest. The vectors also contained the *nos* promoter (*nosP*) to drive the expression of either the kanamycin resistance gene (Kan^R) in pMDC150_35 or the hygromycin resistance gene (*hpt*) in pMDC221 for plant selection. The pMDC221 T-DNA also contained the pBlueScript vector sequence (denoted as grey rectangular box and labelled), which can be used for plasmid rescue procedures [1]. *TE9*, *T3A* and *nosT* are terminator sequences.

C. vulgaris cells from a log-phase culture (OD_{600} = 0.5–1.0) was pre-cultured for 5 days in BBM broth and the cells were then harvested. Prior to co-cultivation, the *C. vulgaris* cells were treated with 500 mg mL^{-1} lysozyme and 500 mg mL^{-1} cellulase for 18 h at 28 °C. The treated microalgae cells were washed with induction medium (IM) broth (BBM + 100 μM acetosyringone, pH 5.6) before being subjected to *Agrobacterium*-mediated transformation. For co-cultivation, each *C. vulgaris* culture was incubated with two *A. tumefaciens* LBA4404 strains, one carrying the activator vector pMDC150_35S and the other carrying the recombinant responder vector, i.e., either pMDC221_GFP (as the positive control), pMDC221_yoeBGFP or pMDC221_pezTGFP and plated on IM media with 3 days incubation. Following that, the transformed *C. vulgaris* were plated on BBM containing 500 μg mL^{-1} cefotaxime for 3 days before transferring to non-selective BBM agar for 7 days for cell recovery. All the steps above were carried out in the dark. Following that, the cells were harvested and spread on BBM agar containing 50 μg mL^{-1} kanamycin (for pMDC150-derived recombinants) and 20 μg mL^{-1} hygromycin (for pMDC221-derived recombinants) for selection. After 4 weeks, the transformed colonies were randomly selected for PCR analysis to determine the insert of transgenes.

Table 1
List of primers used.

Primer name	Primer sequence (5′-3′)
pezT_F	CACCATGATTGGAAAGAACA
pezT_R	GGATCCTTTTTCAAGTAATTC
yoeB_F2	GCTACTCAAGTTTACAGAAG
yoeB_R2	CACGCTATCTCCATCCATC
pezT_F*	CACCATGGAAATCCAAGAT
GFP_R*	TTATAATCCCAGCAGCTGTT
pezT_F2	GCAAGGAATCTTCGTTCACTG
pezT_R2	TCCTTCTCTACCTGACTCCAC
GFP_F*	GGATCCATGGTAGATCTGA
GFP_scR	CAGCTGTTACAAACTCAAGAAG
18S_F**	CCTGCGGCTTAATTTGACTCAACACG
18S_R**	TAGCAGGCTGAGGTCACGTTCG

Note: Asterisk (*) and (**) indicates the primers were obtained from reference [22,26], respectively; other primers were designed in this study.

2.4. Genomic DNA extraction and PCR analysis

The cell lines that were transformed with (1) pMDC221_yoeBGFP together with pMDC150_35S and (2) pMDC221_pezTGFP together with pMDC150_35S were designated as *C. vulgaris* ($yoeB_{Spn}$-GFP) and *C. vulgaris* (pezT-GFP), respectively. Microalgal colonies were randomly selected from selective BBM media for validation by PCR. Genomic DNA was extracted from the selected colonies using Wizard® Genomic DNA Purification Kits (Promega, USA). Each PCR reaction consisted of 100 ng DNA template, 1 × MyFi reaction buffer (Bioline, USA), 0.4 μM of both reverse and forward primers, 0.4 U of MyFi DNA polymerase (Bioline) and sterile deionized distilled water in a final volume of 25 μL. The PCR cycling conditions used in this study were as follows: initial denaturation of DNA template at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, primer annealing at 57 °C for 15 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min. PCR for the detection of the $yoeB_{Spn}$ transgene was carried out using yoeB_F2 and yoeB_R2 primers (Table 1) while for the *pezT* transgene, the primers used were

pezT_F and GFP_R (Table 1). Primers used for the detection of the *GFP* transgene were GFP_F and GFP_scR (Table 1).

2.5. Culture selection and maintenance of transformed C. vulgaris cell lines

Two transgenic lines each from the PCR-positive colonies for *yoeB$_{Spn}$* [*C. vulgaris* (yoeB$_{Spn}$-GFP)] and *pezT* [*C. vulgaris* (pezT-GFP)] transgenes were randomly selected for further experiments. All the selected transgenic lines were cultured alternately on selective media (BBM containing kanamycin and hygromycin) and then non-selective media (BBM with no supplemented antibiotic) with a three-month duration for each sub-culture, to ensure the stability of transgenes. After a year of culture maintenance, the effects of transgene expression on cell morphology and viability as well as transgene stability were determined.

2.6. Detection of GFP activity and toxin expression in causing cell lysis in 17-β-estradiol treated transformed C. vulgaris cells

Selected single colonies of transformed *C. vulgaris* cells were inoculated into BBM broth and were grown to log phase ($OD_{600} = 0.5–1.0$). The cell numbers were then standardized to 5×10^6 cells mL^{-1} by diluting the original cultures with BBM broth. The standardized cells (1 mL each) were distributed into 1.5 mL microcentrifuge tube, followed by the addition of 100 μM 17-β-estradiol. The effect 17-β-estradiol on cells morphology and cells viability was observed at 0.5, 6, 12 and 24 h time points. The experiment was carried out in five replicates for each time points.

The effect of 17-β-estradiol induction on cell morphology was determined by using GFP fluorescence detection. From each time point following 17-β-estradiol induction, a 5 μL aliquot of the treated cells from the transformed *C. vulgaris* (GFP), *C. vulgaris* (yoeB$_{Spn}$-GFP) and *C. vulgaris* (pezT-GFP) were each transferred onto glass slides. Each sample was viewed under bright field and fluorescence microscope at 1000× magnification. The fluorescent imaging was carried out using Leica DM LB2 (Leica, Germany). GFP fluorescence was visualized with an I3 filter (470-nm excitation filter/525-nm barrier filter).

