Algal Research 26 (2017) 323–329

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Chemical regulation of *Fea1* driven transgene expression in *Chlamydomonas reinhardtii*

Paula Barjona do Nascimento Coutinho*, Christine Friedl, Rainer Buchholz, Stephanie Christine Stute

Institute of Bioprocess Engineering, Friedrich-Alexander-Universität Erlangen-Nürnberg, Paul-Gordan-Strasse 3, 91052 Erlangen, Germany

ARTICLE INFO

Keywords:
Chlamydomonas
Inducible transgene expression
Fea1 promoter
mCherry
Flow cytometry
Chelator deferroxamine

ABSTRACT

Inducible promoters can provide regulated gene expression allowing the biosynthesis of gene products at most suitable moments of cultivation. In this study, parameters of induction and deactivation of the iron-responsive *Fea1* promoter (Allen et al., 2007) were investigated in the green alga *Chlamydomonas reinhardtii*. Our results indicate that the construct used, *ble-2A-mCherry*, can be expressed successfully by the *Fea1* promoter under iron-deficient conditions. The fluorescence signals of the fluorescent protein mCherry obtained via flow cytometry were detectable at different intensities in response to concentrations of iron ranging from 0 μM to 20 μM in media. We also demonstrate that the addition of the iron chelator deferroxamine (DFO) to iron-replete media leads to promoter activation, resulting in the increase of mCherry fluorescence. Reversibility of promoter induction is detected already within 3 h after transferring the cells to iron-replete chelator-free media. In this case, the progressive decrease in mCherry fluorescence can reach, within 48 h, as low as 5% of the fluorescence observed in a 40 h – treatment with DFO. Cell viability after DFO treatment is not affected up to a concentration of 100 μM of the chelator, which enables the establishment of a cyclic process of induction and repression for the production of recombinant proteins under the control of the *Fea1* inducible promoter.

1. Introduction

Several transgenes have been successfully expressed from *C. reinhardtii* nuclear and chloroplast genomes [3,4]. However, poor expression of heterologous genes from the nuclear genome has posed a major obstacle to the development of this green alga as an industrial platform. Possible explanations for this limitation include weak promoters, unfavorable position of genome integration, unadapted codon-usage and transgene silencing.

Several attempts have been made towards the development of methods of genetic engineering in order to achieve satisfactory gene expression. They include, among others, the implementation of a variety of selection markers, reporter genes and promoter systems as well as their adaptation to fit the codon usage preferences of *C. reinhardtii* [3].

The challenge of poor transgene expression from the nuclear genome in *C. reinhardtii* has been circumvented by transcriptionally fusing the coding region of 6 different fluorescent proteins to the *sh-ble* antibiotic resistance gene via the 2A self-cleaving peptide from the foot and mouth disease virus. Expression was under the control of the robust constitutive *Hsp70/Rbcs2* tandem promoter [2].

In contrast to constitutive promoters, inducible promoters provide a regulable switch that enables targeted gene expression at most suitable times of the cultivation. They allow the controlled and reversible expression of genes in *C. reinhardtii* by changes in cultivation parameters, such as growth media composition. Examples of inducible promoters in *C. reinhardtii* are the *Nit1* promoter, activated by ammonium starvation and stimulated by nitrate [5–9]; the *Fea1* promoter, activated by iron deficiency [1]; and the *Cyc6* promoter, activated by copper deficiency [10]. The expression of the *MetE* gene is regulated by levels of vitamin B_{12} [11] and riboswitch mechanisms are involved in the regulation of thiamine biosynthesis by physiological concentrations of this vitamin [12].

Controlling gene expression is of great interest when, for example, the objective is the formation of products with low stability. Triggering product formation by induction of gene expression shortly before harvest can minimize yield losses caused by product decay or degradation by e.g. proteases. Other applications of controlled promoter activation are the conditional silencing of essential genes and the expression of potentially toxic gene products at high levels only after reaching high cell densities [10].

