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Introduction of Yeast Genes into the Green Alga, *Chlamydomonas reinhardtii*, to Promote Algal Flocculation

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Introduction of Yeast Genes into the Green Alga,
Chlamydomonas reinhardtii, to
Promote Algal Flocculation

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ABSTRACT

The ability of the flocculin (*FLO*) gene family, from the yeast *Saccharomyces cerevisiae*, to induce flocculation in green algae was investigated. Three genes of interest, *FLO9*, *FLO10*, and *FLO11*, were transformed into the high transgene-expressing *Chlamydomonas reinhardtii* strains, UVM4 and UVM11. Successfully transformed strains that contained the *FLO9* gene were identified and confirmed by PCR. Of these, several showed a potential flocculating phenotype. Western Blot analysis on soluble extracts from the transformed strains identified candidate protein bands of the expected molecular weight in two of the transformed strains. Transformation and identification of transformants containing the *FLO10* and *FLO11* genes is in progress. Availability of naturally flocculating algal strains is expected to reduce the overall cost of biofuel production from algae.

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I. INTRODUCTION

The U.S. Department of Energy (DOE) Biomass Program seeks to meet the Renewable Fuels Standard (RFS) established in 2007, under the Energy Independence and Security Act (EISA), by blending 36 billion gallons of renewable fuels with gasoline by 2022. Of this, 21 billion gallons must consist of advanced biofuels (i.e. not corn starch-based fuels). Biofuels from microalgae could help to fill a portion of this quota. However, production of microalgal biofuels requires several technical and biological improvements before it can be performed in an environmentally and economically viable manner. Harvesting of algal biomass from its liquid growth media is one aspect which adds to the overall cost of biofuel production. This step currently accounts for approximately 20% of the total cost of production.¹ The current method for harvesting algal biomass involves a chemical-mediated cell aggregation step (also called flocculation) followed by mechanical approaches, which include centrifugation, filtration, dissolved air flotation, or gravity sedimentation.¹ Flocculation can be performed using a variety of chemical compounds such as chitosan, metal salts, or cationic polymers or by adjusting the pH of the media.² This step leads to aggregation of cells into clusters that separate from the media by either rising to the top or sinking to the bottom. Chemical methods, although very effective at aggregating cells, lead to additional downstream processing problems. These include interference of the added chemical flocculant in the extraction and conversion process, as well as preventing recycling of water to be used in subsequent batches of algal cultivation, thus further adding to the cost of production.

Flocculation can also be stimulated biochemically by the activity of FLO

proteins, also called flocculins. These proteins are known to bind to mannose residues on the surface of other cells.³ Extensive studies of flocculation in the yeast, *Saccharomyces cerevisiae*, have had important industrial outcomes, particularly in enabling easier separation of yeast from the fermentation broth in the brewing industry. The pathways for synthesis of flocculin proteins can be triggered in yeast by stress or nutrient depletion.⁴ A total of five structural genes, namely *FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11*, have been reported to be involved in yeast flocculation.

Green algae can produce a variety of sugars in their cell walls, which include glucose, mannose, galactose and arabinose. Although the necessary sugar component is found in algae, the proteins required for binding to these cell wall sugars, namely the FLO proteins, are absent. The goal of this project is, therefore, to express the FLO proteins in the model green alga, *Chlamydomonas reinhardtii*, by introduction of the yeast *FLO* genes using a nuclear transformation approach and demonstrate the flocculation ability of the transformed strains.

The UVM4 and UVM11 strains of *C. reinhardtii* were used due to their high nuclear transgene- expressing properties.⁵ Upon successful expression of the FLO proteins in their cell walls, it is expected that these proteins will bind to cell wall sugars on neighboring cells and initiate flocculation, thereby potentially eliminating the need for addition of chemical flocculants. If the algae could settle to the bottom or float to the top of the media, as observed in yeast, this could reduce or eliminate dependence on mechanical means of separation of the algal biomass from the media. This biological mechanism (flocculation) is expected to allow for progress toward a process with a net energy gain and an economically viable end product.

