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Chlamydomonas reinhardtii for recombinant somatotropin production: an alternative expression vector

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Abstract

The human growth hormone (hGH) is a therapeutic protein widely used in medicine for treating growth disorders and metabolic deficiencies. In this study, we aimed to express recombinant human growth hormone (rhGH) in the chloroplast of *Chlamydomonas reinhardtii*, using the endogenous psbD promoter to regulate expression. The optimized hGH gene fused to a FLAG tag was cloned into the GH-psbH expression vector, which was integrated into the chloroplast genome through homologous recombination at the psbH locus. Transformed colonies resistant to kanamycin were screened by PCR, and homoplasmic lines were confirmed. Western blotting revealed the synthesis of a recombinant protein with a molecular weight of approximately 23.6 ± 0.3 kDa, consistent with the predicted size of rhGH-FLAG. The best-performing clone showed a 4.5-fold higher accumulation compared to the reference colony. The recombinant protein was successfully purified by affinity chromatography, yielding an estimated concentration of 1.33 mg/L. These results demonstrate the effectiveness of the psbD promoter in driving heterologous protein expression in *C. reinhardtii* chloroplasts, maintaining photosynthetic capacity and achieving higher accumulation levels than previously reported promoters. This study supports *C. reinhardtii* as a sustainable and promising platform to produce human therapeutic proteins.

Introduction

The growth hormone, also known as somatotropin, is a peptide physiologically synthesized and secreted to the anterior pituitary gland of vertebrate animals. This is an essential hormone that stimulates cellular multiplication for tissue growth, especially bones and muscles, besides being essential for inducing some cellular type's differentiation (1). The growth hormone lack or reduced levels in humans is associated with hypopituitarism, which affects not only growth, but also metabolic processes and the immunological system, causing diseases that are generally treated by synthetic hormone supplementation (1,2).

Thanks to biotechnological advances, since the 70s, it was possible to apply the recombinant DNA technology to express desired molecules in living organisms, using them as factories for valuable proteins production (3). Since then, recombinant human growth hormone (rhGH) has been synthesized in bacteria, it was soon considered safe for human treatment and commercially feasible (2,4). Nowadays, other organisms are studied for their capacity to efficiently produce human-interest substances.

Microalgae are among the living systems studied for recombinant protein production, and they are promising due to their physiological characteristics and sustainability. Microalga is a term that englobes a heterogeneous group of unicellular and photosynthetic microorganisms, which commonly includes eukaryotic algae and prokaryotic cyanobacteria (5). These microorganisms are attractive for their capacity of growing in a relatively cheap culture media and using natural sources, such as sunlight and atmospheric carbon dioxide (6,7). Besides, microalgae are not affected by human pathogens, and therefore, many species are already considered safe for human consumption (8).

Chlamydomonas reinhardtii is a green microalga, commonly used as a model in genetic engineering studies (6,9). This species is notable for its capacity to perform some crucial post-translational modifications in proteins, such as disulfide bridges and glycosylation (6,10). *C. reinhardtii* also features a large chloroplast size, containing a high number of plastid genome copies, which confers to it the capability to accumulate the recombinant protein(9,11).

Heterologous protein expression efficiency in *C. reinhardtii* was already been reported in diverse scientific studies (12,13). So, in recent years, efforts have been made to optimize its accumulation levels, including genetic transformation technique as a promising strategy to increase recombinant protein accumulation (14–16). It has been reported that the chloroplast can accumulate more recombinant protein than the proteins expressed by the nuclear genome(15,17). Additionally, transformations are more stable in the chloroplast, since in this organelle the heterologous DNA insertion occurs through homologous recombination, allowing it to integrate in a specific locus (9). Furthermore, the promoter sequence, an essential regulatory element for heterologous gene expression, plays a meaningful role in the recombinant protein accumulation levels (18). The psbA promoter is triggered by light and it stands out among the promoters already studied in *C. reinhardtii*, when compared to the expression rate driven by atpA promoter, as an example (19).

