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A new approach to express transgenes in microalgae and its use to increase the flocculation ability of *Chlamydomonas reinhardtii*

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Abstract The choice of strong efficient promoters is a critical step in the development of efficient transformation systems for microalgae; however, the physiological and genetic diversity among microalgae groups makes very difficult to develop standard universal plasmids for a wide number of microalgal species as has been achieved for higher plants. Here, we propose a new approach to express transgenes in microalgae: cotransformation with two naked promoterless genes, a selectable antibiotic-resistant gene and a gene of our interest. These genes are randomly inserted into the nuclear genome, where their transcription relies on their adequate insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene. In a high percentage of the transformants obtained, both genes are, not only adequately incorporated in the nuclear genome, but also efficiently transcribed and translated. This transformation method is validated in the model microalga *Chlamydomonas reinhardtii* with the bleomycin-resistant gene from *Streptoalloteichus hindustanus* (*ShBLE*) as gene of interest, and it is employed to express a flocculin gene from *Saccharomyces bayanus* (*SbFLO5*), which is responsible for the flocculation process in yeasts. *Chlamydomonas reinhardtii* transformants exhibited self-flocculation abilities between 2- and 3.5-fold higher than the control untransformed strain. The successful cotransformation of *C. reinhardtii* with two promoterless genes opens doors for the establishment of a universal transformation system based on endogenous promoters, applicable to any microalgal species.

Keywords *APHVIII* · *Chlamydomonas reinhardtii* · Cotransformation · *FLO5* · Genetic transformation · Promoterless gene · Flocculation

Introduction

Microalgae are a heterogeneous group of photosynthetic microorganisms with high ecological importance and an enormous biotechnological potential (Enzing et al. 2014). The use of microalgae for the commercial production of carotenoids, polyunsaturated fatty acids (PUFAs), or other high added-value compounds is well established (Borowitzka 2013; Scaife et al. 2015), and in the last years, there has been an increasing interest in microalgae as a feedstock for the production of biofuels (Wijffels and Barbosa 2010; Vanthoor-Koopmans et al. 2013; Benemann 2013). This has made to increase the attention on genetic engineering of microalgae as a potential tool to aim the economically feasible production of bulk materials and to enhance the productivity of the high added-value compounds (León and Fernández 2007; Georgianna and Mayfield 2012; Scranton et al. 2015).

Genetic engineering represents, according to many researchers, the most promising strategy for the improvement of microalgae (Lee et al. 2008; Radakovits et al. 2010; Larkum et al. 2012; Scaife et al. 2015), but until recently, routine genetic manipulation has been limited to a few species (i.e., the classical model microalgae: *Chlamydomonas*

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reinhardtii, *Volvox cateri*, and *Phaeodactylum tricornutum*). The lack of strong promoters and other regulatory sequences is, besides low efficiency of expression and unstability of transgenes, the main difficulty found to aim the genetic transformation of new microalgal strains (León-Bañares et al. 2004; Jinkerson and Jonikas 2015).

Many attempts to optimize the genetic transformation of new microalgae species have been based on heterologous promoters typically used in higher plants, such as the 35S promoter from the cauliflower mosaic virus (*CaMV35S*p) or the nopaline synthase promoter (*NOS*p) from *Agrobacterium tumefaciens*. In many cases, typical plant plasmids, such as pBI121 (Talebi et al. 2013), pBI221 (Tan et al. 2005), or those from pCAMBIA series (Kathirsesan et al. 2009; Guo et al. 2013; Úbeda-Mínguez et al. 2015) have been used, directly or with minor modifications, for transformation of microalgae. These heterologous promoters have the enormous advantage of being universal. They can, in principle, be used for genetic transformation of any microalgal species, but transformation efficiencies are generally low, and the transgenes are usually unstable. The best transformation efficiencies and the most stable transformants are obtained with endogenous promoters. Examples of this are the successful stable transformation of *Nannochloropsis* sp. (Kilian et al. 2011) or *Nannochloropsis gaditana* (Radakovits et al. 2012), achieved using violaxanthin and β-tubulin endogenous promoters, respectively; the transformation of *Haematococcus pluvialis* submitted to particle bombardment with a modified version of phytoene desaturase (*PDS*) that confers resistance to the herbicide norflurazon directed by its own *PDS* promoter (Steinbrenner and Sandmann 2006) or the genetic transformation of *Ostreococcus* with the luciferase reporter gene under the control of several endogenous promoters (Corellou et al. 2009). But, finding strong efficient promoters for each new species to be transformed is not an easy task.

We propose a new approach to express transgenes in microalgae, the random non-homologous integration of nacked promoterless genes in their nuclear genome. In this approach, transgenes are randomly inserted into a position of the genome where their transcription relies on its adequate insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene. The expression of promoterless marker genes has been previously reported both in higher plants (Yamamoto et al. 2003) and in microalgae (Sizova et al. 2001; Díaz-Santos et al. 2013), and we have demonstrated its usefulness for the selection of strong promoters through promoter trapping (Vila et al. 2012). The originality of the approach that we present here is the cotransformation of microalgae with two promoterless genes: a selectable antibiotic-resistant gene and a gene of our interest. Interestingly, we have observed that in a high percentage of the transformants obtained, both the antibiotic-resistant and the gene of interest are not only adequately incorporated in the nuclear genome but also transcribed and translated.