II. MATERIALS AND METHODS

A. Culture Preparation and Maintenance

The UVM4 and UVM11 strains of *Chlamydomonas reinhardtii* were grown in 100mL of Tris-acetate Phosphate (TAP) media (pH 7.2). These strains were obtained from Dr. Ralph Bock's lab in Germany. Liquid cultures were grown at 25°C under continuous irradiance (80 $\mu\text{mol m}^{-2} \text{s}^{-2}$ PAR) under shaking conditions.

B. Linearization of Plasmid DNA

Each of the *FLO* genes was inserted into the pChlamy_1 plasmid (Life Technologies Corp., USA) such that they were under control of the RbcS2/Hsp70A promoter. The concentration of DNA was estimated by measuring absorbance at 260 nm and 280 nm. This concentration was used to calculate the volume of solution needed to obtain 50 μg of DNA. The restriction digestion mixture consisted of 50 μg of plasmid DNA, 5 μL of NEB4 buffer, 0.5 μL of 100X bovine serum albumin (BSA), 1 μL of the restriction enzyme XmnI and 1X Tris-EDTA (TE) buffer to make up the volume to 50 μL . This solution was incubated overnight at 37°C. An extraction was performed using 50 μL of phenol:chloroform (1:1) mixture by centrifugation at 13,300 RPM for 10 min. The top, aqueous layer was transferred to a new tube and mixed with one-tenth volume of 3M sodium acetate and 3 volumes of chilled 100% ethanol. The solution was incubated at -20°C for a minimum of 20 min to allow DNA precipitation to occur. The solution was centrifuged at 13,300 RPM for 10 min. The supernatant was discarded and the pellet was dried. The pellet was dissolved in 50 μL TE buffer. The concentration of DNA was calculated by measuring absorbance at 260 and 280 nm in a

spectrophotometer.

C. Transformation

Cells were grown to a minimum density of 2×10^6 cells/mL, estimated using a hemacytometer. Based on the cell density, the volume of culture required to yield 10^8 cells per transformation condition was calculated. The cells were then pelleted at 3,500 x g for 5 minutes and resuspended in TAP media supplemented with 40 mM sucrose (TAP + sucrose), to a final cell density of 4×10^8 cells/mL. Approximately 400 ng of linearized DNA (*FLO9*, *FLO10*, or *FLO11*) was added to a 4 mm electroporation cuvette containing 250 μ L of the cell suspension. The mixture was incubated on ice for 10 minutes. Cells were electroporated using an exponential electric pulse of 0.8 kV at a capacitance of 25 μ F. Cells were then transferred into a 14mL Falcon tube containing 10 mL TAP + sucrose and placed on a rocker for 16- 18 h under low light conditions to allow the cells to recover. Cells were pelleted at 3,500 x g for 5 minutes and resuspended in 200 μ L of TAP media. About 80 μ L was plated onto each of 3 TAP containing hygromycin plates (10 μ g/mL). The plates were incubated at 25°C in low light conditions (15-30 μ mol m⁻² s⁻² PAR).

D. Genomic DNA Extraction

Individual colonies that appeared on transformation plates (after 7 days of incubation) were transferred to fresh TAP + hygromycin plates, using sterile toothpicks (see FIG. 1). This was to ensure that all colonies that would be screened for the presence of the *FLO* genes were true hygromycin-resistant transformants. Ten individual colonies from the restreaked plates were then inoculated into TAP media and allowed to grow for 3-4 days. This combined culture of 10 colonies would constitute a

“pool”. Genomic DNA was extracted from each of these pools using a phenol:chloroform:isoamyl alcohol extraction protocol. Briefly, cells were lysed in sodium dodecyl sulfate-EDTA buffer (SDS-EB), followed by an extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The top aqueous layer was transferred to a new tube to which 1 μ L of RNase A (10 mg/mL) was added, and the solution was incubated for 1 h at 37°C. Another round of phenol:chloroform: isoamyl alcohol (25:24:1) extraction was performed. The top aqueous layer was transferred to a new tube, chilled 100% ethanol was added, and the solution was incubated at -80°C for a minimum of 30 min to allow DNA precipitation to occur. The solution was centrifuged and the DNA pellet was washed with chilled 70% ethanol. The pellet was dried and resuspended in 50 μ L of TE buffer. A total of approximately 15-20 pools were extracted for each of the *FLO* gene transformation conditions.