Previous scientific studies reported rhGH expression efficiency from *C. reinhardtii* plastid genome modification. However, the expression levels driven by the psbA promoter were not detected due to its attenuation mechanism (20). Therefore, this promoter efficient use depends on the removal/silencing of the psbA gene, which encodes the D1 protein of photosystem II. This condition results in an organism deficient in its photosynthetic apparatus and some strategies have been reported to restore photosynthetic conditions, such as the reintroduction of the psbA gene regulated by the psbD promoter, as well as the use of psbA gene from other photosynthetic organisms (21,22). In the present study, to address these challenges, rhGH synthesis was regulated by the endogenous psbD promoter to verify this recombinant protein expression and accumulation.

Materials and methods

Microorganism, culture medium, and cell maintenance conditions

In this study, all the experiments were carried out with the microalga *Chlamydomonas reinhardtii* CC-400 cw15 mt+ (www.chlamy.org). It is a mutant cell wall deficient strain, or a significantly reduced cell wall strain compared to the wild type. The microorganism was cultivated in Tris-Acetate-Phosphate (TAP) culture medium (23). The micronutrients required for cell growth were supplied by Hutner's trace elements, a micronutrient solution proposed by Hutner, and developed based on the nutritional requirements of *Pseudomona* (24). The inoculum and liquid maintenance culture were performed in 125 ml Erlenmeyer flasks containing 50 ml of TAP medium. The cultures were monitored until the cells reached exponential growth phase, under the following conditions: at 25 ± 1°C, light intensity of 50-60 µmol photons m^{-2} s^{-1}, and orbital shaking at 110 rpm. CC400 strain maintenance was also performed on

Petri dishes with solid TAP medium, prepared with 1.5% bacteriological agar. The plates were maintained at 25 ± 1°C and 60 µmol photons m^{-2} s^{-1}, and after growth, they were stored under low light conditions. Genetically transformed strains maintenance was performed in plates containing solid TAP medium, with 150 µg/ml kanamycin antibiotic

Plasmid vector

A plasmid vector named GH-psbH was cloned from fragments obtained through the enzymatic digestion of two previously synthesized vectors. Fragments were gel-purified and ligated by a reaction mediated by T4 DNA ligase. The smaller fragment corresponds to the optimized gene sequence that encodes human growth hormone, fused to a C-terminal FLAG sequence, while the larger fragment corresponds to the other structural elements of the expression vector for *C. reinhardtii,* and bacterial replication sequences derived from the pUC57 vector. The ligation reaction product was cloned into *E. coli* Subcloning Efficiency™ DH5α from Thermo Fisher Scientific. The extracted plasmid was analyzed through enzymatic digestion and sequenced by Sanger sequencing.

Chloroplast transformation using glass beads

This technique was performed according Economou *et al.* (2014) modified protocol. 300 µl of cell culture at ~ 2 x 10^8 cells/ml and 5 µg of DNA plasmids were added to each 15mL falcon tube. The tubes were shaken in a vortex mixer at maximum speed for 15 seconds. After shaking, 3 ml of TAP medium containing 0.5% (w/v) bacteriological agar was added to each tube. Finally, the material was spread on the surface of a Petri dish containing TAP medium with 1.5% agar and 150 µg/ml of kanamycin. The plates were incubated at 25 ± 1°C and 1-5 µmol photons m^{-2} s^{-1} of light intensity, and after 12 hours, the light intensity was increased to 50-60 µmol photons m^{-2} s^{-1}. The genetic transformation was performed in quadruplicate, with a negative control that did not include the plasmid vector.

Screening for positive gene and homoplasmic colonies

The resistant colonies were screened for gene positives by the polymerase chain reaction (PCR) technique. The forward primer 5’-GTGATGACTATGCACAAAGCAG-3’ anneals at the end of the psbD promoter sequence, and the reverse primer 5’-CTTCTAAACGACCCATTAATGTTTG-3’, in the middle of the gene of interest. Positive gene colonies were streaked repeatedly on selective medium until homoplasmic condition was achieved. Positive gene colonies were streaked repeatedly on selective medium until homoplasmic condition was achieved, in which all copies of the chloroplast genome carry the same genetic variant. To confirm the condition, a PCR reaction was performed with two sets of primers: the first designed for amplification of the 16S ribosomal gene region as a positive control, 5'-CCGAACTGAGGTTGGGTTTA-3' and 5'-GGGGGAGCGAATAGGATTAG-3' (25), and the second for the psbH genomic amplification. The latter corresponds to the forward primer 5’-GGGGACGTCCTAATATAAATATG-3’ and the reverse primer 5’-TTTTATTTAACACAAACATAAAATATAAAAC-3’. Microalgal DNA was extracted using Chelex 100 resin (Bio-Rad), as described by Economou et al. (2014).