Furthermore, we have successfully used this approach to express a flocculent gene from *Saccharomyces bayanus* in *C. reinhardtii* and obtain transformants with higher autoflocculation ability. Self-flocculation of microorganisms is a biological process in which cells are spontaneously aggregated to form large flocs. This process has been thoroughly studied in brewing or wine fermentative yeasts (Govender et al. 2008; Garcia Sánchez et al. 2012), and it is very important from the engineering point of view if we consider that microalgal harvesting can be 30 % of the total cost of algal biomass production (Salim et al. 2012).

Material and methods

Chlamydomonas reinhardtii partially cell wall-deficient strain 704 (*cw15*, *arg7*, mt+) was kindly provided by Dr. Roland Loppes (Loppes et al. 1999) and cultured photomixotrophically in liquid or agar-solidified Tris-acetate-phosphate (TAP) medium (Harris 2009) at 25 °C under continuous white light irradiation of 100 μmol photons m^{-2} s^{-1}. The *Escherichia coli* strain used for in vivo amplification of DNA was DH5α, cultured in LB medium as previously described (Sambrook and Russell 2001). *Saccharomyces bayanus* var. *uvarum* CECT 1969 was kindly supplied by the Department of Genetic (University of Seville) and cultured in yeast extract peptone dextrose (YPD) medium containing the following components: peptone (20 g L^{-1}), yeast extract (10 g L^{-1}), and glucose (20 g L^{-1}) dissolved in 1 L of demineralized water, at 28 °C, pH 4.5 and aerated by shaking at 150 rpm. For solid medium, agar at 1 % was added.

Isolation of genomic DNA The extraction of genomic DNA from *S. bayanus* was performed following the protocol described by Hoffman and Winston (1987) with minor modifications. Algal genomic DNA was isolated as previously reported (León et al. 2007). For screening of transformants, algal genomic DNA was isolated by the GeneJET genomic DNA purification kit from Life Technologies.

RNA extraction from *C. reinhardtii* and reverse transcription Isolation of total RNA was performed with the RNAeasy plant MiniKit of Qiagen according to the instructions of the manufacturer. Single-strand complementary DNA (cDNA) was synthesized from total RNA according to the SuperScript II RNase H-reverse transcriptase manual (Invitrogen) and used as template for real-time PCR reactions.

Nuclear transformation of *C. reinhardtii* Transformation was carried out using the glass bead method of Kindle (Kindle 1990), with minor modifications. *Chlamydomonas reinhardtii* cells were grown until the middle of the exponential phase of growth (about 1.6×10^6 cells mL^{-1}), harvested by centrifugation, and resuspended in fresh TAP medium to obtain a 100-fold concentrated cell suspension. The concentrated cell suspension (0.6 mL) was added to a conical tube containing 0.3 g of sterile glass beads (0.4–0.6 mm diameter), 0.2 mL of 20 % polyethylene glycol (MW8000), and the indicated quantities of the chosen promoterless genes or plasmids. Cells were vortexed for 8 s and resuspended in 50 mL of fresh sterile TAP medium. After 16 h of incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP solid medium plates with paromomycin (30 μg mL^{-1}). Transformed colonies were visible after 4 or 5 days.

Polymerase chain reaction Standard PCR amplification was performed from 1 μL of template DNA in a total volume of 25 μL containing 10 pmol of each primer, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 2.5 μL of specific 10 × buffer (containing 2.5 mM $MgCl_2$), and 1 % dimethylsulfoxide (DMSO). The PCR program was 0.5 min at 96 °C, 0.5 min at annealing temperature, and 1.5 min at 72 °C for 30 cycles.

Preparative PCR was performed scaling up standard PCR to 2.4 mL. The mixture was then distributed in 96 wells of a PCR plate, which were simultaneously amplified in a standard thermocycler. The content of all the wells was pulled up, and after electrophoretic analysis of a 10 μL aliquot to confirm right amplification, the DNA was extracted with phenol/chloroform and precipitated with ethanol. The obtained DNA was resuspended and further purified to remove dNTPs and primers present in the reaction by pipetting the sample with the Diffinity Rapid tips from SIGMA. The resulting DNA was quantified by UV spectroscopy, using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific).

Quantitative real-time PCR and analysis of the transcript level Real-time PCR was performed on a Mx3000P Multiplex Quantitative PCR System from Stratagene using the Brilliant SYBR Green QPCR Master Mix (Stratagene). Each determination was carried out in triplicate using as template the cDNA synthesized from total RNA using the SuperScript II RNAse H-reverse transcriptase, as previously described, and 10 pmoles of the indicated primers in a final volume of 20 μL. Cycling conditions were 10 min at 95 °C for activation of the hot start Taq polymerase and 40 cycles for the melting (30 s at 95 °C), annealing (30 s at 60 °C), and extension (30 s at 72 °C). The fluorescence measurement was made at the end of the annealing step. A dissociation curve (30 s at 95 °C, 30 s at 55 °C, and 30 s at 95 °C) was applied at the end of the amplification reaction. The ubiquitin ligase (Phytozome v10.2: cre03.g159200.t1.1) was used as housekeeping gene control. Expression of this gene was previously shown to be constitutive under the different conditions used (Vila et al. 2008). Primers UBQLFOR and UBQLREV amplify a 161 bp fragment. The forward primer annealing region is in the joint between two contiguous exons, so genomic DNA amplification is avoided.