FIG. 1. Linear restreaking of colonies for PCR screening

E. PCR Based Screening

The presence of *FLO* genes (*FLO9*, *FLO10*, and *FLO11*) in the pools were identified by performing two PCR reactions. Reaction one was performed using pChlamy-F1 as the forward primer and FloX-R1 as the reverse primer, where X represents the different *FLO* genes being tested. Reaction two was performed using

FloX-XbaI-F and FloX-R1 primers. Whereas the first primer set amplified a region of the inserted plasmid that corresponded to the Hsp70A/RbcS2 promoter region through the 3' end of the individual *FLO* gene (*FLO9*, *FLO10*, or *FLO11*), the second primer set was a gene-specific primer set that corresponded specifically to the individual *FLO* gene (*FLO9*, *FLO10*, or *FLO11*). Electrophoresis on a 1% agarose gel was performed to separate the PCR products. Pools that showed positive PCR products were divided into “subpools” of 2-5 colonies and inoculated into liquid media, as done previously. Genomic DNA extraction and PCR screening was repeated with these subpools. Those subpools that showed positive PCR amplification products were further down-selected for the identification of individual colonies that contained the desired *FLO* gene. Colonies within the positive subpools were inoculated individually. The genomic DNA extraction and PCR screening process was again repeated for these individual colonies. Those colonies that showed the correct size PCR product were restreaked onto fresh TAP+hygromycin plates and maintained for further phenotypic studies

F. Phenotype Screening

Samples of each transformed strain were inoculated into 10 mL of TAP media in a 25 mL flask and grown at 25°C under continuous irradiance ($80 \mu\text{mol m}^{-2} \text{s}^{-2}$ PAR) under shaking conditions. Samples were qualitatively examined under a microscope at 200X and 400X magnification and photographed.

G. Whole Cell Protein Extraction

Samples of each transformed strain were inoculated into 100 mL of TAP media and grown at 25°C under continuous irradiance ($80 \mu\text{mol m}^{-2} \text{s}^{-2}$ PAR) under shaking conditions. Cells were pelleted at 3,500 x g for 5 min and resuspended in 10

mL of cold protein extraction buffer containing a 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and a 1:500 dilution of protease inhibitor cocktail. Cell suspensions were incubated on ice. While on ice, the cells were sonicated using 10 pulses of 30s each with 30s between pulses. Cells were centrifuged at 6,000 x g for 10 min and the supernatant was transferred to a new tube. Protein from the supernatant was clarified by sequential centrifugation at 11,000 x g and then 15,000 x g for 10 min each. The protein concentration was estimated using a Bradford assay.

H. Protein Gel Electrophoresis and Western Blot

Based on the protein concentration, 25 µg of each protein sample was loaded into a 4% SDS-polyacrylamide stacking gel over a 7.5% SDS-polyacrylamide separating gel. Two replicate gels were run for each sample: one for Coomassie blue-staining and the other for Western blotting. Gels were run at 90V for 2 h.

Staining was performed by placing in a 0.2% Coomassie blue solution for 30 min. The gels were destained by placing in a 40% ethanol/10% acetic acid solution with shaking for two cycles of 15 minutes each, followed by placing in a 10% acetic acid solution overnight with shaking.

Prior to Western blotting, proteins were transferred to a nitrocellulose membrane using a semi-dry blotter. Three pieces of Whatman paper were placed on the blotter. The membrane was placed on top of the paper, followed by the gel, and three additional pieces of Whatman paper on top. All items (Whatman papers, the gel, and the membrane) were dipped in transfer buffer prior to placing. The semi-dry blotter was run at 25V for 1 h.

Western Blotting was performed using the following procedure. The membranes

were incubated with 10 mL of blocking buffer containing 5% BSA and 0.1% Tween-20 in tris-NaCl solution (TBS) for 10 min on a rocker. The blocking buffer was discarded and antibody buffer (0.1% BSA and 0.1% Tween-20 in TBS) containing a 1:2,500 dilution of the rabbit anti-FLO antibody was added. The membrane was incubated in the antibody solution for 1 h on a rocker. The membrane was washed for 3 cycles of 5 min in wash buffer (1% Tween-20 in TBS). The membrane was then incubated on a rocker in antibody buffer containing a 1:20,000 of goat anti- rabbit horse radish peroxidase-conjugated antibody. The membrane was washed with wash buffer for 3 cycles of 5 min each. 1 mL of SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) was added to the membrane. The membrane was visualized using a FluorChem Q chemiluminescence imager.

