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High throughput screening of β-glucuronidase (GUS) reporter in transgenic microalgae transformed by *Agrobacterium tumefaciens*

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ABSTRACT

GUS (β-glucuronidase) is a chemiluminescent reporter gene that has been used in *E. coli*, fungi, yeast, mammalian cells, plant cells and microalgae. Currently, the GUS gene fusion system used for detection of GUS enzyme activity is carried out by cell lysis and requires 500 μl cell lysate. Hence, GUS activity cannot be assessed by high throughput screening (HTS) method. This study addresses this by using a HTS technique to quickly isolate transgenic strains (shown using *Chlorella vulgaris*) expressing high GUS activity in a 96 well microplate format. In this technique, quantitative results were obtained without carrying out cell lysis and all the experiments were carried out in a 96 well microplate. The method developed is cost effective, less labor intensive and can be carried out in a timely manner.

For this, a new GUS reporter vector pBIN + TetR + TetO was developed, followed by transformation (*Agrobacterium tumefaciens*), screening and characterization of the transgenic *C. vulgaris*. In the screening study, strain number 18 showed the highest fluorescence intensity (16,988 ± 1168). The GUS enzyme was found to be stable for > 8 h for intact cell and lysed cell studies. The optimum concentration of Triton X-100 to release the product (4-Methylumbelliferone) into buffer was 0.1% and the fluorescence intensity was 28,397 ± 787. The values of K_m and V_{max} of the recombinant GUS for lysed cells were 0.1304 ± 0.0101 mM and 0.35 ± 0.004 pmol 4-MU/min/ml of crude cell lysate respectively.

1. Introduction

Microalgae are a diverse group of oxygenic, photosynthetic eukaryotic and prokaryotic microorganisms. They thrive in a wide range of environments, growing on simple nutrients, and carbon dioxide [1]. Potential autotrophic growth, frequently high levels of intracellular lipids, novel pathways, and enzymes make microalgae interesting candidates for biotechnological and industrial applications [2]. However, for industrial application of microalgae, the ability to use genetic engineering is advantageous [3]. The number of genetically engineered microalgae is very few when compared to yeast, bacteria, plants, and mammalian system [4]. This shows the development of new molecular tools and techniques are necessary to realize the full potential of microalgae [5].

In the development of transgenic microalgae, the gene of interest is typically inserted into the microalgae genome by homologous and non-homologous integration. To date, most of the transgenic microalgae developed are by non-homologous integration [6]. The non-homologous integration has been carried out using several transformation techniques like electroporation, particle bombardment, glass bead method, polyethylene glycol, conjugation, and the *Agrobacterium* system [4] [5]. Some of the drawbacks of current molecular techniques in transgenic microalgae are gene silencing, random integration, codon usage, and low transgene expression levels [7, 8]& [9].

The selection of proper high recombinant protein expressing microalgae is a challenge due to variation in transgene expression level and random integration into the genome. The foremost technique currently used to isolate high expression transgenic microalgae is by RT-PCR. But the mRNA used in RT-PCR is vulnerable to degradation and contamination, and the cell lysis is needed to isolate it. In this method 30 to 50 transgenic microalgae are used for screening [10, 11]. Some of the drawbacks of this technique are that it is expensive, and that the success of the experiment depends on the primers and their design. The screening is further labor intensive, and involves several experimental steps and research expertise [12].

High-throughput screening (HTS) is an approach used mainly in drug discovery and has gained widespread acceptance over the last decade. HTS is a process of carrying out large numbers of assays with small sample, substrate, and buffer volumes. HTS in biotechnological applications are used in drug development [13], isolating transgenic

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microbes expressing high level of recombinant protein [14], to carryout enzyme kinetics and characterization studies [15], to study the effects of different chemicals and bio-chemicals on individual cells [16, 17], and to quantify the expression of reporter and marker proteins in transgenic strains [18].

The β-glucuronidase reporter protein hydrolyzes 4-MUG (4-Methylumbelliferyl β-D-glucuronide) to 4-MU (4-Methylumbelliferone). The 4-MU is a fluorescence molecule which emits light at 460 nm when excited at 365 nm. This can be exploited for calculating the GUS activity, and protein expression levels. Several transgenic strains have been studied with β-glucuronidase reporter protein expression. Some of the microbes are *Escherichia coli* [19], *Mycobacterium tuberculosis* [20], *yeast*, and *filamentous fungi* [21]. The system has also been used in mammalian cells [22], extensively in plant cells [23] and for the past decade in microalgae [24].

The *A. tumefaciens* transformation technique has been successfully applied in *C. vulgaris* [25], *Chlamydomonas reinhardtii* [26], *Dunaliella salina* [27], *Haematococcus pluvialis* [28], *Schizochytrium* [29], *Isochrysis galbana*, and *Isochrysis* sp.

The GUS histochemical assay with X-gluc is utilized for screening of transgenic strain for quantitative analysis of β-glucuronidase [27]. Currently, the conventional method used for isolating the proper construct is by GUS histochemical analysis. The method is tedious and susceptible to biased interpretation of the results since the observations are made under the microscope. Also, to quantify GUS activity, cell lysis is necessary which requires a cell lysate volume of ~500 μl.

Triton X-100 has been used extensively in molecular biology studies for permeabilization of cell membranes [30]. However, the effect on florescence intensity, permeabilization of 4-MUG and 4-MU has not been studied in intact microalgae. It is necessary to study the effect of Triton X-100 on the permeability of 4-MUG through the cell membrane and release of 4-MU into the assay buffer. Hence, we have tried different concentration of Trion X-100 to determine its effect on 4-MU release into the assay buffer.