Self-flocculation assays Flocculation experiments were run in small cylindrical glass tubes (20 mL) with a total final volume of 10 mL. The initial optical density of the microalgal cultures was determined by measuring at 660 nm and their volume adjusted with fresh culture medium to obtain the same cell density, with an absorbance value around 1, in all tubes. The *C. reinhardtii* cultures were left without agitation during the settling period. To follow the kinetic of sedimentation and calculate the self-flocculation percentage for each transformant and for the wild type, small culture aliquots were withdrawn from the top part of the tube and optical density at 660 nm (OD_{660}) was measured in a spectrophotometer (Ultrospec 3100 pro) at 0, 15, 45, 90, 180, and 360 min after starting the experiment. Each flocculation assay was run in triplicate (n=3). The self-flocculation percentage was calculated according to Eq. 1, adapted from Alam et al. (2014):

$$Self\text{-}flocculation\ (\%) = \left(1-\frac{A}{B}\right) \times 100 \qquad (1)$$

where A is the OD_{660} taken at each sampling time and B is the OD_{660} taken at time zero.

Results

Cotransformation of *C. reinhardtii* with two promoterless genes

The efficiency of the simultaneous coinsertion of two promoterless genes in the genome of *C. reinhardtii* is studied using as selectable marker gene the aminoglycoside-3-phosphotransferase encoding gene (*APHVIII*) from *Streptomyces rimosus*, which confers resistance to paromomycin (Sizova et al. 2001), and the *BLE* gene, which encodes the bleomycin-binding protein from *Streptoalloteichus hindustanus* (*ShBLE*), as gene of interest (GOI). The bleomycin-binding protein inhibits in vitro DNA cleavage by the antibiotic bleomycin. Both promoterless genes were obtained by preparative PCR, as is detailed in the "Material and methods" section, using *APHVIII* and *BLE* specific primers (Table 1). *Chlamydomonas reinhardtii* was transformed using different *BLE/APHVIII* ratios. The quantity of the selectable marker gene (*APHVIII*) was

Table 1 Oligonucleotides used in this work

Primer	Sequence	*T* (°C)	Use and amplicon size
BLEFOR BLEREV	TTTACAAGAGGATCCACTCA ACATCTT GAGCTCGTCGAC GTCGGTTAGTCCTG	50 59	*BLE* amplification (726 bp)
APHVIIIF APHVIIIR	CGCCCTCCCCGGATCCGAA GAAACCCACGAGCTCCAACCC TACCC	57 57	*APHVIII* amplification (917)
BLECORFOR BLECORTREV	CGACTTCGCCGGTGTGGTC C ACGAAGTGCACGCAGTTGC	58 57	*BLE* detection (400 bp on genomic; 250 on cDNA)
PARACORFOR PARACORREV	GAGGATCTGGACGAGGAGCG GAA CCCTCAGAAGAACTC GTCCAACAGC	62 61	*APHVIII* detection (359 bp)
FLO5FOR FLO5REV	GATTAGCACCACTAAAAAAA ATGACAA CTGCAGCAAAG AAAAGATACACAGATAC	54 52	*FLO5* amplification and detection (3026 bp)
UBQLFOR UBQLREV	GTACAGCGGCGGCTAGAGGCA C AGCGTCAGCGGCGGTT GCAGGTATCT	60 60	*UBQL detection* (*161 bp on cDNA*)

maintained at 1 μg, while the quantities of the *BLE* gene ranged between 1 and 10 μg. The number of putative transformants per reaction was quantified after 5–7 days (Table 2). The transformation efficiency when *C. reinhardtii* is transformed with the plasmid pSI103 (Sizova et al. 2001), where the *APHVIII* gene is under the control of the strong tandem chimeric *HSP70A/RBCS2* promoter, has also been included as a positive control. The mean transformation efficiency for transformations carried out with the promoterless *APHVIII* gene is about 8-fold lower than the transformation efficiency obtained when the selectable marker gene is under the control of the *HSP70A/RBCS2* promoter (Table 2).

Thirty paromomycin-resistant transformants (10 for each of the *BLE/APHVIII* gene proportions) were randomly selected and cultured in TAP medium with paromomycin (15 μg mL^{-1}). Genomic DNA was isolated from each of the selected transformant, and the integration of *BLE* and *APHVIII* genes was checked by PCR (Fig. 1). We observed that for a *BLE/APHVIII* ratio of 10, all the transformants that incorporated the selectable marker gene also incorporated the GOI in their genomes. When the genes' ratio decreased to 3:1 or to 1:1, the number of *BLE* positive transformants decreased to 70 and 40 %, respectively (Table 2).