In order to determine the effect of 17-β-estradiol on cells viability, a modified Miles and Misra method [27] was carried out independently for *C. vulgaris* (GFP), *C. vulgaris* (yoeB$_{Spn}$-GFP) and *C. vulgaris* (pezT-GFP) cell lines. A 20 μL aliquot from each 17-β-estradiol-treated sample was dropped on solid BBM media for each time points. Colonies appearing after 7 days were calculated for colony forming unit (CFU)/mL values. All the data were expressed as the mean ± SD and statistical significance was set at $p = 0.05$. The *t*-test was used to statistically analyze the samples' differences using Microsoft Excel® 2013.

2.7. RNA extraction and reverse transcriptase PCR

RNA extraction was carried out for the 17-β-estradiol-treated transgenic *C. vulgaris* cells using the GF-1 Total RNA extraction kit (Vivantis Technologies, USA). Following that, M-MuLV reverse transcriptase (Thermo Scientific) was used to reverse-transcribe RNA into complementary DNA (cDNA) using the gene specific primer, GFP_scR (Table 1). The components for the first strand cDNA synthesis were as follows: 0.5 μg of total RNA, 0.4 μmol of GFP_scR gene specific primer, 1× MyFi reaction buffer, 20 U of RiboLock RNase Inhibitor (Thermo Scientific), 0.5 mM of dNTP mix, 200 U of RevertAid Reverse Transcriptase (Thermo Scientific) and sterile distilled deionized water added to a final volume of 20 μL. The mixture was incubated at 37 °C for 1 h for first strand cDNA synthesis. The first strand cDNA was used as DNA template for PCR to confirm the presence of *yoeB$_{Spn}$* and *pezT* transcripts in the transgenic *C. vulgaris* lines. The following primers were used to amplify the cDNA; yoeB_F2 and yoeB_R2 for *yoeB$_{Spn}$* transgene; and pezT_R2 and pezT_R2 for *pezT* transgene (Table 1). The 18S rRNA was used as the housekeeping control and amplified using 18S rRNA-specific primers (Table 1). Each PCR reaction consisted of 100 ng DNA template, 1× MyFi reaction buffer (Bioline), 0.4 μM of both reverse and forward primers, 0.4 U of MyFi DNA polymerase (Bioline) and sterile deionized distilled water in a final volume of 25 μL. The PCR cycling conditions were as follows: initial denaturation of DNA template at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, primer annealing at 57 °C for 15 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min.

2.8. Determination of transgene stability in C. vulgaris

A single colony was randomly selected from the primary selective plate of both *C. vulgaris* (yoeB$_{Spn}$-GFP) and *C. vulgaris* (pezT-GFP) transformants and was cultured and maintained as described in Section 2.5 for about one year. Following that, the transgenic YG_1 and PG_1 cells were sub-cultured for five consecutive passages in alternating antibiotic selection pressure (one generation grown in the presence of kanamycin and hygromycin, and the next generation without the antibiotics selection pressure). After the consecutive alternate subculturing, colonies from the final subculture were randomly selected for DNA extraction and PCR analysis. The presence of the *yoeB$_{Spn}$* and *pezT* transgenes was determined by PCR amplification as described in Section 2.4.

3. Results

3.1. Successful co-transformation of the two-component expression vectors into C. vulgaris with functional GFP expression

Colonies of putative *C. vulgaris* transformants that were co-transformed with both pMDC150_35S and pMDC221_GFP vectors were successfully recovered from the kanamycin and hygromycin selection

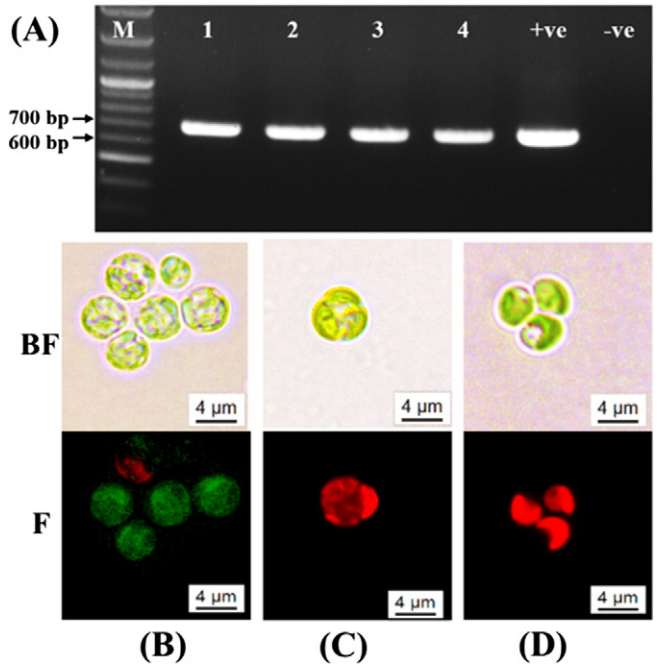


Fig. 2. PCR analysis and expression of GFP in transformed *C. vulgaris* (GFP). (A) PCR amplification of the 691 bp *GFP* gene fragment from four randomly selected *C. vulgaris* (GFP) transformants (lanes 1–4) with plasmid pMDC221_GFP as the positive control (lane marked "+ve") and genomic DNA of wild-type *C. vulgaris* as the negative control (lane marked "−ve"); Lane M is the 100 bp DNA marker (Vivantis). GFP fluorescence detection for (B) *C. vulgaris* (GFP) cells treated with 17-β-estradiol after 6 h; (C) untreated *C. vulgaris* (GFP) cells and (D) wild-type UMT-M1 cells. Image observed under fluorescence (F) and Bright field (BF) microscope as indicated. GFP fluorescence in *C. vulgaris* (GFP) at other time points is shown in Supplementary Fig. S1.

plates. The presence of *GFP* reporter gene with the expected band size of 691 bp was confirmed by PCR-amplification and a transformant designated as *C. vulgaris* (GFP) was selected to serve as a positive control (Fig. 2A). The amplified product of the *GFP* transgene was absent in the negative control, wild type *C. vulgaris* (Fig. 2A). This indicated that the *GFP* transgene had been successfully transferred into *C. vulgaris* via *Agrobacterium tumefaciens*-mediated transformation.

