A novel, robust and mating-competent Chlamydomonas reinhardtii strain with an enhanced transgene expression capacity for algal biotechnology

Polina Dementyeva a, Robert A. Freudenberg a, Thomas Baier a, Kristin Rojek a, Lutz Wobbe a, Bernd Weisshaar b, Olaf Kruse a, Robert A. Freudenberg a

a Algae Biotechnology and Bioenergy, Faculty of Biology, Bielefeld University, Universitätsstrasse 27, 33615, Bielefeld, Germany
b Faculty of Biology, Genetics and Genomics of Plants, Bielefeld University, Bielefeld, Germany

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ABSTRACT

In the future, algae biotechnology could play an important role in sustainable development, especially with regard to the production of valuable chemicals. Among the established laboratory strains with efficient transgene expression, there are none that have demonstrated the required robustness for industrial applications, which generally require growth at larger scale. Here, we created a robust and mating-competent cell line of the green microalga Chlamydomonas reinhardtii, which also possesses a high transgene expression capacity. This strain shows a comparably high resistance to shear stress by accumulating increased amounts of biomass under these conditions. As a proof-of-concept, a high phototrophic productivity of cadaverine from CO₂ and nitrate was demonstrated by efficiently expressing a bacterial L-lysine decarboxylase. In contrast to other established strains, this novel chassis strain for phototrophic production schemes is equipped with the traits required for industrial applications, by combining mating-competence, cell wall-mediated robustness and high level transgene expression.

1. Introduction

Green biotechnology could play an essential role in the reduction of CO₂ emissions. Within these concepts, metabolically-engined microalgae could become renewable and environment-friendly sources of diverse carbon-based compounds [1] and fuels [2]. Among biotechnologically suitable microorganisms, microalgae are especially promising, since they are photoautotrophic and readily cultivateable in cheap water-based media, using sunlight and carbon dioxide [3]. Metabolic engineering and synthetic biology approaches can substantially expand the range of products obtainable from microalgae [4] and increase their photoautotrophic productivity [5].

Chlamydomonas reinhardtii represents a well-established model organism for basic research in the field of photosynthesis research [6] and also microalgal biotechnology, including metabolic engineering [7–9], since for many fast-growing microalgal species suitable for large-scale cultivation, a molecular toolbox is not available [10].

In 2009 Neupert et al. created Chlamydomonas cell lines capable of efficiently expressing transgenes, by applying UV mutagenesis and subsequent selection of high transgene expressors based on their improved resistance towards the antibiotic emetine [11]. Since then, the UVM4/11 expression strains have successfully been used for the high-level expression of various nuclear transgenes [7,9,12,13].

Although, these strains efficiently express transgenes, UVM4/11 cell lines have two main disadvantages complicating their application in biotechnology. Firstly, the lack of a cell wall renders these strains susceptible to shear stress and thus unsuitable for cultivation at a larger scale, which is normally accompanied by intense mixing based on stirring or gassing with high flow rates [14]. Secondly, their mating inability prevents crossing experiments as a fast and efficient way of removing antibiotic selection markers or adding further genetic traits.

Recently, phototrophic production of the diamine cadaverine (1,5-pentanediamine) via decarboxylation of L-lysine, catalyzed by the enzyme L-Lysine decarboxylase (CadA, EC 4.1.1.18), was reported for C. reinhardtii, using UVM4-derivative strains [9]. 1,5-pentanediamine is used as a building block for the synthesis of (bio-) polyamides, which have excellent material properties and find application as medicinal plastics, fibers for textiles, or films and coatings [15,16].

In order to transfer sustainable cadaverine production into a robust C. reinhardtii phototrophic production chassis, we applied UV-
mutagenesis to the cell wall-containing and nitrate-assimilating wild-type CC-1690 [17]. Here, we demonstrate that an iteration of UV mutagenesis and selection of improved transgene expressors, based on their enhanced resistance towards the antibiotic Zeocin, can be used to equip robust C. reinhardtii wildtype strains with the ability to express transgenes at a high level, which is a prerequisite for their application in sustainable production schemes.