Table 2 Efficiency of cotransformation with two promoterless genes

Selectable marker (*APHVIII*, μg)	Gene of interest (*BLE*, μg)	Transformantion frequency	*Ble* positive (%)
1 (pSI103)	–	140±10 (*n*=5)	–
1	10	8±2 (*n*=3)	100
1	3	14±3.6 (*n*=3)	80
1	1	18±6.5 (*n*=3)	40

C. reinhardtii was transformed with different ratios of *BLE* and *APHVII* genes and selected in the presence of paromomycin. The number of colonies obtained per transformation reaction is the average of three independent transformation experiments. Mean and standard deviation values are shown. The efficiency of the transformation with the plasmid pSI103 in which the *APHVIII* gene is under the control of the tandem *HSP70A/RBCS2* promoter has been included for comparison. The presence of the *APHVIII* and the *BLE* genes in the nuclear genome of *C. reinhardtii* was checked by PCR, and the percentage of transformants that showed *BLE* insertion was designed as *Ble* positive

The Ble^R phenotype of the *BLE/APHVIII* cotransformants was investigated. Thirty paromomycin-resistant *C. reinhardtii* transformants obtained by cotransformation with the *BLE/APHVIII* promoterless genes, which had previously shown to have both genes correctly integrated in their genomes, were inoculated in TAP liquid medium with paromomycin (15 μg mL^{-1}) and grown for about 5 days. The biomass in each tube was evaluated by measuring the absorbance at 660 nm and their volume adjusted with fresh sterile TAP medium to obtain the same biomass concentration in all the tubes. TAP-agar plates with paromomycin (30 μg mL^{-1}) and bleomycin (10 μg mL^{-1}) were spotted in an ordered array with 10 μL drops from each tube and grown for several days (Fig. 2). After 7 days, 36 % of the transformants were able to grow vigorously in the presence of the antibiotic bleomycin, showing that the *BLE* gene is not only inserted in the genome of *C. reinhardtii* but also efficiently expressed in many of the cases. These cotransformation rates are similar to those described for cotransformation with independent genes preceded by promoters (Christou and Swain 1990)

Two of the Pm^R Ble^R transformants (1-2 and 1-4) were further investigated to check the stability of their phenotypes over a period of 6 months. Both transformants were kept in two different selective medium: TAP-agar plates with

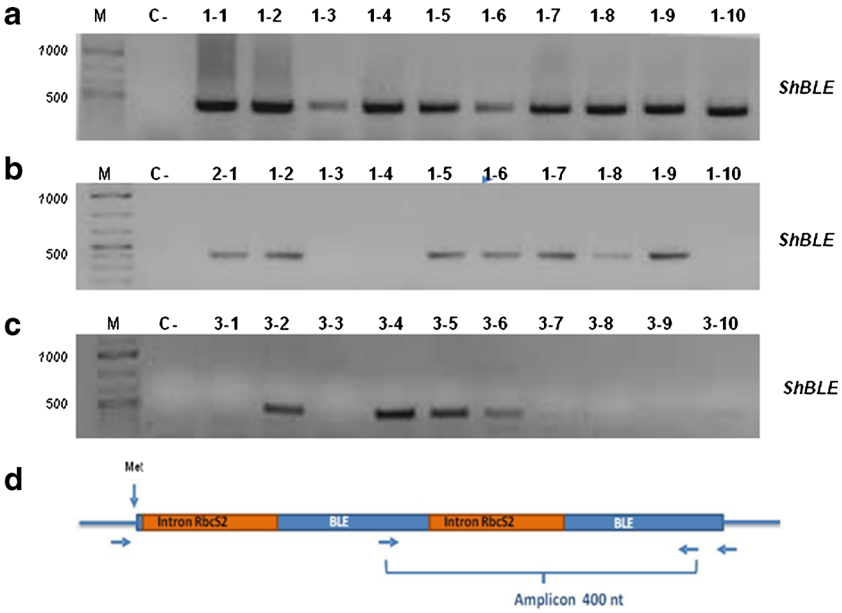

Fig. 1 PCR detection of *ShBLE* gene in *C. reinhardtii* transformants. Genomic DNA from several *C. reinhardtii* transformants obtained by cotransformation with *BLE/APHVIII* naked promoterless genes was subjected to PCR using primer specific for a 400 bp fragment from the *ShBLE* gene. The transformants shown were obtained with *BLE/APHVIII* ratios of 10:1 (**a**), 3:1 (**b**), and 1:1 (**c**). *C−* stands for the negative control without template. *M lane* is the molecular-weight size marker. **d** Schematic representation of the *BLE* gene, indicating the PCR primers used for its cloning and detection

paromomycin (30 μg mL^{-1}) and TAP-agar plates with bleomycin (10 μg mL^{-1}). Every month, both clones of both transformants were inoculated in TAP liquid medium until a OD_{660} of 1, and 10 μL drops of these cultures were placed on TAP-agar plates with bleomycin (10 μg mL^{-1}) + paromomycin (30 μg mL^{-1}). Both, Pm^R and Ble^R, phenotypes were stable in the two transformants for all the period studied, even when maintained without the corresponding antibiotic selective pressure (Fig. 3). These transformants were also subjected to real-time PCR to evaluate the *BLE* transcript level, which was of the same order of magnitude than that of *C. reinhardtii* transformants obtained with pSP124S plasmid (Stevens et al. 1996), in which the *BLE* gene is under the control of the strong quimeric promoter *HSP70A/RBCS2*.