To investigate if the two-component XVE-based expression system was functional in *C. vulgaris*, GFP fluorescence was determined for *C. vulgaris* (GFP) cells in the presence and absence of the 17-β-estradiol inducer. GFP fluorescence could clearly be observed in *C. vulgaris* (GFP) cells following induction with 17-β-estradiol but was absent in untreated *C. vulgaris* (GFP) cells and in 17-β-estradiol-treated wild-type *C. vulgaris* cells (Fig. 2B–D and Supplementary Fig. S1). There were no observable differences between the morphology of cells that displayed GFP fluorescence and that of the wild-type cells. Thus, the two-component XVE expression system is functional in *C. vulgaris* and expression of the *GFP* transgene did not cause any observable morphological changes to the transformed microalgae. Furthermore, the detection of GFP fluorescence in *C. vulgaris* (GFP) cells also indicated successful co-transformation of the activator vector pMDC150_35S, along with the responder vector pMDC221_GFP into *C. vulgaris*.

3.2. Detection of the $yoeB_{Spn}$ and pezT toxin transgenes in transformed C. vulgaris lines

Six *C. vulgaris* (yoeB$_{Spn}$-GFP) transformant colonies were randomly selected from the selective BBM plates for PCR analysis. The 223 bp $yoeB_{Spn}$ (Fig. 3A) and 691 bp *GFP* (Fig. 3B) transgene bands were present in all the colonies tested. Similarly, four randomly selected *C. vulgaris* (pezT-GFP) transformant colonies showed the expected 1494 bp amplification product for the *pezT-GFP* fusion transgene (Fig. 3C). Two lines of cells from the *C. vulgaris* (yoeB$_{Spn}$-GFP) transformants were selected and named YG_1 and YG_2 whereas two lines from *C. vulgaris* (pezT-GFP) were named PG_1 and PG_2. The *C. vulgaris* (GFP) line with only *GFP* and no toxin transgene was used as positive control together with the selected lines of *C. vulgaris* (yoeB$_{Spn}$-GFP) and *C. vulgaris* (yoeB$_{Spn}$-GFP), for further experiments after a year of culture maintenance. The original untransformed wild-type (WT) *C. vulgaris* was included as negative control.

3.3. Morphology of transgenic C. vulgaris cells expressing the $YoeB_{Spn}$ and PezT toxins

Transgenic *C. vulgaris* (yoeB$_{Spn}$-GFP) lines, namely YG_1 and YG_2, emitted GFP fluorescence at all sampled time-points following 17-β-estradiol induction (Fig. 4A). Interestingly, all the GFP-fluorescent cells showed abnormal morphologies indicative of cellular damage and lysis except for cells of the YG_1 lines which still displayed normal cell morphologies 0.5 h after induction. Nevertheless, cellular damage was apparent in YG_1 cells 6 h after induction and thereafter. Similar results were obtained for the *C. vulgaris* (pezT-GFP) transgenic lines (Fig. 4B). PG_1 and PG_2 lines exhibited GFP fluorescence at all time points after 17-β-estradiol induction and all GFP-fluorescent cells showed signs of cell damage and lysis, particularly 6 h after induction and beyond (Fig. 4B). The affected cells were discolored due to chlorophyll loss and clumped together into a mass. The effects were most prominent in samples taken 24 h after induction in which the cell contents were found dispersed into the surrounding environment (Fig. 4B). Relative to the tested transgenic *C. vulgaris* cells, the wild-type *C. vulgaris* cells treated with 17-β-estradiol showed normal cellular morphologies and exhibited only red chlorophyll auto-fluorescence. Likewise, *C. vulgaris* (GFP) cells also showed normal morphologies up to 24 h after 17-β-estradiol induction (Fig. 2A and Supplementary Fig. S1).

3.4. The effect of $YoeB_{Spn}$ and PezT toxin expression on cell viability

The *C. vulgaris* (yoeB$_{Spn}$-GFP) cell lines YG_1 and YG_2 treated by 17-β-estradiol showed GFP expression and a reduced cell viability (Fig. 5A). After 6 h of induction, the viability of YG_1 cells relative to the negative control (i.e., wild type treated with 17-β-estradiol) showed a reduction of 57.8% and viability continued to decrease to 72.7% 24 h after 17-β-estradiol induction (Fig. 5A). The reduction in cell viability was greater for YG_2 cells, with a reduction of 91.9% recorded at 30 min after 17-β-