III. RESULTS

A. PCR Based Screening for *FLO9* Transformants

Based on the presence of a 3,900 bp PCR fragment during gel electrophoresis, 11 out of 18 UVM4 transformant pools were determined to possess colonies that contained the *FLO9* gene. 28 subpools were generated from these pools, 10 of which were determined to possess colonies that contained the *FLO9* colonies. Ultimately, seven UVM4 colonies were identified as positive transformants for *FLO9* (FIG. 2). These were UVM4-9-54, UVM4-9-100, UVM4-9-101, UVM4-9-119, UVM4-9-120, UVM4-9-127, and UVM4-9-128 (FIG. 2).

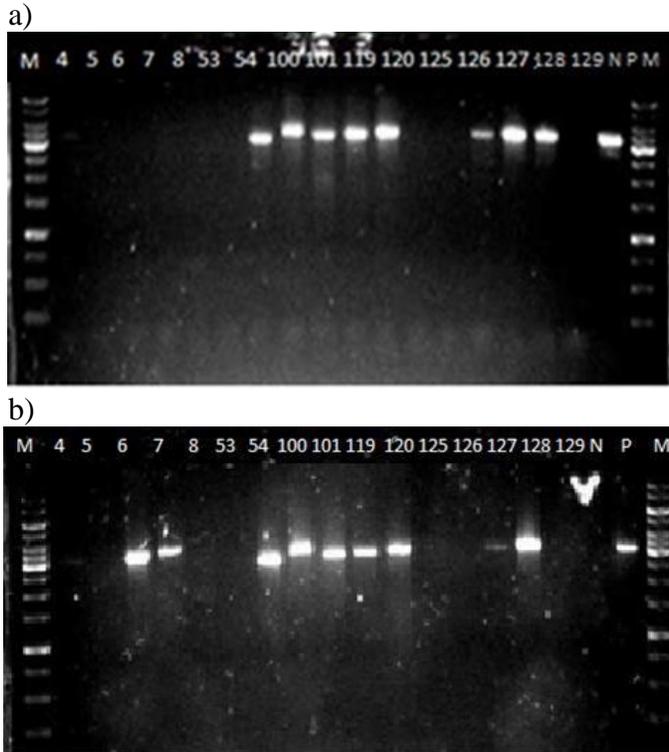


FIG. 2. Gel electrophoresis results for *FLO9* transformation of UVM4 colonies, with primer set 1 (pChlamy-F1 & Flo9-R1) (a) and primer set 2 (Flo9-XbaI-F1 & Flo9-R1) (b). M: molecular weight marker, Numbers: strain (UVM4-9-X), N: negative control, P: positive control

Based on the presence of a 3,900 bp PCR fragment during gel electrophoresis, 11 out of 14 UVM11 transformant pools were determined to possess colonies that contained the *FLO9* gene. Six of these pools were selected and divided into 16 subpools. Ultimately, five UVM11 strains were identified as positive transformants for *FLO9* (FIG. 3). These were UVM11-9-25, UVM11-9-26, UVM11-9-27, UVM11-9-60, and UVM-9-63.