Total soluble proteins extraction and quantification

Colonies were transferred to 50 ml TAP liquid medium and incubated at 25 ± 1°C, 20 µmol photons m^{-2} s^{-1} of light intensity, at 110 rpm in a rotating shaker for 4 days. Culture was centrifuged at 2060 x g for 10 minutes, and the cells were resuspended in 500 µl of Tris-Buffered Saline Tween (TBST). The samples were sonicated twice for 15 seconds at 20% amplitude, with a 1-minute cooling interval in between. After centrifugation, the supernatant, which contains the total soluble proteins, was collected and stored at -20 ºC.

Soluble proteins were quantified using the DC Protein Assay kit (Bio-Rad), which is performed by a reaction according to Lowry et al., 1951. Colorimetric reaction was performed in a 96-well transparent microplate and evaluated using an Infinite Tecan microplate reader. To correlate absorbance at 750 nm with the concentration of total soluble proteins, a calibration curve was constructed using the following concentrations of bovine serum albumin (BSA) diluted in TBST: 250, 500, 750 and 1000 µg/ml.

Western blotting analysis

The analysis was performed by adding 50 µg of total soluble proteins per well of a 12% SDS-PAGE gel. The proteins were transferred from the gel to a nitrocellulose membrane in a chamber containing the transfer buffer (25 mM TRIS, 192 mM Glycine and 20% methanol) at 80V and 1.0 mA for 50 minutes. The membrane was blocked in 100 ml of TBS-T solution with 5% milk and incubated for 1 hour. Then, the membrane was incubated overnight at 4°C with the primary antibody Anti-FLAG IgG produced in rabbit from Merck (MFCD02262912) (1:5000 in TBST). After washing, the secondary antibody anti-rabbit IgG produced in goat and conjugated with HRP from Thermo Scientific (A27036) (1:10000 in TBST) was applied. The incubation with the secondary antibody was performed for 1 hour at room temperature. For chemiluminescence detection, 1 ml of Pierce ECL substrate A and B from Thermo Scientific (32106) were applied to the membrane and exposed using a ChemiDoc XRS+ system (Bio-Rad).

Purification

The culture intended for the purification step was carried out according to the parameters described previously but performed in a volume of 100 mL in a 250 mL Erlenmeyer flask. The extraction of total soluble proteins was performed from a resuspension in 1 mL of TBST. Total soluble proteins were subjected to affinity chromatography using Pierce™ Spin Columns - Snap Cap (69725), loaded with anti-DYKDDDDK resin (A36803), both from Thermo Fisher Scientific. Initially, the column was prepared by adding 200 µL of TBST and 50 µL of the resin (25 µL of settled resin). After centrifugation for 1 minute at 1,000 × g, the resin was resuspended in 10 volumes of bed and centrifuged again. The process was repeated twice, and then 600 µL of sample was added. The column was shaken on a rocker for 20 minutes at room temperature and then centrifuged again. Two washes were performed with 250 µL of phosphate-buffered saline (PBS), followed by elution with 0.1 M glycine buffer at pH 2.8, for a final volume of 200 µL. The eluted was neutralized using 30 µL of 1 M Tris at pH 8.5. Samples obtained at each purification step were analyzed by SDS-PAGE (12%) and Western blotting, using 20 µL of each

sample per well. The concentration of recombinant protein produced was estimated by measuring the material in the eluate post-purification using OD280.