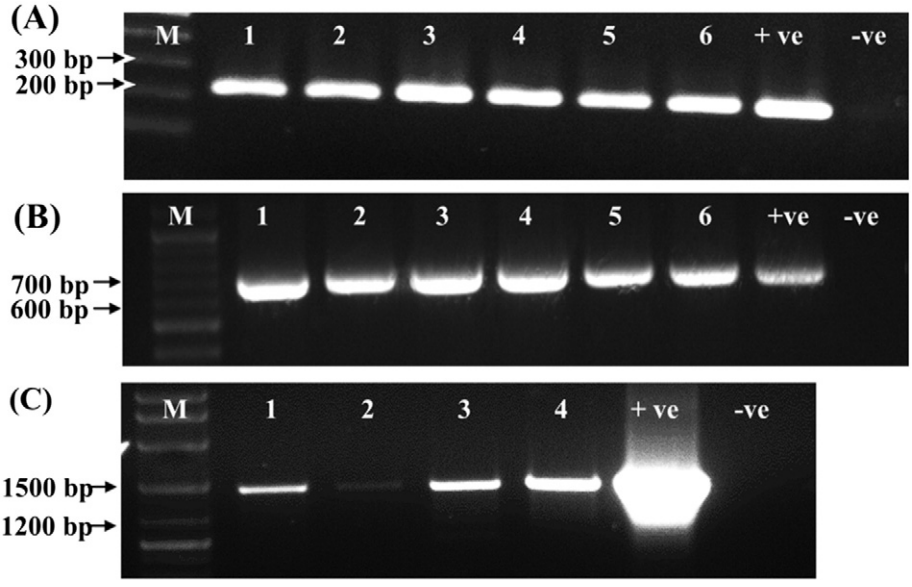
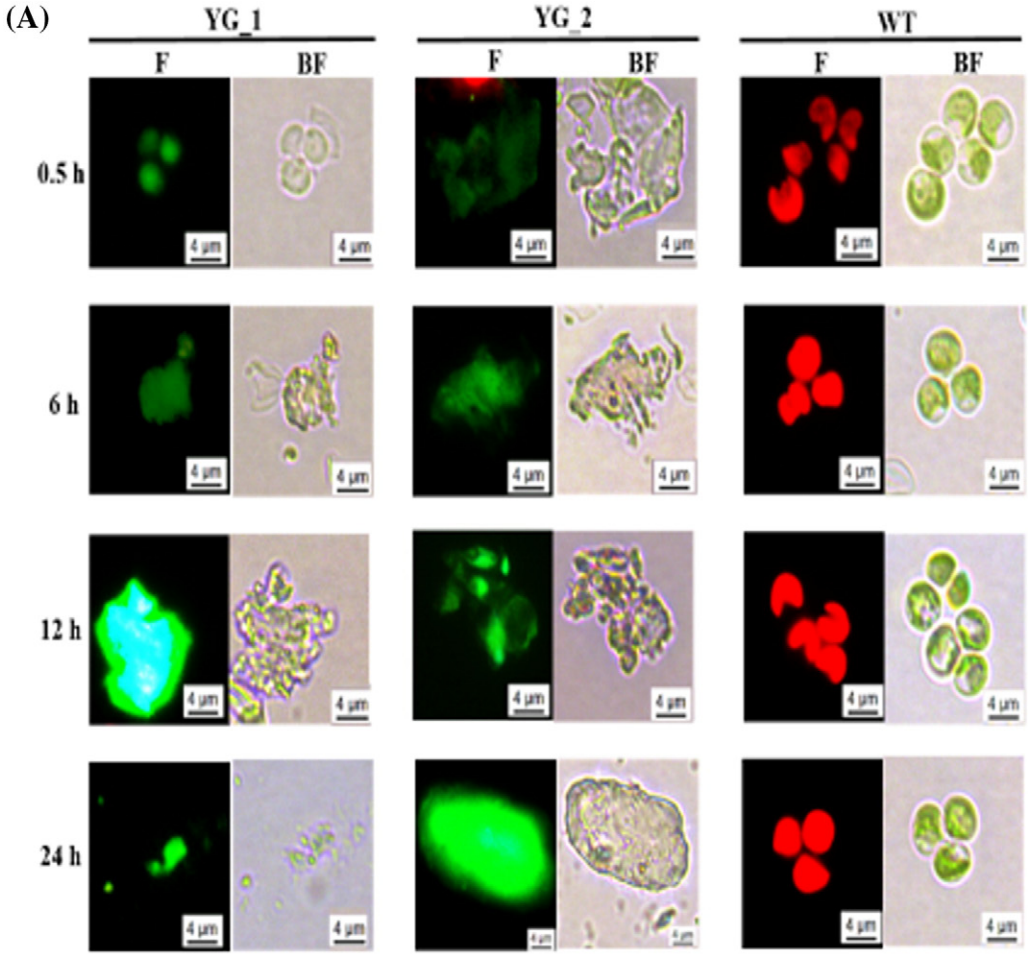
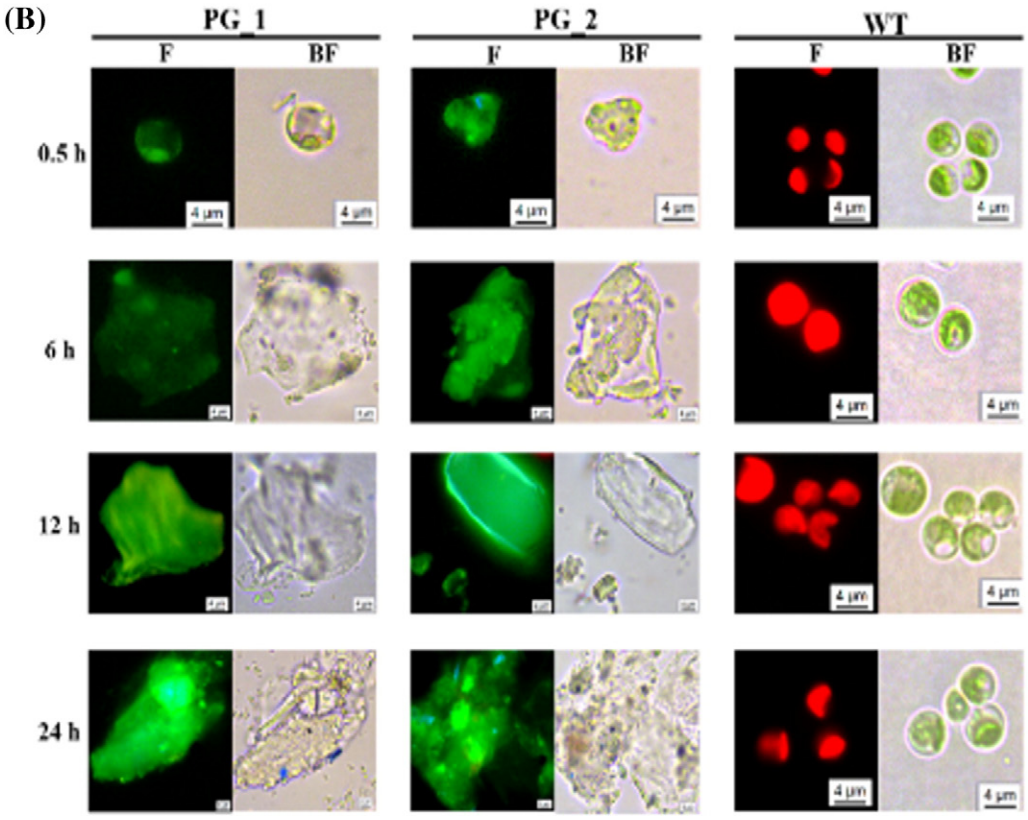


Fig. 3. PCR detection of the $yoeB_{Spn}$ and *pezT* toxin genes in transformed *C. vulgaris* strains. PCR amplification of the (A) $yoeB_{Spn}$ toxin gene and (B) *GFP* gene from *C. vulgaris* (yoeB$_{Spn}$-GFP) transformant lines (lanes 1–6) with plasmid pMDC221_yoeBGFP as the positive control (lane marked "+ ve"). PCR amplification of the (C) *pezT-GFP* fusion gene (band size of 1494 bp) from *C. vulgaris* (pezT-GFP) transformed lines (lanes 1–4) with plasmid pMDC221_pezTGFP as the positive control (lane marked "+ ve"). The negative control (lane "− ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).