2. Materials and methods

2.1. Algal strains and culture conditions

The Chlamydomonas reinhardtii cell wall-containing strain CC-1690 [17] was used for UV mutagenesis experiments and UVM4 was used as a reference strain for transgene expression at a high level [11]. Throughout the manuscript wildtype (WT) refers to strain CC-1690. These strains and derivative cell lines were cultivated at 20 °C under constant illumination (250–350 μmol photons m⁻² s⁻¹) by using either TAP medium [18] for mixotrophic growth or HS medium [19] and high cell density medium [9] for phototrophic growth.

2.2. Vector design and cloning

Cloning of vector DNA was performed using the C. reinhardtii MoClo Toolkit [20]. A Shbl selection marker (pCM0-077) was assembled with the PSAD promoter (pCM0-016) and FDX1 terminator (Einhaus et al., 2021) as described previously [20] and the corresponding vector was used for initial transformation of CC-1690. UV-treated transformants were further transformed with pOpt2_Clover_Paro [7] to quantify gene expression based on fluorescence measurements. The E. coli lysine decarboxylase cadA (Uniprot: P0A9H3) was optimized for C. reinhardtii nuclear expression (as described in [21]) and fused to mRuby2 (NCBI: AFR60232) as previously described [9] and the resulting plasmid was used in combination with the aphVII hygromycin resistance gene (pCM0-073), [22] to probe heterologous cadaverine production.

2.3. Transformation of C. reinhardtii CC-1690

Nuclear transformation of C. reinhardtii was performed using the electroporation method according to Yamano et al. with some modification [23]. First, CC-1690 was cultivated in TAP medium to the early logarithmic phase (OD₆₈₀ = 0.2 – 0.4 or 1 – 2 × 10⁶ cells mL⁻¹). Then, cells were harvested by centrifugation at 1,000 × g, the resulting pellet resuspended in electroporation buffer (100 mM sucrose, 1.5 mM phosphate buffer, pH 7.4) to a final concentration of 5 – 10⁸ cells mL⁻¹. For each transformation 2 μg of linearized plasmid DNA were mixed with 50 μl of cell suspension (2.5 – 5 × 10⁷ cells) and the mixture was transferred to a 2 mm electroporation cuvette (Biorad). For electroporation, the square wave protocol was used with 400 V pulses and a duration of 5 ms. Then, the mixture was transferred to regeneration medium (40 mM glucose in TAP medium) and incubated with gentle shaking (55 – 65 rpm) for 40 h. The selection of transformants was conducted for 7 – 10 days on TAP agar plates with different concentrations of the antibiotics: Zeocin (5 μg mL⁻¹), Paromomycin (10 μg/mL) and Hygromycin b (20 μg/mL) under 150 – 200 μmol m⁻² s⁻¹ continuous light.

2.4. Zeocin resistance assay

Nuclear transformation of parental strain CC-1690 was performed using a Shbl selection marker [20] and individual transformants obtained were isolated on fresh Zeocin containing agar plates (5 μg mL⁻¹). To determine the level of Zeocin resistance in individual strains, different cell numbers (10⁴ / 10⁵) were spotted on TAP agar plates, containing distinct concentrations of Zeocin (5, 60, 100, 150, 200 μg mL⁻¹) and their growth was analysed. After UV mutagenesis, colonies were selected based on an elevated tolerance towards Zeocin (400, 600, 800, 1000, 1200 μg mL⁻¹).
2.5. UV mutagenesis

The selected low resistance strain (hereafter named ‘40p’) was grown in 25 mL of TAP medium to a cell density of $2.4 \times 10^6$ cells mL$^{-1}$. Cells were harvested by centrifugation (1,000 × g, 5 min) and the pellet was resuspended in 5 mL of TAP medium. For each sample, 0.25 mL corresponding to $3 \times 10^6$ cells were spread over agar plates containing either 150 μg/mL or 200 μg/mL Zeocin. Agar plates were then placed on a transilluminator with distance between cell suspension and UV lamp of 2 cm. Cell suspensions were directly exposed to UV light (312 nm) and different exposure times applied (1, 3, 5, 10, 15, 20, 25, 30, 40 min). Following mutagenesis, cells were transferred to darkness for 24 h. Finally, UV treated cells were incubated for 3 weeks on TAP agar plates at 100–150 μE m$^{-2}$ s$^{-1}$ continuous light illumination and regenerated colonies were tested for an elevated Zeocin tolerance by assessing growth on TAP agar plates containing different concentrations of Zeocin (400, 600, 800, 1000, 1200 μg mL$^{-1}$).