Expression of the promoterless *FLO5* gene from *S. bayanus* by cotransformation in *C. reinhardtii*

We have demonstrated that cotransformation with two promoterless genes is a valid approach for the expression of foreign genes in microalgae. This is especially interesting for microalgal species for which no specific plasmid or promoter regions have been isolated, and it is also useful for genes difficult to subclone due to their long sizes or to instability or toxicity of their products to bacteria. The *FLO* genes from *S. bayanus*, which encode for specific cell surface lectin-like glycoproteins called flocculins, are long and have a large number of tandem repeats in their central domain, which makes their cloning

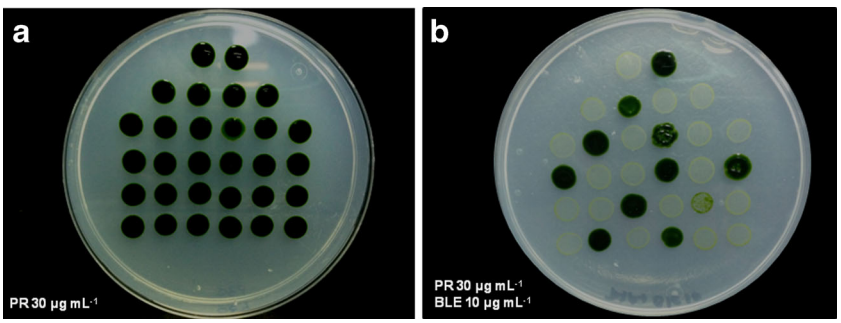

Fig. 2 Ble^R phenotype in *C. reinhardtii BLE/APHVIII* cotransformants. *Chlamydomonas reinhardtii BLE/APHVIII* cotransformants with both promoterless genes correctly integrated in their genomes were cultured in TAP liquid medium with paromomycin (15 μg mL^{-1}) and grown for about 5 days. After normalizing the biomass in each tube by addition of fresh sterile TAP medium, TAP-agar plates with paromomycin (30 μg mL^{-1}) (**a**) and bleomycin (10 μg mL^{-1}) + paromomycin (30 μg mL^{-1}) (**b**) were spotted in an ordered array with 10 μL drops from each tube and grown for several days. After 7 days, the number of viable transformants was evaluated. The 36 % of the studied transformants were able to grow vigorously in the presence of both antibiotics, showing that the *BLE* gene is not only inserted in the genome of *C. reinhardtii* but also efficiently expressed

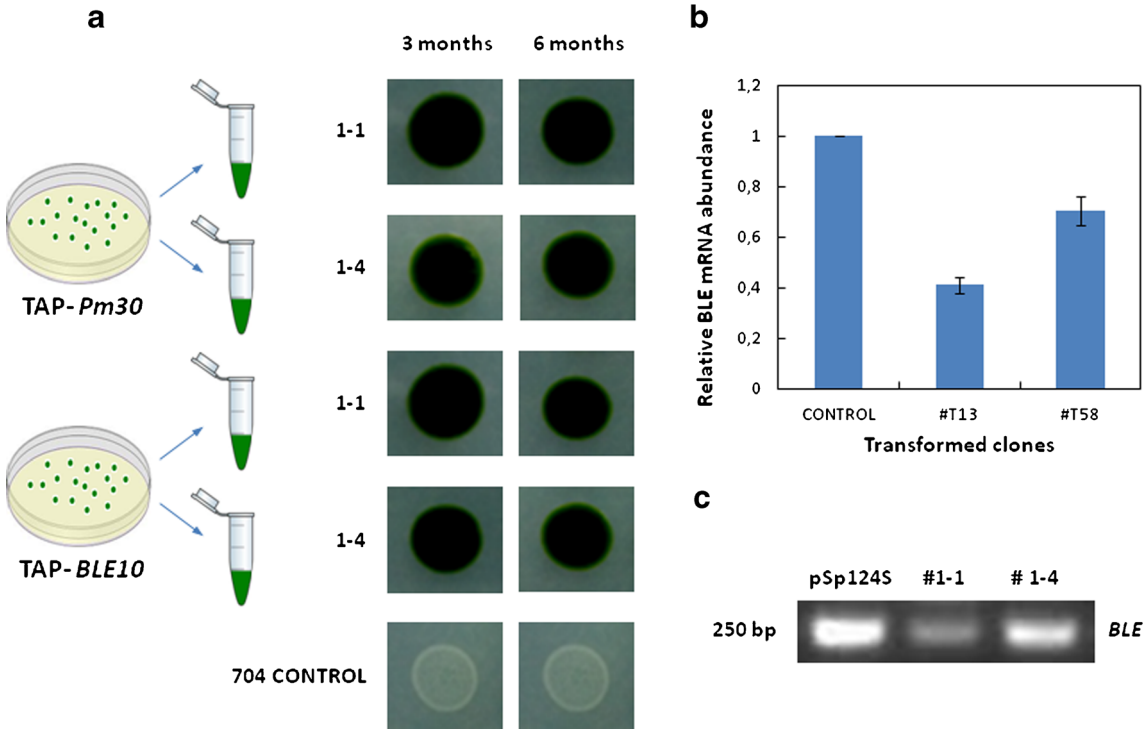

Fig. 3 Evaluation of the phenotypic stability of the transformants. Two of the Pm^R Ble^R transformants (1-2 and 1-4) were maintained for a long period in TAP-agar plates with paromomycin or bleomycin and were periodically tested for their antibiotic resistance phenotype by spotting 10 μL drops of each transformant in TAP-agar plates supplemented with both paromomycin (30 μg mL^{-1}) and bleomycin (10 μg mL^{-1}) (**a**). The *BLE* transcript level of the same transformants was evaluated by real-time PCR (**b**) and end-point PCR (**c**) 6 months after the transformation and compared with the *BLE* transcript level in a transformant obtained with the pSP124S plasmid

and propagation in bacteria a difficult task (Goossens and Willaert 2010). Some flocculins have shown to play a critical role in floc-forming ability of yeast (Govender et al. 2008), and we have recently demonstrated that the addition of the flocculant yeast *S. bayanus* to cultures of *C. reinhardtii* induces their flocculation (Díaz-Santos et al. 2015). Therefore, we propose the expression of *FLO* genes from *S. bayanus* in *C. reinhardtii* as an approach to obtain autoflocculating *C. reinhardtii* transformants.