(A)
YG_1
YG_2
WT
F
BF
0.5 h
6 h
12 h
24 h
4 μm


(B)
PG_1
PG_2
WT
F
BF
0.5 h
6 h
12 h
24 h
4 μm

estradiol induction, and by 24 h after induction, cell viability had dropped to 99.9% of the value of the control cells. The CFU/mL values for the positive control *C. vulgaris* (GFP) cells (that expresses only GFP) showed a gradual increase in the CFU/mL values (Supplementary Fig. S2-A), indicating that the viability of the *C. vulgaris* (GFP) cells was not affected by 17-β-estradiol-treatment. The *C. vulgaris* (pezT-GFP) transgenic lines (PG_1 and PG_2) treated with 17-β-estradiol showed a 99.9% and 96.3% reduction in the number of viable cells relative to the control (i.e., wild type treated with 17-β-estradiol), even at 30 min after induction and this was sustained up to 24 h post-induction (Fig. 5B). There were no apparent differences in the reduction of cell viability observed for either of the PG_1 and PG_2 lines up to and including the 24 h post-induction time point.

3.5. Determination of $yoeB_{Spn}$ and pezT transcripts in transgenic C. vulgaris lines

Total RNA was isolated from the transgenic lines after induction of the transgenes by treatment with 17-β-estradiol. RT-PCR analysis indicated the presence of the $yoeB_{Spn}$ transcripts in YG_1 and YG_2 lines after 17-β-estradiol induction with the expected amplicon size of 223 bp (Fig. 6A). However, RT-PCR was only able to be performed on the total RNA of samples up to 12 h after induction as there was insufficient RNA for RT-PCR at the later sample times. In the case of the UMT M1 (pezT-GFP) transgenic cells, the *pezT* transcript was successfully detected by RT-PCR (with the expected band size of 663 bp) from the PG_1 line at all time points following induction (Fig. 6B). In contrast, there was insufficient total RNA recovered from the PG_2 line for RT-PCR assay due to PezT toxin-induced cell lysis (Fig. 5). The *$yoeB_{Spn}$* and *pezT* transcripts were not detected in the wild-type cells (Fig. 6).

3.6. Stability of transgenes in transgenic C. vulgaris lines

Transgene stability is crucial in the development of transgenic microalgal systems. After maintaining the transgenic culture *C. vulgaris* ($yoeB_{Spn}$-GFP) and *C. vulgaris* (pezT-GFP) for about one year and subjecting the cells to a series of 5 consecutive subcultures (~75 days) of alternating presence and absence of antibiotic-selection pressure (i.e., with kanamycin and hygromycin, followed by antibiotic-free media), PCR analysis confirmed that the *$yoeB_{Spn}$* and *pezT* genes were still present in the respective transgenic *C. vulgaris* transformants (Supplementary Fig. S3). After about 1 year maintenance and five serial passages with and without antibiotics, treatment of the transgenic *C. vulgaris* with 17-β-estradiol led to GFP fluorescence and cell lysis in the transgenic cell lines (Figs. 4 and 5).

4. Discussion

In this study, a two-component XVE-based inducible plant expression system that was initially developed for the model plant, *Arabidopsis thaliana* to test the lethality of the $YoeB_{Spn}$-GFP and PezT-GFP fusion proteins [22] was successfully applied in the microalga, *C. vulgaris*. To date, *A. tumefaciens*-mediated genetic transformation of some algae has been well established [25,28,29]. Previous reports on inducible gene expression systems in microalgae have been limited to introducing a single vector into microalgae, such as the expression of chloramphenicol acetyltransferase (*CAT*) [30] and heat shock protein 90 (HSP 90) [31] reported for *C. vulgaris*.

The current study provides a first demonstration that a two-component system is feasible in *C. vulgaris* and this can be useful for the "non-leaky" conditional expression of detrimental gene products. The XVE-based expression system appeared to be tightly regulated in *C. vulgaris* as GFP fluorescence was not detected in the absence of 17-β-estradiol-treatment (Fig. 2C), similar to the previous findings in *A. thaliana* [22]. Co-transformation of the activator pMDC150_35S and responder pMDC221_GFP vectors into *C. vulgaris* was successfully performed by *A. tumefaciens*-mediated transformation despite the presence of the *C. vulgaris* rigid cell wall which usually renders the cell impermeable to foreign DNA [13]. In the current study, the inclusion of lysozyme and cellulase enzymatic treatment stage to degrade the trifluoroacetic-glucosamine polymer cell wall layer prior to *Agrobacterium*-mediated transformation was used to aid the simultaneous transformation of two different vector cassettes into *C. vulgaris*.

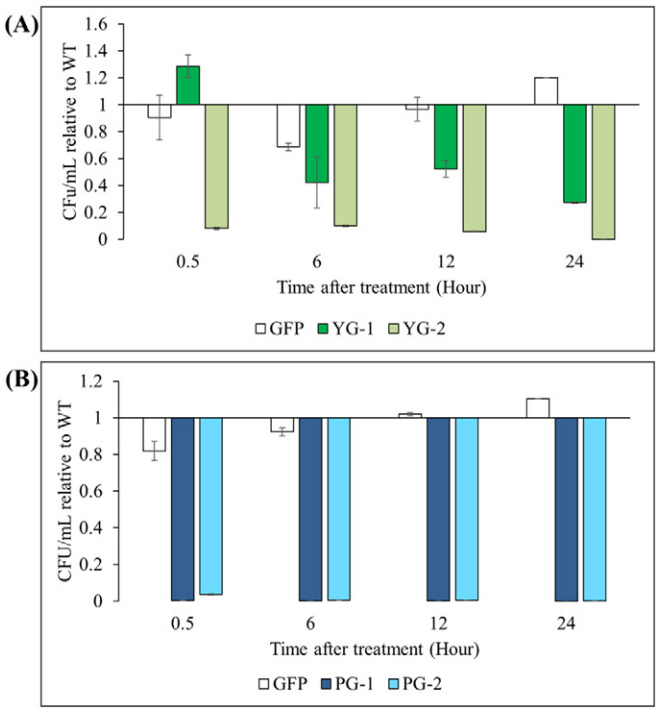


Fig. 5. The viability of transgenic *C. vulgaris* UMT-M1 cell lines after 17-β-estradiol induction. The effects of (A) $YoeB_{Spn}$ toxin in *C. vulgaris* ($yoeB_{Spn}$-GFP) and (B) PezT toxin in (pezT-GFP) cells. The randomly selected cell lines for UMT-M1 ($yoeB_{Spn}$-GFP) were designated YG_1 and YG_2 and for *C. vulgaris* (pezT-GFP) were designated PG_1 and PG_2. The transgenic *C. vulgaris* UMT-M1 (GFP) was included as a control to show that GFP expression did not have any adverse effects on transgenic *C. vulgaris*. All the CFU/mL obtained were shown relative to the wild type (WT), which was set to 1. Values are indicated as means ± SD.