2.6. Fluorescence microplate reader assay

Cells were grown in TAP medium until late log phase and 100 μL of culture transferred to wells of a black 96-well plate (Greiner 96 Flat Bottom Black Polystyrol (GRE96 fb chimney). For fluorescence readings, a microplate reader Tecan Infinite M200 (Tecan, Männdorf, Switzerland) was used and readings acquired by using the following excitation wavelengths and emission filters: 480 / 515 nm for the detection of Clover fluorescence and 559 / 600 nm for mRuby2-fluorescence detection. As a blank, TAP medium was used. Reporter protein fluorescence was normalized against the chlorophyll absorbance at 680 nm. Normalized fluorescence values were also determined for the wildtype, which were used as a reference (set to 1).
2.7. Protein quantification via Western Blot analyses

The strains of interest were grown to an OD_{680} of 0.9–1 in 20 mL TAP medium and then cells were harvested by centrifugation (3000 x g for 3 min). After that, proteins were extracted by resuspending pelleted cells in 200 μl of lysis buffer (60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT). Protein concentrations were quantified using the amido black assay [24]. Samples containing 10 μg of total protein fractions were denatured at 95 °C for 5 min, separated via Tris-glycine-SDS-PAGE in 8% (w/v) polyacrylamide gels and transferred to 0.45 μm Protran Nitrocellulose membranes (Amersham) using transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol). Immunochemical protein detection was performed with HA-tag polyclonal antibody (Thermo Fisher Scientific) using the Thermo Scientific Pierce ECL Western blotting substrate. Signals were visualized to 0.45 μm Protran Nitrocellulose membranes (Amersham) using transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol). Immunochemical protein detection was performed with HA-tag polyclonal antibody (Thermo Fisher Scientific) using the Thermo Scientific Pierce ECL Western blotting substrate. Signals were visualized using the FUSION-FX7 detection system (Peqlab, Germany).

2.8. Fluorescence microscopy

The randomly selected colonies harboring plasmids carrying the reporter genes were grown on TAP plates with appropriate antibiotic during 1–2 days and then fluorescence signals were analysed with Leica M205 FA fully motorized fluorescence stereo microscope as previously described [25]. The visualization of the Clover fluorescence was performed at an excitation of 450–490 nm and at an emission of 505–515 nm. The following parameters were used to detect mRuby2 fluorescence: excitation at 540–580 nm and emission at 589–599 nm.

2.9. Algal growth in HSM medium and assessment of cadaverine production

Strains were grown in HSM medium under constant illumination with a light intensity of 100–200 μE m⁻² s⁻¹ and vigorous (vvm=2) bubbling with carbon dioxide-enriched air (3% (v/v) CO₂) for two weeks, with samples taken at days 1, 2, 3, 4, 5, 7, 11 and 14. For each sample, biomass, cell numbers and the optical density at 680 nm were determined. Polyamines were extracted and quantified, as previously described [9].

2.10. Mating and tetrad separation of the UV-mutagenized CC-1690

Mating experiments were performed as previously described [26]. For mating experiments, strains were grown on TAP agar plates for 2–3 days and gametogenesis was induced by nitrogen depletion and formation of dykaryons was observed microscopically.

3. Results and discussion

3.1. UV-mutagenesis and Zeocin selection yield in a Chlamydomonas reinhardtii wildtype strain with increased reporter expression