FLO5 is one of the smallest functional flocculin proteins, and the genomic gene which encodes this flocculin lacks introns, allowing its easy isolation from the yeast genome. It was isolated by PCR, using the genomic DNA from the yeast *S. bayanus* var. uvarum as template and a high fidelity polymerase. Three micrograms of the *FLO5* gene and 1 μg of the *APHVIII* gene, both obtained by preparative PCR as is detailed in "Materials and methods" section, were used for cotransformation of *C. reinhardtii*. An average of 20 paromomycine-resistant transformants per experiment were obtained, which means an efficiency of 2×10^{-7} transformants $cell^{-1}$ μg^{-1} DNA. Twenty of these transformants were further investigated for insertion of the *FLO5* gene. Genomic DNA from each ot the selected transformants was isloted and used as template for PCR analysis with *APHVIII* and *FLO5* specific primers (Table 1). In all the transformants studied, a 360 bp fragment corresponding to the *APHVIII* gene was found, and 25 % of the cotransformants showed the 3026 bp band corresponding to the *FLO5* gene (Fig. 4). The identity of the amplified *FLO5* product was further confirmed by sequencing of the PCR product.

The five cotransformants which had incorporated both genes in their genomes were subjected to expression analysis. The transformants were cultured in TAP medium; total RNA was isolated, and single-strand cDNA was synthesized from the total RNA and used as template for PCR. Electrophoresis showed the presence of the expected fragment corresponding to the *APHVIII* gene in all the transformants analyzed and the presence of the expected *FLO5* gene in three of them (Fig. 5). These data confirmed that a high number of the transformants with the *FLO5* gene integrated in their genome were able to efficiently express it, thanks to the own endogenous regulatory regions of the microalga. In this case, coexpression is higher than that observed for cotransformation with *BLE/APHVIII* genes. The mechanisms of action of bleomycin-binding protein, which interacts with the antibiotic and cancels its effect by sequestering it and not by a catalytic degradation (Lumbreras et al. 1998), make that only transformants with high levels of bleomycin-binding protein can survive in the presence of the antibiotic.

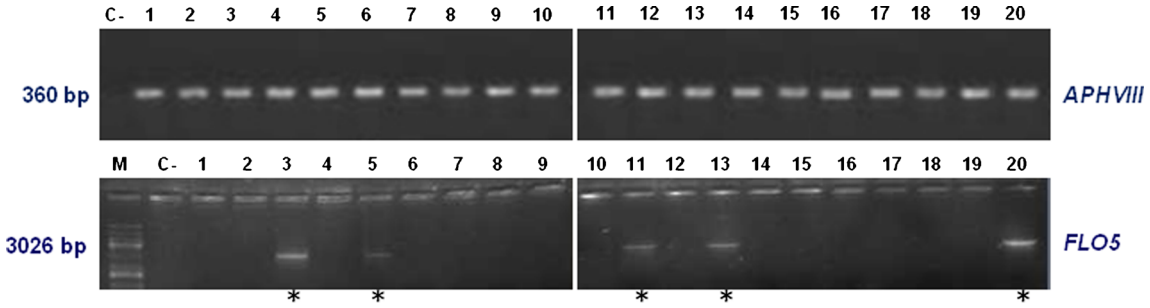

Fig. 4 PCR analysis of genomic DNA from *APHVIII/FLO5* cotransformants using *APHVIII* (**a**) and *FLO5* (**b**) specific primers. In *lanes 3*, *5*, *11*, *13*, and *20*, a 3026 band corresponding to the *FLO5* gene can be seen. *C−* is the negative control without template DNA. *M* is the molecular-weight size marker. *Asterisks* indicate the *FLO5* positive transformants

Self-flocculation assays of *C. reinhardtii* CrFLO511, CrFLO513, and CrFLO520 transformants

The selected *C. reinhardtii* cotransformants, CrFLO511, CrFLO513, and CrFLO520, were subjected to a self-flocculation activity test, as is described in the "Material and methods" section. The kinetics of sedimentation was followed by measuring the optical density at 660 nm in the top part of the tubes, and the self-flocculation percentage was calculated according to Eq. 1. While wild untransformed *C. reinhardtii* exhibited very low ability of spontaneous flocculation, less than 15 % after 360 min, CrFLO511, CrFLO513, and CrFLO520 showed self-flocculation percentages between 36 and 52 % at the same settling period (Fig. 6), indicating an enhancement of the self-flocculation ability between 2- and 3.5-fold in relation with the wild type and confirming that the insertion of the *FLO5* gene into the genome of these *C. reinhardtii* cotransformants generates self-flocculation phenotypes.