Results

GH-psbH vector was cloned following the methods described above, resulting in a plasmid vector with the expected fragmentation pattern for enzymatic digestion and the correct gene sequence of interest, as assessed through sequencing (data not shown). The expression vector that recombined with *C. reinhardtii* chloroplast genome is illustrated in Figure 1. This vector targets the chloroplast genomic region known as psbH, with insertion between the tnrE1 gene, which encodes a transfer RNA, and the psbH gene, which encodes a protein of the photosystem II reaction center. The vector contains two homologous regions at the insertion site, the psbD promoter for the gene of interest regulation, gene sequence of rhGH gene sequence, 5' aphA promoter that regulates kanamycin resistance gene (aphA-6) expression, as well as 3' UTR regions (rbcL) following each gene present in the expression vector. Although the 3’ psbH homology region present in the expression vectors contains the psbH gene sequence, the insertion does not cause any deletion or gene silencing.

After the cloning steps by using bacterial competent strains, the plasmid vector was transformed into *C. reinhardtii* chloroplast genome. Resistant colonies were selected on TAP medium containing 150 µg/ml of kanamycin and it resulted in only four antibiotic-resistant colonies. All colonies were detected as gene positive by colony PCR with 630 bp band amplification (Figure 2a). Gene positive colonies were seeded on Petri dishes containing 150 µg/ml of kanamycin, and this streaking process was performed in three rounds of plate replication, aiming for the chloroplast genome, which has high ploidy (26), to reach the condition of homoplasmy. After three cycles, a PCR test for homoplasmy was conducted on 18 randomly collected colonies.

None of the 18 colonies amplified the second band of 343 bp, related to the presence of unmodified copies of the chloroplast genome; thus, all colonies were considered homoplasmic. In figure 2b, 8 of the 18 tested colonies are represented, where amplification of the control region 16s RNAr, which has 513 bp, is observed in all wells.

From the 18 homoplasmic colonies, 8 of them were cultured for total soluble proteins extraction and recombinant product detection by Western blotting. The cultures grown under light conditions of approximately 20 ± 0.7 µmol photons m^{-2} s^{-1} showed an average concentration of total soluble proteins of 0.21 ± 0.01 g/L. The relative molecular weight and detected bands intensity were estimated by Image Lab software (Bio-Rad), with the lowest intensity band considered as the reference (colony 1). For all colonies, a prominent and intense band was observed, with an average molecular weight of 23.6 ± 0.3 kDa (Figure 3a). As each sample was added at equal protein concentration it is possible to compare the protein accumulation between them. The correlation indicated that colony 4 shows a higher accumulation of detectable protein, 4.53 times greater than the reference (Figure 3b).

The colony with the highest relative intensity (colony 4) was cultured for the recombinant product purification by affinity chromatography in a centrifuge column. Both, the protein extract and the flow-through exhibit a high protein density, making it impossible to distinguish bands (Figure 4a). In the eluted sample, two close size bands were observed, with a molecular weight between 22-25 kDa. This result is similar to that seen in the colony screening by Western blotting. In Figure 4b, the WB membrane was exposed for 167.551 seconds, capturing the signal accumulation before pixel saturation occurred. It was observed that the purified recombinant protein interacts with the anti-FLAG antibody, resulting in a prominent band. The concentration of rhGH was approximated at 1.33 mg/L of culture; however, it is important to acknowledge potential product loss during the purification process.

Discussion

In this work, the expression of human growth hormone (hGH) in the chloroplast of *Chlamydomonas reinhardtii* was investigated, with the aim of further assessing this microalga as a biological platform for recombinant protein production. This microalga has been extensively studied as an alternative expression system, due to its ability to perform post-translational modifications necessary for the correct folding and functionality, with patterns comparable to those found in human cells. In addition, this microalgal system enables the production of recombinant proteins free from antigens that may be present in mammalian cell-based platforms, while exhibiting rapid growth and eliminating the need for large arable areas, as required by higher plant systems (27,28).

Another relevant advantage of *C. reinhardtii* is its reliance on renewable resources, such as sunlight and atmospheric carbon dioxide, which contributes to avoiding environmentally harmful waste generation (9,27). From a bioprocess perspective, microalgal cultivation for recombinant protein production offers relatively low operational and capital costs, as it relies on simple aqueous media with the potential for recycling, thus supporting the feasibility for industrial-scale production in modern photobioreactors (28,29). Although limitations related to low productivity have been reported in some cases, recent advances in molecular engineering strategies and process optimization approaches indicate that substantial improvements in recombinant protein yields are achievable (9,30).