The *Fea1* gene codes for an iron-binding protein that is secreted to the periplasmatic space. There, this protein is responsible for

* Corresponding author.
E-mail address: paula.coutinho@fau.de (P. Barjona do Nascimento Coutinho).

http://dx.doi.org/10.1016/j.algal.2017.08.006
Received 5 October 2016; Received in revised form 9 July 2017; Accepted 1 August 2017
Available online 09 August 2017

concentrating iron in the vicinity of transmembrane proteins that will then transport it into the cytoplasm [1]. The *Fea1* promoter has been used for the conditional expression of reporter proteins such as ARS [1,13], luciferase [14] and mCherry (this work) in iron deficiency conditions.

The time-related response of the *Fea1* promoter to different iron concentrations is essential in order to estimate its performance. This knowledge is important since harvesting the cells at a time point in which accumulation of the product is at its maximum is indispensable for an economic and sustainable production of recombinant proteins, but has not been addressed in the literature before.

Therefore, in this work, we demonstrate that the inducible *Fea1* promoter is able to confer sufficient levels of transgene expression, allowing not only cell survival during antibiotic selection but also the generation of mCherry fluorescence. Following, the effect of different iron concentrations in the culture media was investigated over time for 3 different clones. Finally, we show the induction of the *Fea1* by the addition of the iron chelator deferroxamine to the media and its reversibility.

2. Materials and methods

2.1. Algal strain and culture conditions

Cell-wall deficient *Chlamydomonas reinhardtii* strain cc400, obtained from the *Chlamydomonas* Resource Center, was used as recipient strain for the transformation with the *Fea1_ble-2A-mCherry* construct.

Stock cultures were maintained in tris-acetate-phosphate TAP media [15] agar slants or plates added with 1% sorbitol and propagated twice on a rotatory shaker at 20 °C and ~50 μmol photons $m^{-2} s^{-1}$ before use.

For experiments in liquid media, a modified TAP in which the trace elements are added individually from stock solutions was used [16]. The trace elements and their concentrations corresponded to the Hutner's trace elements solution with exception for experiments with altered iron concentration. Iron was added at the concentrations indicated in each experiment.

In experiments in which it was necessary to avoid carryover of iron or the chelator deferroxamine (DFO) from the starter culture, cells were collected by centrifugation (10 min.; 10 °C; 720*g*) washed once in iron-free TAP medium, and inoculated into the desired iron concentration.

In order to displace any residual metal ions, all glassware was treated with 6 N HCl overnight and washed with distilled water prior to use as described by [17]. High purity chemicals were used for making stock solutions for media preparation.

2.2. Plasmid construction

In order to measure promoter activity and strength, the reporter gene *mCherry* was used. Plasmid pBR9, kindly provided by Dr. Beth Rasala from the San Diego Center for Algae Biotechnology and Division of Biological Sciences, University of California, San Diego, contains the *mCherry* gene, specifically codon-optimized for *Chlamydomonas* nuclear expression, fused to the antibiotic resistance gene *sh-ble* via the foot and mouth disease virus peptide FMDV 2A under the control of the constitutive tandem promoter *Hsp/Rbcs2* [2]. The embodied 2A self-cleaving peptide [18] leads to the separation of the mCherry protein and thus to the accumulation of two separate proteins.

The iron responsive inducible promoter, *Fea1* was PCR-amplified from the plasmid p5′*Fea1-ARS2* [1], available from Chlamydomonas Resource Center, with forward and reverse primers TACGtctagaAGGACAGAGTGCGTGTG and GACTcatatgTGGTTAACTGTGCGACG, respectively. Lower case letters indicate nucleotides added to introduce recognition sites for restriction enzymes *Xba*I and *Nde*I into forward and reverse primers, respectively.