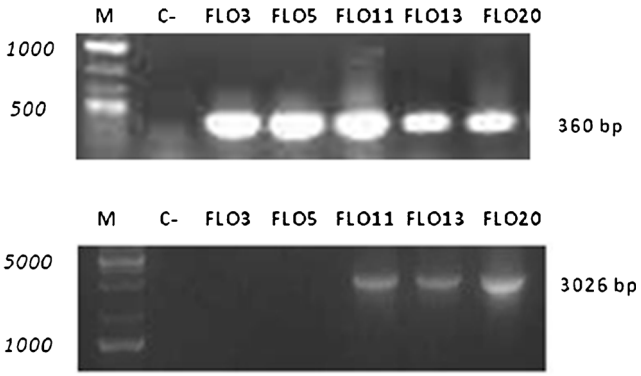

Fig. 5 Analysis of *FLO5* transcription in the selected *APHVIII/FLO5* cotransformants. mRNA obtained from the five clones that had shown to contain the *FLO5* gene in their genomes was reverse transcribed and products amplified by PCR using *FLO5* specific primers. *C−* is the negative control without template DNA. *M* is the molecular-weight size marker

Discussion

Cotransformation with two naked promoterless genes allows the successful integration in the nuclear genome and the efficient expression of both genes at high frequency

The fact that naked promoterless genes were integrated in nuclear genome and efficiently expressed at high frequency provides a new approach for the robust and stable expression of transgenes in microalgae with many advantages: Transgenes are expressed under the control of endogenous promoters, reducing the risk of silencing events; transgenes are integrated in the genomic environment of the promoter, which guarantees the presence of enhancers or other cis- or trans-regulatory sequences, and the method can be applied to any microalgal species, even those for which no endogenous promoters or specific expression plasmids have been designed, without the need of subcloning.

This new cotransformation approach has also some drawbacks, such as the need of high quantities of the DNA insert, the low efficiency of the transformation, or the possibility of collateral undesirable effects as a consequence of multiple gene insertion in many sites of the genome. Screening is essential to select those transformants able to express higher percentages of the protein encoded by the gene of interest. This screening could seem to be a handicap of this transformation approach, but in fact, it is an inevitable step in transformation of microalgae, since large variability of expression has been observed among transformants obtained in the same transformation events, even for transformants obtained with traditional plasmids, which contain strong promoters.

Although initially, the probability that a second promoterless gene is correctly genome-integrated and efficiently expressed would seem to be very low, we have demonstrated here that coexpression events happen at high frequency. A detailed analysis of *Chlamydomonas* genome can help us to understand the base of the cotransformation with two promoterless genes. The nuclear genome of *C. reinhardtii* comprises approximately 111 megabase arranged in 17

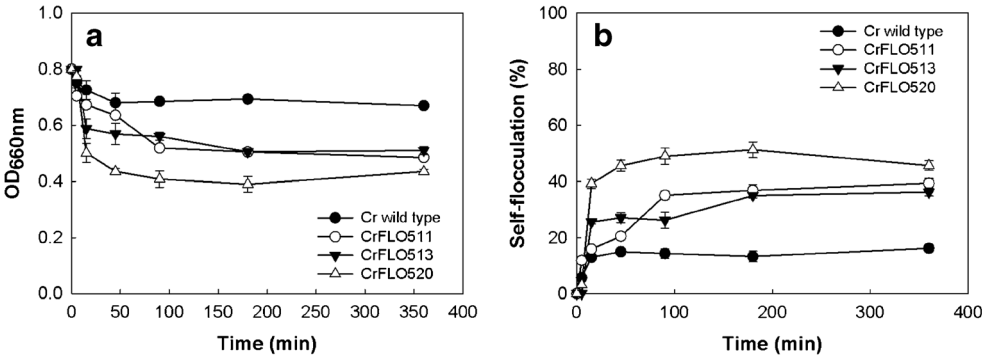

Fig. 6 Analysis of the sedimentation kinetic (**a**) and the self-flocculation percentage (**b**) in the three selected *APHVIII/FLO5 Chlamydomonas reinhardtii* cotransformants and in the control untransformed strain

linkage groups with 17,728 protein-encoding loci (Merchant et al. 2007). If we estimate an average number of 1768 protein-encoding nucleotides per gene, we can conclude that more than 31.3×10^6 bases of *C. reinhardtii* genome (17,737 genes×1768 nt=31.3×10^6 bases) are coding nucleotides. This means that 23.8 % (almost one fourth) of the nuclear genome of *C. reinhardtii* ($31.3\times10^6/111\times10^6=0.28$) are coding nucleotides.

Considering that insertion of our gene can take place in any of the three different reading frames and in two possible orientations and only one of them is adequate for gene expression, about 1 of each 24 insertions would allow the adequate expression of the foreign gene of our interest (Eq. 2). This means that, in theory, if more than 24 copies of a gene were inserted in the genome of the microalga, the transcription of this gene would be 100 % ensured. In practice, there will be a number of insertions that will cause lethal insertional mutations or unfunctional fusion proteins, so the real percentages will be not so optimistic but will be certainly high.

$$probability = protein\ encoding\ nucleotides \times 3\ ORF \times 2\ orientations = \frac{1}{4} \times \frac{1}{3} \times \frac{1}{2} = \frac{1}{24} \quad (2)$$

The successful cotransformation of *C. reinhardtii* with two promoterless genes opens doors for the establishment of a universal transformation system based of endogenous promoters applicable to any microalgal species; in fact, this approach could be used to any species. The higher the percentage of coding nucleotides in a species is, the easier it will be to express promoterless genes in it. In Table 3, statistic data of the genomes of different organisms are shown. Most microalgae, as well and some higher plants, have coding densities similar to those of *C. reinhardtii*. In organisms with highly compacted genomes, such as the marine picoeukaryote microalga, *Ostreococcus tauri*, with very low percentage of introns and short genes, or the model yeast *Saccharomyces cerevisiae*, the protein-encoding nucleotides represent around 50 % of the genome. The large size of the genomes of superior eukaryotes makes that the percentage of protein-encoding nucleotides decrease until values of 10 and 1 % in *Mus musculus* and *Homo sapiens*, respectively (Table 4).