As shown in the results (Figure 3), the recombinant growth hormone was successfully expressed. The expected theoretical molecular weight for human growth hormone, according to DrugBank, is 22.13 kDa. However, this theoretical value does not account for the FLAG peptide added for detection, resulting in an expected molecular weight of approximately 23.37 kDa. The observed value closely matches this theoretical expectation, confirming successful expression (Figure 3).

Somatotropin had already been successfully expressed in *Chlamydomonas reinhardtii*, using the psbH insertion site, but with different endogenous promoters (20). Wannathong et al. 2016 (20) evaluated the psbA sequence as a promoter, a well-studied photosynthetic promoter for expression in *C. reinhardtii*. However, the use of the psbA promoter requires the knockout of the psbA gene, which encodes the D1 protein of photosystem II. The accumulation of D1 protein leads to attenuation of the psbA promoter

through a negative feedback mechanism (31,32). Consequently, Wannathong et al. did not detect growth hormone expression when using this promoter.

The psbD promoter, applied in this work, does not require the knockout of photosystem-associated genes, preserving the photosynthetic capacity of the mutant microalga and avoiding costs related to the addition of an organic carbon source to the culture medium, which would be necessary for the growth of photosynthetically deficient cells (32). Although an organic carbon source was added in our experimental setup, the use of this promoter retains the potential for cultivation without supplementation, offering a cost-reduction advantage. Some studies have sought to identify the best regulatory elements for *C. reinhardtii*, since they significantly affect gene expression (33–35). It is important to note that regulatory elements can result in expression variability depending on the associated gene, highlighting the necessity of empirical optimization for each sequence of interest (19).

Somatotropin is a simple-chain protein, composed of 191 amino acids, arranged in a specific three-dimensional configuration that allows interaction with target cell receptors (36). It is initially synthesized as a pre-hormone with a signal peptide, which is cleaved during secretion process. This hormone is predominantly composed of alpha helices, organized in a compact conformation. Its tertiary structure is arranged in a unique fashion with left-twisted helical bundles, where the four alpha helices are positioned (37,38). This conformation allows the formation of two internal disulfide bonds between cysteine residues (Cys53–Cys165 and Cys182–Cys189), aiding in molecular integrity, although it has been reported that such disulfide bonds are not essential for the protein's function but may play an important role in its proper folding and stability (38,39).

Western blotting analysis revealed an intense band at the expected molecular weight, alongside a second, lower-intensity band (Figura 3a). Such results may indicate the formation of a structural variant of somatotropin, which could be later analyzed with high-resolution physicochemical techniques to assess the possible variation type.

The presence of multiple immunoreactive species with similar molecular weights has been previously reported for somatotropins produced in heterologous systems(40–42). A range of structural variations for somatotropin has already been reported and characterized, resulting from modifications during transcription, translation, and post-translation, or from degradation and/or aggregation products. Among these various reported modifications, there are: variations in trisulfide bonds, with the formation of a third cysteine linkage; non-incorporation of amino acids in bacterial platforms; addition of analog amino acids; N-terminal modifications with acetylated derivatives; enzymatically and non-enzymatically cleaved forms; oxidized forms and those with removal of chemical groups; and dimeric forms or high-mass aggregates (40–42). Product-related impurities can arise from variations in the manufacturing process or exposure to stressful conditions such as extreme pH and temperature, typically independent of the expression system(43).

The presented results show that the gene encoding human growth hormone insertion into the *psbH* site of the chloroplast genome of *Chlamydomonas reinhardtii* was successful. The transformed colonies

showed detection of recombinant protein synthesis regulated by the photosynthetic promoter *psbD*, with a molecular weight compatible with that expected for somatotropin. The observed variation in molecular weight in purified material should be further investigated to precisely characterize the recombinant protein.

Wannathong et al. (2016) reported the production of rhGH at a concentration of 0.5 mg/L in *Chlamydomonas reinhardtii* cultures. The authors demonstrated that rhGH expressed in *C. reinhardtii* exhibits biological activity, as evidenced by the induction of proliferation in the rat lymphoma cell line Nb2-11. Additionally, they highlighted an interesting feature of the regulatory element *psaA*: its ability to drive recombinant synthesis in *E. coli*. Based on this, they suggest the use of the *psaA* promoter as a preliminary tool for testing recombinant protein expression prior to transformation in *C. reinhardtii*. Earlier studies had already explored the expression of rhGH in the microalgal platform *Chlorella*, under different promoters, reporting concentrations ranging from approximately 0.2 to 0.6 mg/L (44). Although obtained with a different species and under distinct experimental conditions, these results laid important groundwork for the use of microalgae in biopharmaceutical protein production, paving the way for subsequent advances with *C. reinhardtii*.