To add the capability of expressing transgenes at a high level to the robust C.reinhardtii wildtype strain CC-1690 [17], the following strategy was applied (Fig. 1). First, strain CC-1690 was transformed with a SABie containing plasmid [20] conferring resistance against the antibiotic Zeocin [27, 28] and a transformant displaying only a low resistance level was isolated. This strain was transformed with a nuclear expression construct for the expression of a Clover green fluorescent protein reporter alone or a red fluorescent (mRuby2) reporter protein fused to the...
l-lysine decarboxylase CadA. To obtain mutant strains expressing transgenes at a higher level by inactivating or circumventing the transgene silencing mechanisms inherent to *Chlamydomonas* [29–31], UV-mutagenesis was applied. UV mutagenized strains were then tested for an elevated resistance towards Zeocin. The Zeocin resistance level is proportional to the amount of expressed Zeocin-binding protein, since it forms a 1:1 complex with the antibiotic, which prevents DNA cleavage [32]. Zeocin resistance can be thus used as a proxy for the transgene expression capacity of UV mutants. UV mutants showing an elevated antibiotic resistance level were then also transformed with the fluorescent Clover reporter and transformants screened for high reporter expression levels. After obtaining a strain with retained robust phototrophic growth and high transgene expression capacity, this strain was transformed with a lysine decarboxylase (CadA)-mRuby2 reporter fusion construct as a proof-of-concept application for phototrophic cadaverine production.

To isolate the starting strain with a low Zeocin resistance level, 95 Zeocin-resistant transformants were subjected to growth assays on agar plates containing Zeocin concentrations in the range of 5–200 μg/mL of the antibiotic. Among all transformants, 11 showed little growth on 100 μg/mL Zeocin and could not grow at all on 150 μg/mL of the antibiotic (Fig. S1).

Among them, strain 40p showed the lowest resistance level and was selected as the starting strain. To this starting strain, which is further on designated “low resistance strain”, we applied UV-mutagenesis to obtain mutants with an altered transgene expression capacity.

Among the isolated UV mutants seven could survive on up to 1200 μg/mL Zeocin and we focused on one of these mutants (Fig. S2; 7a; CC-1690***), designated “CC-1690***” in further experiments.

Along with UVM4, CC-1690*** was subjected to growth analyses under phototrophic conditions in a flat panel bioreactor (Fig. 2A) with vigorous bubbling (gas flow rate of 2.5 vvm, 1.7 % (v/v) CO₂) to test the shear force resistance of both strains. The final biomass yield noted for the cell wall-containing strain CC-1690*** was about two-fold higher compared to the cell-wall deficient [11] UVM4 strain (1.46 ± 0.03 g L⁻¹ for CC1690*** vs. 0.70 ± 0.07 g L⁻¹ for UVM4 at day 5).

It is well-known, that cell wall-reduced *C. reinhardtii* strains cannot resist the high shear forces resulting from vigorous mixing in bioreactors [33]. The much better performance of CC-1690*** compared to UVM4 should therefore result from the fact that CC1690*** contains a wildtype-like robust cell wall, whereas UVM4 does not.

CC-1690*** and the low resistance strain were transformed with a construct for the expression of a Clover green fluorescent protein (Fig. 2B). For each transformation 25 distinct transformants were analysed in regard to their Clover expression based on fluorescence emission (Figs. 2C and S3). The Clover fluorescence emitted by UV strain-derived transformants was significantly \( P < 0.05 \) higher than in those derived from the low resistance strain.

### 3.2. The novel UV strain displays a UVM4-like capacity to express nuclear transgenes

The UV strain, its progenitor the low resistance strain and UVM4 were then transformed with a nuclear expression construct (Fig. 3A) encoding...
a fusion of the lysin decarboxylase CadA [9,15,16] and the fluorescent reporter mRuby2 [34]. After transformation, transformants were randomly picked, obtaining a broad spectrum of transgene expression levels (Fig. 3B), which is due to positioning effects, as a result from random integration of expression constructs into the nuclear genome of *C. reinhardtii* [35].

Fluorescence levels in transformants derived from CC-1690*** (11 out of 200 transformants analysed) and UVM4 (11 out of 288 transformants) were significantly (*P* < 0.05) higher when compared to those derived from the low resistance strain (Fig. 3B). Although, the median fluorescence and the upper quartile range was higher in UVM4-derived transformants, fluorescence differences between the two transformant populations were insignificant according to a Student’s *t*-test (*p* < 0.01, two-tailed hypothesis). Therefore the UV strain displays a high transgene expression capacity, comparable to that of strain UVM4, a strain which along with UVM11 was for many years the only option, if high level transgene expression in *C. reinhardtii* was required. The nuclear genomes of both strains, UVM4 and UVM11 carry mutations in a gene encoding the Sir2-type histone deacetylase SRTA [36], which were shown to be causative for the high transgene expression phenotype. Sequencing of the SRTA gene in CC-1690*** confirmed that UV mutagenesis did not cause mutations in its coding sequence, pointing at mutations in other genes as the cause for higher transgene expression in this strain. Future experiments will aim at identifying the genotype responsible for the phenotype of CC-1690***.