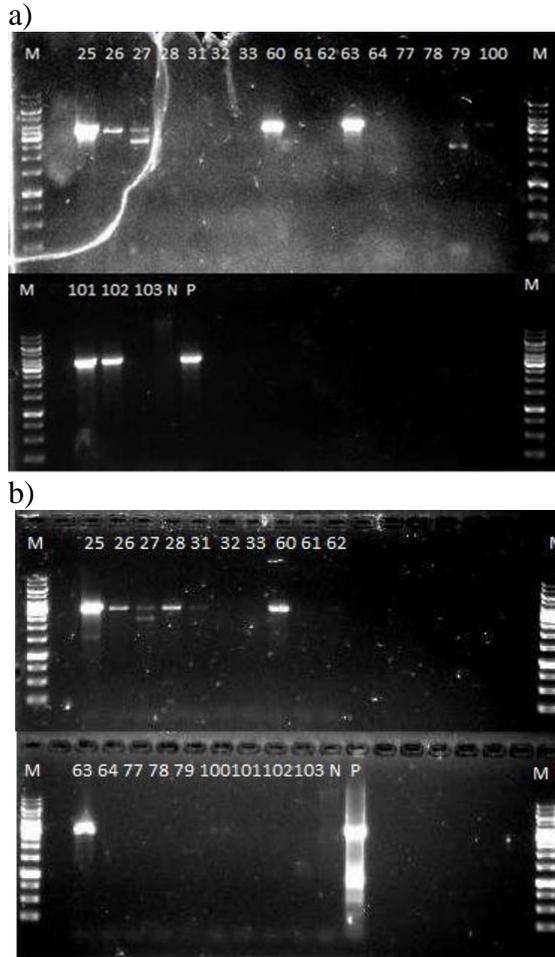


FIG. 3. Gel electrophoresis results for *FLO9* transformation of UVM11 colonies, with primer set 1 (pChlamy-F1 & Flo9-R1) (a) and primer set 2 (Flo9-XbaI-F1 & Flo9-R1) (b). M: molecular weight marker, Numbers: strain (UVM11-9-X), N: negative control, P: positive control

B. Microscopy and Protein Analysis of *FLO9* Transformants

Qualitative microscopy of the *FLO9* transformants in the UVM4 strain showed small clusters of cells (3-8 cells/cluster) in the UVM4-9-100, UVM4-9-120, and UVM4-9-128 strains in comparison to the wild-type UVM4 cells, which showed separated individuals cells (FIG. 4). Qualitative microscopy of the *FLO9* transformants in the UVM11 strain showed similar small clusters of cells in the UVM11-9-25, UVM11-9-26, and UVM11-9-63 (FIG. 5).

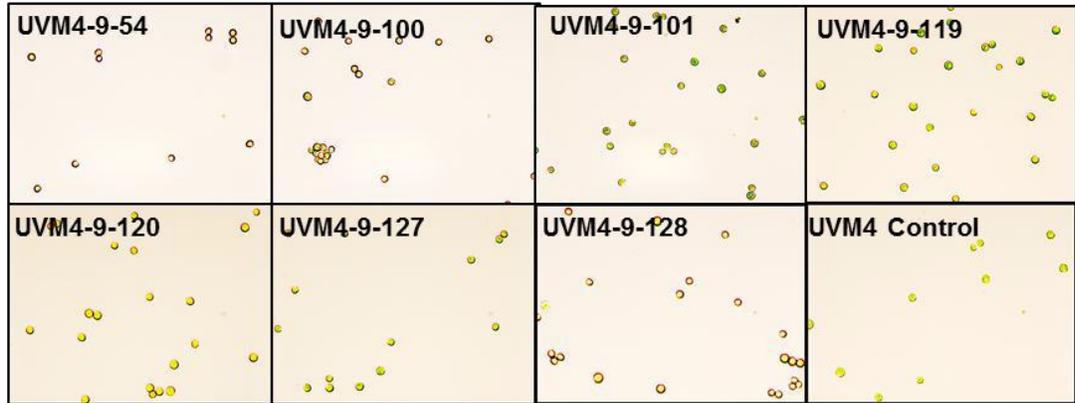


FIG. 4. Qualitative microscopy images (400X magnification) of strains transformed with the *FLO9* gene compared to the untransformed UVM4 strain.