Both the $YoeB_{Spn}$ and PezT toxins were cloned as translational fusions with GFP in this study. GFP has been widely used to detect the gene localization and expression in various species of marine microalgae [32,33]. As shown previously when studying expression of $YoeB_{Spn}$-GFP in the terrestrial plant *A. thaliana* [22], expression of GFP alone in *C. vulgaris* did not lead to any morphological changes or significant reduction in cell viability while *C. vulgaris* cells expressing the $YoeB_{Spn}$-GFP and PezT-GFP fusions showed cellular damage and lysis (Fig. 4) with a significant reduction in cell viability (Fig. 5), indicating the lethality of these bacterial toxins in *C. vulgaris*. Expression of the $YoeB_{Spn}$ toxin in *E. coli* resulted in cell death and an inability to form colonies due to the endoribonuclease activity of the toxin resulting in the cleavage of mRNA and thus, translational inhibition [34], while expression of a $YoeB_{Spn}$-GFP fusion in *A. thaliana* also caused cell death and was closely associated with apoptosis [22]. Based on this, it seems likely that

Fig. 4. Morphology of transgenic and wild-type *C. vulgaris* as viewed under fluorescent (F) and bright-field (BF) microscope at 1000× magnification. The 17-β-estradiol-treated transgenic *C. vulgaris* cells were mounted on glass slides at 0.5, 6, 12 and 24 h post-induction. Altered cell morphologies and green GFP fluorescence were visible in (A) transgenic *C. vulgaris* ($yoeB_{Spn}$-GFP) cell lines YG_1 and YG_2 and (B) transgenic *C. vulgaris* (pezT-GFP) cell lines PG_1 and PG_2. The wild-type *C. vulgaris* (WT) as negative control showed normal cell morphology and chlorophyll auto-fluorescence (red).

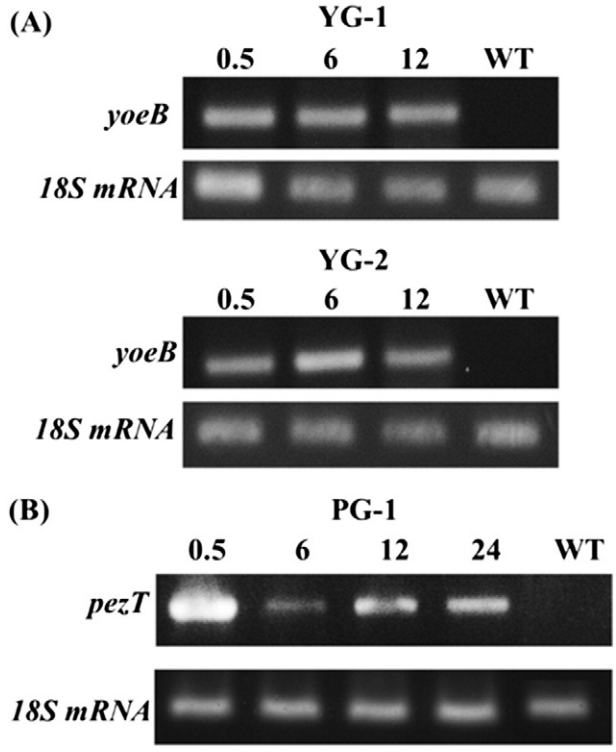


Fig. 6. RT-PCR analysis of the 17-β-estradiol-treated transgenic *C. vulgaris*. (A) Detection of the *yoeB*$_{Spn}$ toxin gene mRNA transcript in the transgenic *C. vulgaris* (yoeB$_{Spn}$-GFP) = lines (YG_1 and YG_2). There was insufficient RNA for RT-PCR 24 h after 17-β-estradiol-treatment; (B) detection of the *pezT* toxin gene mRNA transcript in transgenic *C. vulgaris* (pezT-GFP) (PG_1). The 18S rRNA transcript was used as the housekeeping control for the RT-PCR reactions.

the endonuclease activity of YoeB$_{Spn}$ led to cell death observed in the YG_1 and YG_2 lines of transgenic *C. vulgaris*. Interestingly, even though both YG_1 and YG_2 transgenic lines showed significantly reduced cell viabilities relative to wild-type *C. vulgaris*, YG_1 displayed a slower declining trend compared to YG_2 (Fig. 5A), indicative of perhaps a lower expression level of *yoeB*$_{Spn}$*-GFP* transgene in YG_1 line. The *yoeB*$_{Spn}$*-GFP* transgene might have integrated at different loci in the genomes of the YG_1 and YG_2 transgenic lines, thus leading to the different levels of gene expression [35]. Determining the exact locus of the transgene and quantifying the transcript levels of the *yoeB*$_{Spn}$*-GFP* transgene in the transgenic *C. vulgaris* by quantitative real-time RT-PCR may provide clues to the apparently different toxin expression levels of YoeB$_{Spn}$-GFP in the two transgenic lines. The transgene integration sites were not determined in this study. Integration within chromosomal euchromatin regions, which are known to be transcriptionally active, can promote transgene expression whereas integration within heterochromatin regions can result in suppression of gene transcription instead [36]. For example, the *lacZ* reporter gene was integrated in different loci of the *Saccharomyces cerevisiae* genome and the gene expression was determined from β-galactosidase activity. There was a 8.7 fold difference between the yeast strains with the highest and lowest activity of β-galactosidase, respectively [37].