### 3.3. The novel UV strain efficiently produces the diamine cadaverine under photoautotrophic conditions

Among the analysed UV strain-derived transformants UV_6, UV_10 and UV_3 displayed the highest mean mRuby2 fluorescence (Fig. 4A) and were subjected to further analyses (Fig. 4B) together with UV_1 and UV_2, as strains with an intermediate fluorescence. UV_6, UV_3 and UV_2 showed the highest cadaverin titers in the range of 3.5–3.7 mg L$^{-1}$ after 7 days of cultivation in mixotrophic TAP medium (Fig. 4B) and were further analysed regarding their cadaverine production capacity under photoautotrophic conditions (Fig. 4C) and using the novel 6xP medium [8], developed for high cell density cultivation of *C. reinhardtii*. Under these conditions UV_6 displayed the highest biomass (20.49 g L$^{-1}$ biomass dry weight) and cadaverine accumulation (22.5 mg L$^{-1}$) after
14 days of phototrophic cultivation.

In order to compare the cadaverine production capacity of UV_6 to that present in the best UVM4-derived strain (UVM4_3; Fig. 4A), cultivations in phototrophic HSM medium were performed (Fig. 5), which, in contrast to 6xP medium, contains ammonium instead of nitrate as the nitrogen source [9, 37].

Strain UV_6 accumulated about 20 % more biomass than strain UVM4_3 (1.63 g L\(^{-1}\) in UV_6 vs. 1.38 g L\(^{-1}\) at day five, where biomass accumulation peaked for both strains (Fig. 5A). Cadaverine titers (Fig. 5B) showed a comparable increase in both strains, reaching a similar level (23.2 ± 1.6 mg L\(^{-1}\) in UVM4_3 vs. 22.3 ± 2.8 mg L\(^{-1}\) in UV_6) at day 11, before cadaverine further accumulated until day 14 (34.2 ± 3.1 mg L\(^{-1}\) in UVM4_3 vs. 21.4 ± 6.7 mg L\(^{-1}\) in UV_6). Immuno-detection of the CadA-reporter fusion protein after 3 days of cultivation (Fig. 5C) showed that in UVM4_3 the lysine decarboxylase enzyme accumulated to higher levels, explaining the higher cadaverine titer in UV_6 at day 3. The higher biomass accumulation in UV_6 at day 11 seems to compensate for the lower transegene expression, resulting in comparable cadaverine titers.

Based on the observed traits of the UV strain including high transgene expression capacity (Figs. 2C, 3B, 4A and 5C) and robust growth (Figs. 2A and 5A), it was tested if this strain could be crossed with other \textit{Chlamydomonas} strains of the opposite mating type (Fig. 6).

3.4. The novel UV strain is mating-competent

As expected from the presence of long flagella and the motility of the UV strain, dikaryons (white boxes) possessing four flagella could be identified in microscopic images taken after mixing UV strain (derived from CC1690 mt\(^{-}\)) with strain CC1691 (mt\(^{+}\)). It is well established that almost every dikaryon (>99.9 \%) [38] becomes a zygote, and indeed these dikaryons turned into zygotes (Fig. 6B), showing the typical thick cell wall [39]. The cell-wall deficiency and immotility of UVM4/11 precluded mating experiments [11, 36] representing a major drawback for their use a bioproduction chassis strain. Only recently, UVM11 could be crossed with wildtype strain CC-124 and the progeny retained the capacity to express transegenes at a high level [36]. The mating-competence of the UV strain will facilitate future map-based cloning or whole genome re-sequencing experiments [40] to identify the causative mutation.

4. Conclusion

A new \textit{Chlamydomonas reinhardtii} cell-well- containing strain derived from the robust wildtype CC-1690 was generated by combining UV mutagenesis and subsequent candidate selection based on an increased Zeocin antibiotic tolerance. This novel strain (UV strain) will be further generated by combining UV

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00644.

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