Here a GUS expression vector was constructed and transformed into *C. vulgaris* via *A. tumefaciens*. Also, a new HTS method for intact cell screening of the transgenic *C. vulgaris* expressing β-glucuronidase was developed in a 96 well microplate format. The new technique will enable GUS expressing microalgae screening to be carried out in cost effective, less labor intensive and time effective manner.

2. Materials and methods

2.1. Strains, plasmids, and culture condition

The pUC 19 plasmid was purchased from New England Biolabs (Whitby, ON, Canada) and was used for cloning work. Vectors pTET1/pBin Tet R and pBin HygTx–GUS-INT were kindly provided by Prof. Dr. Christiane Gartz (University of Göttingen, Göttingen, Germany). *Escherichia coli* strain DH5α was purchased from New England Biolabs (Whitby, ON, Canada) and was used for cloning work. Cells were cultured in Luria-Bertani (LB) medium containing 50 μg/ml of ampicillin or 50 μg/ml of kanamycin at 37 °C and 200 rpm. *A. tumefaciens* (LBA 4404) was kindly provided by Dr. Abdelali Hannoufa (Agriculture and Agri-Food Canada, London, ON, Canada). Cells were cultured in Yeast Extract Beef (YEB) medium containing streptomycin 100 μg/ml, rifampicin 20 μg/ml, and kanamycin 50 μg/ml at 25 °C and 200 rpm. Antibiotics, and were of analytical grade. *C. vulgaris* (UTEX 2714) was purchased from UTEX Culture Collection of Algae (Austin, TX, USA). The cells were cultured in Tris-Acetate-Phosphate (TAP) medium at 25 °C, 150 rpm, 90% humid atmosphere, and under 140 μmol $m^{-2} s^{-1}$ white light illumination of 16 h on/8 h off.

2.2. Vector construction

Vector pUC19 + Tx-GUS-INT was generated by double digestion of pUC19 and pBin HygTx–GUS-INT with *Eco*RI and *Hin*dIII. The digested vectors were purified by GeneJet Gel Extraction kit according to manufacturer's instruction (Catalog #K0691). They were gel purified by GeneJet Gel Extraction kit and ligated with Fast ligase enzyme. Fast cloning pack, Fast digest restriction enzymes, PCR clean up kit were purchased from Fermentas (Ottawa, ON, Canada) and Plasmid purification kits (catalog #27104) were purchased from Qiagen (Toronto, ON, Canada). The vector was transformed into *E. coli* by heat shock method and blue white screening was done to isolate the proper construct.

The final expression vector pBin19 + TetR-TetO was generated by PCR amplifying CaMV 35S –GUS-INT-35S 3′ poly tail (GUS gene containing fragment) from pUC19 + Tx-GUS-INT. *Hin*dIII and *Cla*I restriction site were introduced at 5′ and 3′ termini of the GUS gene fragment by PCR, using primers F_HindIII for (5′ –AAA AAA AAG CTT CGT CTA AGA AAC CAT TAT TAT CAT GAC ATT AAC C – 3′) and R_ClaI (5′ –AAA AAA ATC GAT CTT GCA TGC CTG CAG GTC AC– 3′). Oligonucleotides were purchased from UWO Oligo Factory (London, ON, Canada) and Q5 high fidelity polymerase was purchased from New England Biolabs (Whitby, ON, Canada). The GUS gene PCR fragment and pBin Tet R vector were double digested with *Hin*dIII and *Cla*I. They were gel purified by GeneJet Gel Extraction kit and ligated with Fast ligase enzyme to get the final vector pBin19 + TetR-TetO. The vector was transformed into *E. coli* by heat shock method. The precise construct was isolated by carrying out colony PCR and restriction digest analysis.

2.3. Electroporation of A. tumefaciens and transformation of C. vulgaris

Electrocompetent *A. tumefaciens* LBA 4404 cells were transformed with pBin19 + TetR-TetO vector in a Bio-Rad MicroPulser (Hercules, CA). The transformation was carried according to the MicroPulser manual [31]. For transformation of *C. vulgaris*, the Pratheesh et al. protocol was followed [26].

For selection of transformed *C. vulgaris*, the cells were plated on TAP agar plate containing 500 μg/ml cefotaxomine and 30 μg/ml G418 Sulfate. After 1 month of growth on the selection plate, colonies where visible. Single colonies were suspended in 5 ml of TAP media containing 30 μg/ml G418 sulfate for 2 weeks.

2.4. GUS histochemical analysis

The G418 sulfate resistant colonies were analyzed for GUS activity by staining with 5-bromo-4-chloro-3-indoyl b-D-glucuronide (X-gluc). X-gluc was purchased from Sigma-Aldrich (Oakville, ON, Canada). Transgenic *C. vulgaris* culture were pelleted and re-suspended in X-gluc solution and incubated at 37 °C overnight. After incubation, cells were pelleted, bleached with ethanol and analyzed under the microscope.

2.5. Quantitative measurement of GUS activity

GUS activity was measured by monitoring cleavage of 4-MUG to 4-MU by β-glucuronidase enzyme [23, 32]. 4-MUG and 4-MU were purchased from Sigma-Aldrich (Oakville, ON, Canada). The formation of 4-MU was measured by a Tecan M-1000 multimode plate reader (Männedorf, Switzerland) with an excitation of 365 nm and emission at 455 nm. The assay was adapted so that large numbers of samples could be assayed and measured in a 96-well microplate format (Greiner 96 Flat Bottom Black Polystyrol). Microalgae cultures were mixed for 30 s in the plate reader before taking readings. For all the fluorescence intensity measurement the gain was set manual and the *Z*-position to 20,000 μm. All results are typically the mean (± SD) of at least three replicates unless stated otherwise.