Under the bioprocess conditions applied, the psbD promoter yielded accumulation greater than 1 mg/L, demonstrating significantly higher performance within the microalgae-based platform. While bacterial systems often achieve higher productivity levels, they may present limitations in the proper folding of certain proteins. Mammalia system provides robust folding but involves high production costs and complexity. In this context, sustainable platforms such as the microalga *Chlamydomonas reinhardtii* have gained increasing attention, driving research focused on the synthesis of therapeutic proteins using this platform (7,29). More than 20 therapeutic proteins have been successfully expressed in *C. reinhardtii*, underscoring its versatility(29). Despite the promising results obtained in recombinant expression in *C. reinhardtii*, further studies are needed to identify efficient promoters, develop genetically optimized strains, and refine bioprocess parameters to enhance recombinant protein production.

Declarations

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Competing Interests

The authors declare that they have no conflict of interest.

Author Contributions

França Junior: investigation, methodology, analysis, writing – original draft. Pessoa: analysis, writing - review and editing. Filho: investigation, analysis, writing – review and editing. Ferreira-Camargo: project administration, supervision, writing – review and editing.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

All authors are aware of the content and agree with the submission.

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Data availability Statement

Data will be available on reasonable request.

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Figures

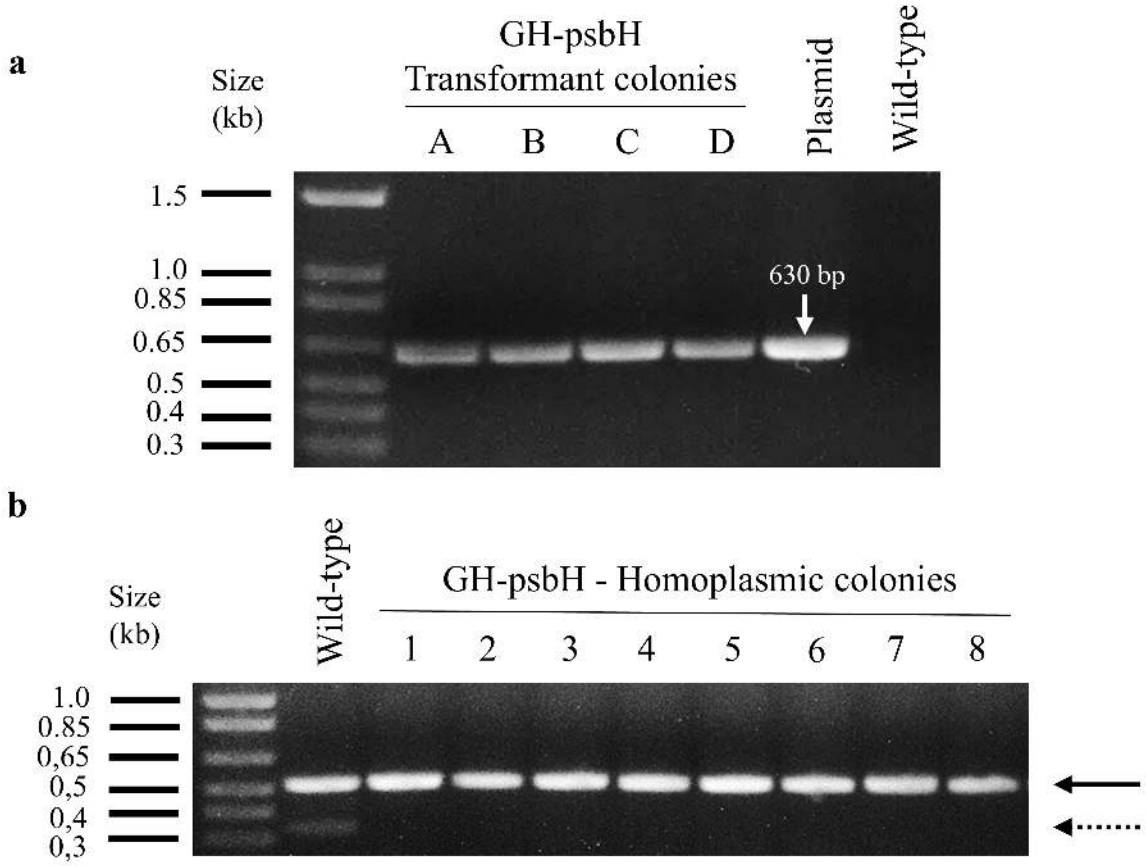
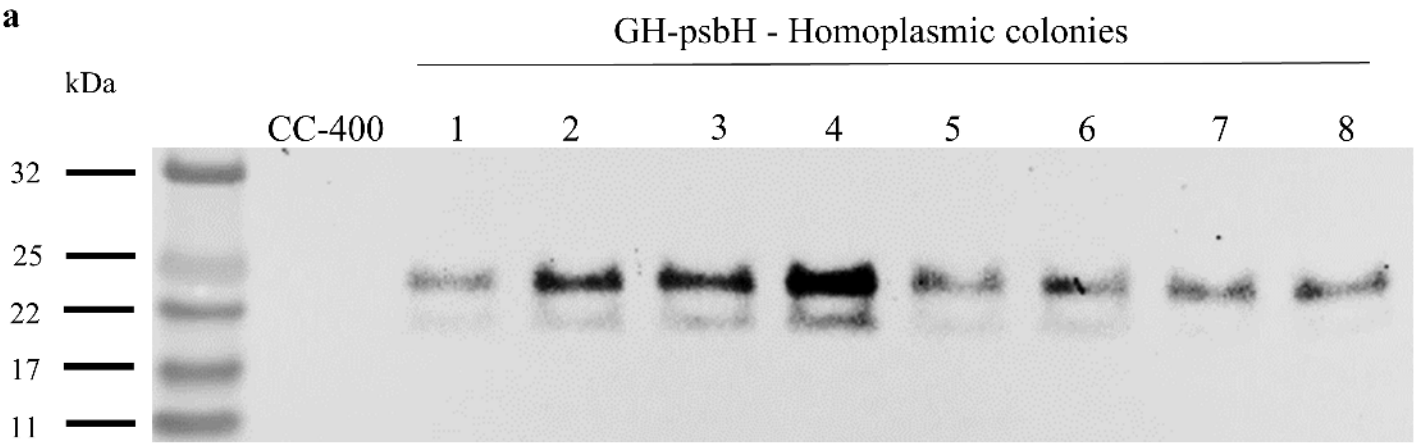


Figure 2

Analysis of genetically transformed colonies. (a) PCR of gene-positive colonies transformed with the GH-psbH expression vector. (b) PCR for evaluating homoplasmy in colonies transformed with the GH-psbH vector. The solid arrow indicates amplification of the 16S RNAr gene sequence, while the dotted arrow indicates amplification of the unmodified genomic psbH region

a



b

Colonies (lane)	Mol. Wt. (kDa)	Rel. Quant.	Total Soluble protein (g/L)	Standard deviation (g/L)
1	23,88	1,00	0,22	±0,02
2	23,88	2,61	0,21	±0,04
3	23,78	2,89	0,22	±0,02
4	23,78	4,53	0,20	±0,01
5	23,78	1,51	0,20	±0,03
6	23,56	1,65	0,22	±0,02
7	23,19	1,41	0,23	±0,03
8	23,24	1,58	0,21	±0,03

Figure 3

Evaluation of recombinant protein synthesis in *Chlamydomonas reinhardtii* homoplasmic colonies. (a) Western blotting of GH-psbH colonies, using the primary antibody Anti-FLAG IgG produced in rabbit and the secondary antibody goat anti-rabbit IgG conjugated with HRP, at ratios of 1:5000 and 1:10000, respectively. (b) Table of observed molecular weight data, total soluble proteins, and relative quantification. Image recording and analysis were performed using Bio-Rad's Image Lab software