This finding about the expression of two independent promoterless genes makes us to think on the possibility that many of the cases described in the literature in which heterologous promoters are directing the expression of marker genes in microalgae could be misinterpreted. Furthermore, reviewing the literature, we can find many examples of microalgal transformations with plasmids containing the *NOS* or *CaMV35S* promoters in which very large quantities of DNA are used. A critical reflection on those results, in the view of the ability of promoterless genes to be randomly expressed under the control of endogenous promoters, leads us to believe that the effective promoter role of those heterologous promotes is demonstrated.

Expression of the yeast flocculin encoding gene, *FLO5*, induces self-flocculation phenotypes in the non-flocculating microalga *C. reinhardtii* In the present work, genome integration and expression at mRNA level of the *FLO5* gene in three of the *C. reinhardtii* transformants

Table 3 Statistics of *Chlamydomonas reinhardtii* genome

	Calculations	Nucleotides
Nuclear genome size		111×10^6
Protein-encoding loci		17,737
Average number of nucleotides per gene		3895
Average length of transcripts		1768
Average length of proteins		497
Protein-encoding nucleotides	$17737\times497\times3$	31.3×10^6
	Calculations	Percentage
Percentage of coding nucleotides	$(31\times10^6\times100)/111\times10^6$	23.8 %

Estimation of the percentage of protein-encoding nucleotides in *C. reinhardtii* nuclear genome. Data obtained from *C. reinhardtii genome assembly (v 5.5)* in the JGI Comparative Plant Genomics Portal phytozome *10.2* (http://phytozome.jgi.doe.gov)

Table 4 Estimation of the percentage of protein-encoding nucleotides in different species

Species	Genome size (Mb)	Chrom.	Protein-encoding loci	Average gene length	Average protein length	Protein-encoding nucleotides (%)	Reference
Microalgae							
C. reinhardtii	111	17	17,748	3895	497	23.8	Merchant et al. 2007
N. gaditana	29		9052	2200	393	36.8	Radakovits et al. 2012
C.subellipsoidea	49	16	9627	3250	427	25.2	Blanc et al. 2012
V. carteri	131.2	14	14,971	5300	558	25.1	Prochnik et al. 2010
O. tauri	12.6	20	7892	1000	260	48.8	Derelle et al. 2006
Other organisms							
S. cerevisiae	12.5	16	5770	2000	379	52.5	Goffeau et al. 1996
A.thaliana	135	5	35,386	2011	447	35.1	Lamesch et al. 2012
M. musculus	280	20	23,000	–	416	10.25	Pruess et al. 2003
H. sapiens	3200	23	35,000	–	469	1.53	Pruess et al. 2003

The formula used is protein-encoding nucleotides (%)=(*N* genes×ave prot size×3)×100/genome size (pb). Information for this table has been obtained from the statistic section of the phytozome genome project (http://www.phytozome.net), from the NCBI (http://www.ncbi.nlm.nih.gov), and from the indicated references

obtained by cotransformation with *APHVIII* and *FLO5* promoterless genes has been proven (Figs. 4 and 5). And, the self-flocculation activity test has shown that the three cotransformants selected show flocculating activities higher than the control untransformed cells (Fig. 6). Previous studies in the non-flocculent *S. cerevisiae* demonstrated that introduction of *FLO1* or *FLO5* genes into non-flocculent *S. cerevisiae* causes constitutive flocculation (Barney et al. 1990; Watari et al. 1990). More recently, placement of *FLO5* under the control of inducible promoters allowed specific flocculation under inductive conditions (Govender et al. 2008); FLO proteins are N-glycosylated by a site-specific process, which is required for proper functioning of the protein (Goossens and Willaert 2010). Therefore, an evident flocculating phenotype in the *C. reinhardtii* transformants selected indicates that the flocculin protein is produced, post translationally processed to yield a functional flocculin and correctly targeted to the cell surface. There are no previous reports about the expression of yeast flocculins in microalgae, but it has been previously demonstrated that nuclear-expressed recombinant proteins are adequately glycosylated in *C. reinhardtii* (Eichler-Stahlberg et al. 2009; Mathieu-Rivet et al. 2014; Mamedov and Yusibov 2011), which has been proposed as a platform for the production of eukaryotic proteins (Rasala and Mayfield 2011).

Conclusions We have demonstrated that cotransformation with two naked promoterless genes is a feasible method to express genes in microalgae without the need of exogenous regulatory sequences. Both the resistance gene and the gene of interest are integrated in the nuclear genome and are efficiently expressed at high frequency. The expression of the transgenes relies on their adequate insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene. Our goal has been the use of this technology to express the flocculing yeast encoding gene, *FLO5*, in *C. reinhardtii* and obtain transgenic microalgae which undergo self-flocculation; this is a totally new approach to deal with problems of microalgal harvesting.

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