In contrast to the two transgenic lines of *C. vulgaris* (yoeB$_{Spn}$-GFP), both the *C. vulgaris* (PezT-GFP) transgenic lines, PG_1 and PG_2, showed equally significant reduction in cell viability within the first 30 min following induction by 17-β-estradiol-treatment (Fig. 5). In its native environment, PezT functions as a kinase that targets UDP-*N*-acetylglucosamine (UNAG), the key intermediate in the synthesis of bacterial peptidoglycan [20]. The overproduction of PezT toxin was reported to be lethal in its native host, *S. pneumoniae* as well as in *E. coli* cells, due to inhibition of peptidoglycan synthesis, resulting in cell autolysis [20]. A homolog of the PezT toxin, the *S. pyogenes*-encoded ζ toxin, was found to trigger cell lysis when expressed in yeast cells due to depletion of UNAG which is required for chitin synthesis in yeast cells [38]. The rigid cell wall of *Chlorella* sp. is composed of *N*-acetylglucosamine chains that are present as chitin-like glycan [39], which is similar to the cell wall composition of bacteria and yeast. Therefore, PezT is likely to target cell wall biosynthesis in *C. vulgaris*, causing its expression to be lethal in *C. vulgaris*.

The *yoeB*$_{Spn}$*-GFP* and *pezT-GFP* transgenes in *C. vulgaris* were expressed following 17-β-estradiol-treatment even after repeated subcultures over a period of more than a year, indicating that the XVE-based two-component inducible expression system is able to produce stable transgenic lines of *C. vulgaris*. Since many species of microalgae have a rapid life cycle, the elimination of transgenes from a sub-population during cell division can lead to loss of the desired trait over a relatively short time scale, particularly in the absence of selection pressure [2]. Therefore, the stability of transgenes introduced into eukaryotic microalgae is an important consideration for feasibility in long term applications. Previous studies have shown promising results including stable expression of GFP in *C. vulgaris* even after 16 consecutive subcultures (about 8 months) with alternating antibiotic pressure [40] and stable GUS activity in *Chlorella ellipsoidea* transformants that had been maintained under alternating antibiotic selection pressure for about 10 months [41].

Differential expression of toxin-antitoxin systems have been proposed as a possible method for gene containment in transgenic yeasts ever since it was found that the expression of the *E. coli*-encoded RelE toxin was lethal in the yeast *S. cerevisiae* due to the RelE-induced cleavage of mRNAs. Conditional expression of the *relE-relB* toxin-antitoxin genes was proposed as a containment system to prevent the accidental release of genetically modified yeast to the environment [42]. Likewise, the pneumococcal YefM-YoeB and PezAT TA systems could be used to develop a containment system for transgenic *C. vulgaris* and other transgenic microalgae: under nutrient-rich conditions in a bioreactor, the expression of the toxin would be repressed by placing the toxin gene under the control of an appropriate nutrient-responsive promoter. If the transgenic microalgae were to escape from the bioreactor into the environment, the nutrient-poor conditions outside of the bioreactor would trigger the expression of the toxin leading to its demise. The lethality of the bacterial toxin leading to the death and lysis of the transgenic microalgae also enabled another potentially useful application in harvesting valuable microalgal cellular contents [8]. Transgenic microalgal cells harboring TA toxins could be induced to lyse at the appropriate time such as when the lipid storage accumulation is at the maximum, thereby facilitating the harvesting and recovery of the useful primary metabolite under optimal conditions. The conditional expression of toxin genes in transgenic microalgae can provide a more controlled environment to either prevent the accidental release of transgenic microalgae or to minimize the cost of producing organic bioenergy compounds.

In conclusion, this study is the first to report the co-transformation and functionality of the XVE-based two-component inducible gene expression system in the eukaryotic microalgae *C. vulgaris*. Using this expression system, we have demonstrated the functional lethality of the bacterial TA toxins YoeB$_{Spn}$ and PezT from *S. pneumoniae* in *C. vulgaris*. It is possible that their corresponding YefM$_{Spn}$ and PezA antitoxins are likewise functional in *C. vulgaris* as well although this has yet to be validated. The functionality of these bacterial TA toxins and the utility of the two-component inducible gene expression system in *C. vulgaris* enable the possibility of new and interesting applications for a conditional lethal system to be developed for *C. vulgaris* and other eukaryotic microalgae. It was also shown that after numerous subcultures, the transgenes were still present and actively transcribed. This study should pave the way for novel applications for bacterial TA systems in

transgenic eukaryotic microalgae such as those that have been developed for other eukaryotic systems [21].

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.07.011.