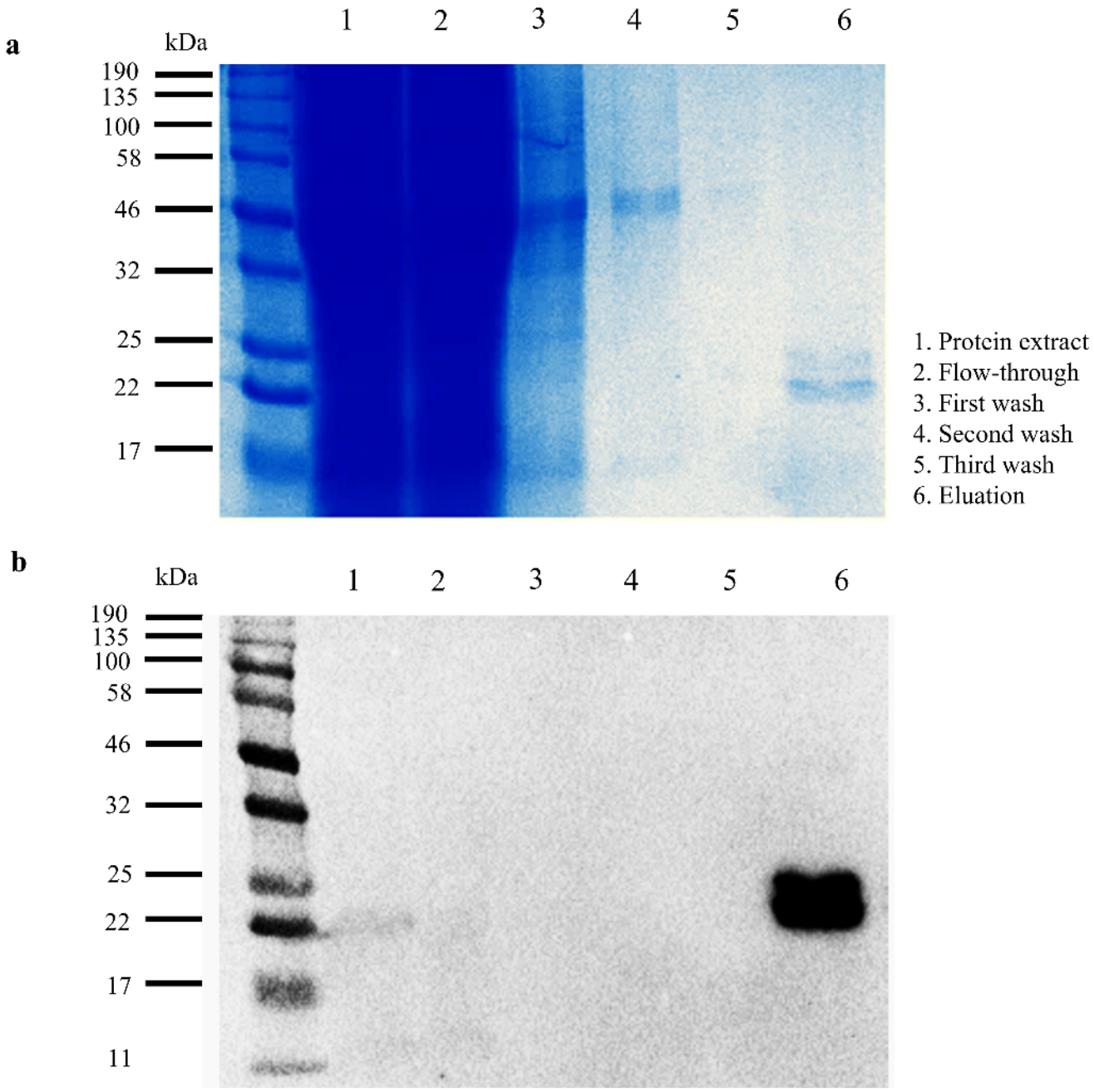


Figure 4

Purification analysis by affinity chromatography. (a) SDS-PAGE with samples from each stage of the purification process. (b) Western blotting of samples from the purification process. The primary and secondary antibodies were applied at ratios of 1:5000 and 1:10000, respectively. A total of 20 µl of sample was added to each well