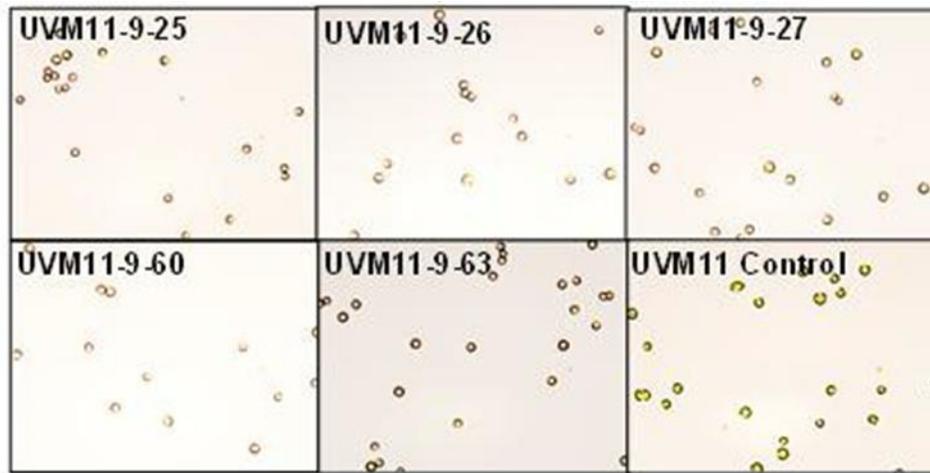


FIG. 5. Qualitative microscopy images (400X magnification) of strains transformed with the *FLO9* gene compared to the untransformed UVM11 strain.

Total soluble protein extracts from both the UVM4 and UVM11 transformant strains showed intact proteins on Coomassie stained gels. Western Blot analysis showed a unique band that corresponded to the expected molecular weight of FLO9 protein (~140 kDa), only in the UVM4-9-119 and the UVM11-9-27 lanes (FIG. 6).

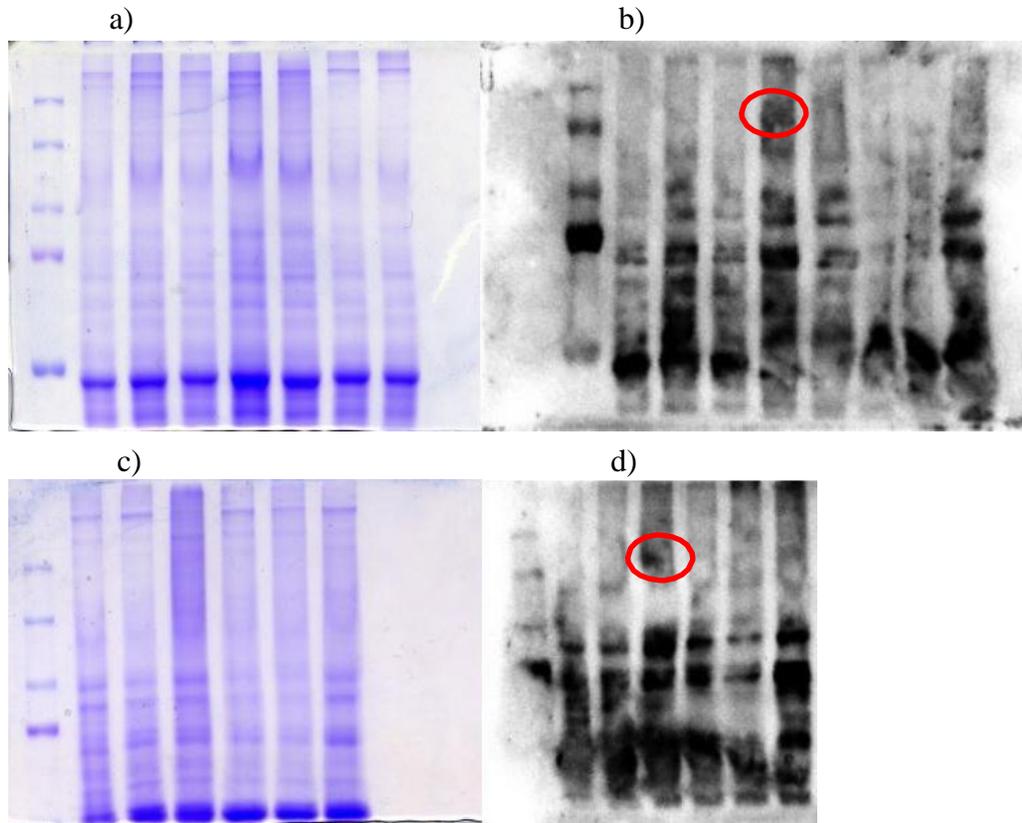


FIG. 6. Coomassie blue-stained SDS-PAGE gels for UVM 4 (a) and UVM11 (b) transformants containing the *FLO9* gene. Western blot for UVM4 (c) and UVM11 (d) transformants containing the *FLO9* gene. UVM4 control is loaded into the last lane of the Western blot. UVM11 controls are loaded into the last lane of the both images. Circles represents the unique protein bands of ~ 140 kDa.