The *Xba*I/*Nde*I fragment containing the *Hsp70/Rbcs2* tandem promoter was excised from the plasmid pBR9, and substituted by the 1207 bp PCR product, consisting of the *Fea1* promoter including the 5′UTR from the plasmid p5′*Fea1-ARS2*. The ligation product resulted in a construct in which the expression of the antibiotic resistance gene *sh-ble* and of the fluorescent protein gene *mCherry* are under the control of the iron-responsive promoter *Fea1*, yielding the construct *Fea1_ble-2A-mCherry*.

2.3. Transformation method

$2 \cdot 10^8$ *C. reinhardtii* cc 400 cells were centrifuged at 720*g*, 10 °C for 10 min from a 3–4 day old culture and resuspended in 300 μL of iron-free TAP media. Cells were vortex-agitated for 20 s at top speed together with 3 μg plasmid DNA, 300 mg of autoclaved glass beads and 100 μL of 20% polyethylene glycol 8000 in 15 mL conical disposable polypropylene centrifuge tubes [19]. 10 mL of iron-free TAP media + 1% sorbitol was added to the tubes. Beads were allowed to settle and cells were transferred to new 15 mL tubes for centrifugation for 10 min at 720*g* and 10 °C. Pellet was resuspended in 20 mL iron-free TAP media + 1% sorbitol and cultivated overnight in 50 mL Erlenmeyer flasks at 20 °C and ~50 μmol photons $m^{-2} s^{-1}$ constant illumination.

Overnight cultures were centrifuged at 720*g*, 10 °C for 10 min. Pellet was resuspended in 0.4 mL iron-free TAP media + 1% sorbitol and spread on selective iron-free TAP + 1% sorbitol-agar plates supplemented with 7.5 μg/mL zeocin covered with 5 mL antibiotics-free TAP + 1% sorbitol agar media.

Selection for *Fea1_ble-2A-mCherry* transformants was performed in two subsequent steps: colonies from iron-free TAP + 1% sorbitol agar plates containing 7.5 μg/mL zeocin overlaid with 5 mL antibiotics-free agar media were transferred to plates containing 15 μg/mL zeocin without the antibiotics-free layer. Surviving colonies were transferred to liquid TAP media added with 2 μM iron and 5 μg/mL zeocin and cultivated in 24-well plates.

2.4. PCR analysis to determine the incorporation of transferred genes

To confirm the incorporation of the transferred DNA into the genome of *C. reinhardtii*, PCR amplification of the 1555 bp fragment, which included the resistance gene *ble* and the reporter gene *mCherry*, was performed with the forward and reverse primers CATATGGCCAAGCTGACCA and CGCTTCAAATACGCCCA, respectively.

PCR reaction parameters were as follows: 95 °C – 3 min; 35 cycles 95 °C – 30 s; 61.5 °C – 30 s; 72 °C – 1 min; 72 °C – 5 min.

Genomic DNA was isolated by the CTAB method: 2 mL of zeocin-selected *C. reinhardtii* cultures were centrifuged in 1.5 mL tubes and resuspended in 500 μL CTAB buffer supplemented with 2% β-mercaptoethanol. After 1 h incubation at 65 °C, DNA extraction was performed by phenol-chloroform-isoamyl alcohol (25:24:1), followed by ethanol precipitation. Wild-type (WT) cells were treated the same way and used as control.

2.5. Flow cytometry analysis

Detection of mCherry fluorescence was acquired on LSRFortessa (BD Biosciences) for experiments described in Sections 3.2 and 3.3 or a Cytoflex S flow cytometer (Beckman Coulter) for the experiment described in Section 3.4. Excitation and emission wavelengths were 561 and 610/20 nm, respectively. Analysis was performed with FlowJo v10.

The number of recorded events was 10,000 and gates were set as follows: The FSC and SSC parameters were obtained using a 488 nm blue laser and were used to eliminate fragments and clumps of algae that can be misread as a single cell. Chlorophyll fluorescence was detected by using 638 and 660/20 nm excitation/emission wavelengths. mCherry fluorescence channel was plotted against chlorophyll and events containing very low chlorophyll signal were excluded in order to eliminate dead or dying cells from the analysis.