References

[1] N. Mallick, S. Mandal, A. Singh, M. Bishal, A. Dash, Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel, J. Chem. Technol. Biotechnol. 87 (2012) 137–145.
[2] M. Yamamoto, M. Fujishita, A. Hirata, S. Kawano, Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae), J. Plant Res. 117 (2004) 257–264.
[3] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96.
[4] K.L. Yeh, J.S. Chang, Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels, Biotechnol. J. 6 (2011) 1358–1366.
[5] K. Bala Amutha, A.G. Murugesan, Biological hydrogen production by the algal biomass *Chlorella vulgaris* MSU 01 strain isolated from pond sediment, Bioresour. Technol. 102 (2011) 194–199.
[6] C. Posten, G. Schaub, Microalgae and terrestrial biomass as source for fuels - a process view, J. Biotechnol. 142 (2009) 64–69.
[7] A.A. Fathi, M.M. Azooz, M.A. Al-Fredan, Phycoremediation and the potential of sustainable algal biofuel production using watewater, Am. J. Appl. Sci. 10 (2013) 189–194.
[8] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel production using wastewater resources, Bioresour. Technol. 102 (2011) 17–25.
[9] R. Harun, M. Singh, G.M. Forde, M.K. Danquah, Bioprocess engineering of microalgae to produce a variety of consumer products, Renew. Sust. Energ. Rev. 14 (2010) 1037–1047.
[10] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other application: a review, Renew. Sust. Energ. Rev. 14 (2010) 217–232.
[11] Y.H. Chen, T.H. Walker, Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol, Biotechnol. Lett. 33 (2011) 1973–1983.
[12] P.M. Schenk, S.R. Thomas-Hall, E. Stephens, U. Marx, J.H. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: high-efficiency microalgae for biodiesel production, Bioenergy Res. 1 (2008) 20–43.
[13] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production, Planta 237 (2013) 239–253.
[14] Y. Yamaguchi, J.H. Park, M. Inouye, Toxin-antitoxin systems in bacteria and archaea, Annu. Rev. Genet. 45 (2011) 61–79.
[15] S.K. Christensen, G. Maenhaut-Michel, N. Mine, S. Gottesman, K. Gerdes, L. Van Melderen, Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the yefM-yoeB toxin-antitoxin system, Mol. Microbiol. 51 (2004) 1705–1717.
[16] F. Hayes, B. Kedzierska, Regulating toxin-antitoxin expression: controlled detonation of intracellular molecular timebombs, Toxins (Basel) 6 (2014) 337–358.
[17] S.J. Unterholzner, B. Poppenberger, W. Rozhon, Toxin-antitoxin systems: biology, identification, and application, Mob. Genet. Elements 3 (2013) e26219.
[18] W.T. Chan, M. Espinosa, C.C. Yeo, Keeping the wolves at bay: antitoxins of prokaryotic type II toxin-antitoxin systems, Front. Mol. Biosci. 3 (2016) 9.
[19] R. Bertram, C.F. Schuster, Post-transcriptional regulation of gene expression in bacterial pathogens by toxin-antitoxin systems, Front. Cell. Infect. Microbiol. 4 (2014) 6.
[20] H. Mutschler, M. Gebhardt, R.L. Shoeman, A. Meinhart, A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis, PLoS Biol. 9 (2011) e1001033.
[21] C.C. Yeo, F. Abu Bakar, W.T. Chan, M. Espinosa, J.A. Harikrishna, Heterologous expression of toxins from bacterial toxin-antitoxin systems in eukaryotic cells: strategies and applications, Toxins (Basel) 8 (2016) 49.
[22] F. Abu Bakar, C.C. Yeo, J.A. Harikrishna, Expression of the *Streptococcus pneumoniae yoeB* chromosomal toxin gene causes cell death in the model plant *Arabidopsis thaliana*, BMC Biotechnol. 15 (2015) 26.
[23] L. Brand, M. Horler, E. Nuesch, S. Vassalli, P. Barrell, W. Yang, R.A. Jefferson, U. Grossniklaus, M.D. Curtis, A versatile and reliable two-component system for tissue-specific gene induction in *Arabidopsis*, Plant Physiol. 141 (2006) 1194–1204.
[24] S.K. Khoo, B. Loll, W.T. Chan, R.L. Shoeman, L. Ngoo, C.C. Yeo, A. Meinhart, Molecular and structural characterization of the PezAT chromosomal toxin-antitoxin system of the human pathogen *Streptococcus pneumoniae*, J. Biol. Chem. 282 (2007) 19606–19618.
[25] T.S. Cha, W. Yee, A. Aziz, Assessment of factors affecting *Agrobacterium*-mediated genetic transformation of the unicellular green alga, *Chlorella vulgaris*, World J. Microbiol. Biotechnol. 28 (2012) 1771–1779.
[26] H.W. Nichols, Growth media-freshwater, in: J.R. Stein (Ed.), Handbook of Psychological Methods, Culture Methods and Growth Measurements, Cambridge University Press, London 1973, pp. 7–24.
[27] D.T. Tran, C.L. Chen, J.S. Chang, Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst, Bioresour. Technol. 135 (2013) 213–221.
[28] T.A. Beacham, S.T. Ali, Growth dependent silencing and resetting of *DGA1* transgene in *Nannochloropsis salina*, Algal Res. 14 (2016) 65–71.
[29] S.C. Kumar, R.W. Misqitta, V.S. Reddy, Genetic transformation of the green alga *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*, Plant Sci. 166 (2004) 731–738.
[30] Y.F. Niu, M.H. Zhang, W.H. Xie, J.N. Li, Y.F. Gao, W.D. Yang, J.S. Liu, H.Y. Li, A new inducible expression system in a transformed green alga, *Chlorella vulgaris*, Genet. Mol. Res. 10 (2011) 3427–3434.
[31] Z. Liu, L. Zhang, Y. Pu, Z. Liu, Z. Li, Y. Zhao, S. Qin, Cloning and expression of a cytosolic HSP90 gene in *Chlorella vulgaris*, Biomed. Res. Int. 2014 (2014) 487050.
[32] A. Miyagawa-Yamaguchi, T. Okami, N. Kira, H. Yamaguchi, K. Ohnishi, M. Adachi, Stable nuclear transformation of the diatom *Chaeteceros* sp, Phycol. Res. 59 (2011) 113–119.
[33] S. Watanabe, M. Ohnuma, J. Sato, H. Yoshikawa, K. Tanaka, Utility of a GFP reporter system in the red alga *Cyanidioschyzon merolae*, J. Gen. Appl. Microbiol. 57 (2011) 69–72.
[34] C. Nieto, I. Cherny, S.K. Khoo, M.G. de Lacoba, W.T. Chan, C.C. Yeo, E. Gazit, M. Espinosa, The yefM-yoeB toxin-antitoxin systems of *Escherichia coli* and *Streptococcus pneumoniae*: functional and structural correlation, J. Bacteriol. 189 (2007) 1266–1278.
[35] C.D. Day, E. Lee, J. Kobayashi, L.D. Holappa, H. Albert, D.W. Ow, Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced, Genes Dev. 14 (2000) 2869–2880.
[36] E.J. Richards, S.C. Elgin, Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects, Cell 108 (2002) 489–500.
[37] D.B. Flagfeldt, V. Siewers, L. Huang, J. Nielsen, Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*, Yeast 26 (2009) 545–551.
[38] U. Zielenkiewicz, M. Kowalewska, C. Kaczor, P. Ceglowski, In vivo interactions between toxin-antitoxin proteins epsilon and zeta of streptococcal plasmid pSM19035 in *Saccharomyces cerevisiae*, J. Bacteriol. 191 (2009) 3677–3684.
[39] E. Kapaun, W. Reisser, A chitin-like glycan in the cell wall of *Chlorella* sp. (Chlorococcales, Chlorophyceae), Planta 197 (1995) 577–582.
[40] B. Yang, J. Liu, B. Liu, P. Sun, X. Ma, Y. Jiang, D. Wei, F. Chen, Development of a stable genetic system for *Chlorella vulgaris* - a promising green alga for CO2 biomitigation, Algal Res. 12 (2015) 134–141.
[41] L. Liu, Y. Wang, Y. Zhang, X. Chen, P. Zhang, S. Ma, Development of a new method for genetic transformation of the green alga *Chlorella ellipsoidea*, Mol. Biotechnol. 54 (2013) 211–219.
[42] D. Andreev, V. Hauryliuk, I. Terenin, S. Dmitriev, M. Ehrenberg, I. Shatsky, The bacterial toxin RelE induces specific mRNA cleavage in the A site of the eukaryote ribosome, RNA 14 (2008) 233–239.