A list of UVM4 and UVM11 transformant strains that were identified to contain the *FLO9* gene is presented in Table 1. Strains that showed putative *FLO9* protein expression or a flocculating phenotype are indicated.

Table 1. Transformants of the UVM4 and UVM11 strains containing the *FLO9* gene that were confirmed by PCR analyses.

UVM4 Transformants	UVM11 Transformants
UVM4-9-54	UVM11-9-25*
UVM4-9-100*	UVM11-9-26*
UVM4-9-101	UVM11-9-27 ⁺
UVM4-9-119 ⁺	UVM11-9-60
UVM4-9-120*	UVM11-9-63*
UVM4-9-127*	
UVM4-9-128*	

*: weak clustering phenotype observed

+ : candidate FLO protein expression

C. Nuclear Transformation of the *FLO10* and *FLO11* Genes

Transformation of UVM4 strain with the *FLO10* and *FLO11* genes yielded colonies on the hygromycin selection plates. The identification of positive for both genes using the PCR- based screening approach is currently in progress. Positive pools have been identified for *FLO10* and subpools are under investigation. Pools are under investigation for *FLO11*. Successful transformation has not yet been achieved for the UVM11 strain with either the *FLO10* or *FLO11* gene.

IV. DISCUSSION

Transformation of the *C. reinhardtii* strains, UVM4 and UVM11, with the *FLO9* gene was successful, although the transformation efficiency was low. A similar preliminary observation was also made in the case of the *FLO10* gene that was transformed into the UVM4 strain. Despite having several hundred colonies on the hygromycin selection plates, the frequency of finding a complete *FLO9* transformant was extremely poor. As low as one in 15 transformed hygromycin-resistant colonies contained the *FLO* gene of interest. As a result, ~150 colonies had to be screened for each strain in order to identify a sufficient number of *FLO* gene- containing transformants.

Although the UVM4 and UVM11 strains are known to be high-transgene expressing strains, they are cell wall-deficient.⁵This means that a protein which is expected to be translocated to the cell wall either will not localize in its expected location or will do so improperly, depending on the availability of a cell wall-like structure. Two *FLO9*-containing strains, UVM4-9-119 and UVM11-9-27, showed the presence of a unique protein band at the expected molecular weight of the *FLO9* protein in the Western blot analysis but lacked a strong phenotype. The absence of a strong phenotype in these strains could be attributed to this cell wall- deficiency. Further confirmation is needed to prove that the unique protein band observed on the PAGE gel was the *FLO9* protein. If this candidate band is confirmed to be the *FLO9* protein, this would mean that although the gene is being expressed, the protein cannot embed in the cell wall. Transformation of the *FLO9* into a different strain with an intact cell wall along with a high-transgene expressing ability could potentially produce a flocculation phenotype. However, currently such a strain combination is unavailable. If a strain with an intact cell wall also expresses the protein but also fails to produce a phenotype, it is possible that the protein cannot embed in an algal cell wall as it does in yeast, possibly due to differences in cell wall composition and/or structure.

Many strains showed a possible low level of flocculation, despite no candidate *FLO9* proteins being observed by Western blotting. These could be due to chance interactions of the cells, rather than a true protein-sugar interaction. Transmission electron microscopy could give a higher resolution imaging of these interactions. True cell-cell interactions could be possible, despite the strains being

cell wall-deficient, if fragments of the cell wall are still present. Therefore, it is possible that there are flocculins embedded in these portions of the cell wall, but the limited coverage was not sufficient to allow for formation of larger clusters. Performing a cell wall protein extraction, following by Western blotting, could provide further information toward explaining the candidate phenotypes. Additionally, mass spectrometry analysis on the unique protein band would help confirm the identity of the FLO9 protein.

Future studies should focus on regulation of *FLO* gene expression and the expressibility of these genes in other algal strains. The effects of age and culturing conditions on FLO protein expression should also be evaluated. Testing *FLO* gene expression under control of regulatable promoters could lead to synchronization of flocculation with the timing of the desired biofuel production. Once these data are acquired, transformation of the FLO genes into high lipid- producing strains of industrial interest would result in reducing harvesting costs and thereby present a significant advancement towards commercialization of biofuels from microalgae.

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