J Appl Phycol
DOI 10.1007/s10811-014-0392-5

Heterologous expression of a *Volvox* cell adhesion molecule causes flocculation in *Chlamydomonas reinhardtii*

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Received: 15 April 2014 / Revised and accepted: 18 August 2014

Abstract Harvesting microalgae at large scales is a significant barrier to economically feasible production of biofuels and other low-cost commodities from microalgal biomass. We have tested a strategy for reducing the costs of microalgal harvest by generating transgenic strains of *Chlamydomonas reinhardtii* that express a heterologous cellular adhesion molecule, Algal-CAM, from the multicellular green alga *Volvox carteri*. Constitutive expression of heterologous Algal-CAM causes *Chlamydomonas* unicells to adhere together and settle out of suspension much more rapidly than controls. Immunoblotting shows the heterologous Algal-CAM to be present in the extracellular matrix of *Chlamydomonas* transformants and possibly cross-linked with native glycoproteins there. We define this form of cell–cell adhesion as genetically engineered (GE) flocculation to distinguish it from other cell flocculation strategies. Future development of this trait can include making expression of Algal-CAM inducible for controlled timing of GE flocculation and exploring regulated expression of additional cell adhesion molecules from the many other multicellular relatives of *Chlamydomonas*. Advanced forms of this technology could lead to production of novel biomaterials from single-celled algae by controlled expression of diverse cell adhesion molecules with different cross-linking properties.

Keywords Flocculation · Cell adhesion molecule · Extracellular matrix · Biotechnology · *Chlamydomonas* · *Volvox*

Introduction

Production of biofuels and other low-cost commodities from microalgae is not yet economically feasible owing to the high costs of their cultivation, harvest, and processing (Craggs et al. 2013; Pragya et al. 2013). Recent advances in harvest and processing technologies, such as dissolved air floatation and hydrothermal liquefaction, offer a reduction of these costs (e.g., Liu et al. 2013) but bulk harvesting of microalgae at large scales remains a primary bottleneck to production of cost-competitive biofuels, animal feeds, soil amendments, and other products from microalgal biomass (Li et al. 2008; Mata et al. 2010; Pragya et al. 2013; Salim et al. 2011; 2014; Spilling et al. 2010; Vandamme et al. 2013; Wijffels and Barbosa 2010).

Flocculation is the aggregation of suspended particles into larger clumps that are more easily separated from a liquid. Originally developed for waste water treatment, flocculation is emerging as an effective approach to microalgal harvest because it can act synergistically with other harvest methods, such as sedimentation, filtration, or dissolved air floatation (Pragya et al. 2013). Flocculation can, therefore, be part of an integrated harvest technology that utilizes multiple harvest methods to dewater microalgal biomass with high efficiency. Harvest methods that do not separate cells from water and thus avoid the cost of harvesting microalgae are also being developed, including direct capture of secreted ethanol, terpenoids, or lipids (Bentley et al. 2013; Wijffels et al. 2013; Zhang et al.

Electronic supplementary material The online version of this article (doi:10.1007/s10811-014-0392-5) contains supplementary material, which is available to authorized users.

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Published online: 20 September 2014

2013). These approaches can be very useful but harvest of whole cells is often preferred, for example when multiple high value products can be extracted from the harvested cells or when removal of cells and cell debris aid in the recycling of culture water.

Several recent studies have demonstrated biological or chemical enhancers that improve the efficiency of microalgal flocculation (Grima et al. 2003; Salim et al. 2011; Spilling et al. 2010; Zheng et al. 2012). Flocculation enhancers act by neutralizing the negative charges on cell surfaces that normally keep them from sticking together. Electrolytic and electrophoresis-based methods have also been shown to enhance flocculation of microalgae by a similar mechanism (Azarian et al. 2007; Chen et al. 2011). None of these enhanced flocculation methods are cost-efficient, however, as large amounts of energy, chemicals, or biological additives must be expended (Craggs et al. 2013; Park et al. 2011). Moreover, in some cases, chemical flocculation enhancers must be removed after harvest before recycling culture water (Vandamme et al. 2013).

An ideal flocculation process would cause microalgae to floc together on command when ready for harvest with little or no input of energy or chemicals. Genetic engineering of algae to flocculate by synthesis of cell adhesion molecules would accomplish this. A similar approach has already been taken in yeast (Govender et al. 2008; Vandamme et al. 2013).

Chlamydomonas reinhardtii and *Volvox carteri* are an effective model system pair for testing genetic engineering of flocculation by cell–cell adhesion. This is true for several reasons. Among these, analysis of the recently sequenced *V. carteri* and *C. reinhardtii* genomes show them to be closely related at the genetic level (Prochnik et al. 2010). The two species also have very similar extracellular matrix (ECM) properties in terms of biochemical composition, fine structure, and self-assembly (e.g., Roberts et al. 1985; Adair et al. 1987). For example, the perchlorate-soluble ECM layers can be stripped from one species, and after dialysis to remove the perchlorate, can re-assemble into functional forms of the same ECM layers on the other species (Adair et al. 1987; Roberts et al. 1985). Moreover, the divergence of multicellular *Volvox* from a unicellular, *Chlamydomonas*-like ancestor 200 million years ago seems to have occurred through a relatively small number of genetic changes that resulted in regulated cell–cell adhesion (Nishii and Miller 2010). This suggests that just a few transgenes from *V. carteri* could be used to create a simple multicellularity in *C. reinhardtii* that is the functional equivalent of flocculation.

The volvocalean ECM is relatively well-characterized (Adair et al. 1987; Adair and Apt 1990; Goodenough and Heuser 1985; Matsuda et al. 1987; Roberts et al. 1985; Voigt et al. 2007; Woessner and Goodenough 1994). It is a self-assembling network of inter-connecting, fibrous glycoproteins that are rich in repeat motifs of serine and hydroxyproline and thus belong to the family of structural proteins known as hydroxyproline-rich glycoproteins (HRGPs). The HRGPs of *Volvox* and *Chlamydomonas* resemble the rod-like extensins of plant cell walls, which are similarly *O*-glycosylated at their hydroxyproline and serine residues (Lee et al. 2007; Miller et al. 1972; Popper et al. 2011). Volvocalean HRGPs differ in that they often possess globular domains on one or both ends of their extensin domains. These bind together and form protein meshes of varying pore size (Goodenough and Heuser 1985; Goodenough et al. 1986; Hallmann 2003; Hallmann 2006; Roberts et al. 1985). In both plants and volvocalean algae, extensin domains become insolubilized on secretion by covalent iso-dityrosine cross-linking to the hydroxyproline-rich environment of their ECMs (Fry 1982; Waffenschmidt et al. 1993).

During embryonic development, *V. carteri* exhibits regulated cell–cell adhesion mediated primarily by HRGPs of the ECM, one of which is the Algal-CAM of this study (Ertl et al. 1992; Huber and Sumper 1994; Kirk 1998). Algal-CAM was discovered by generating antibodies that disrupted cell–cell adhesion during *V. carteri* embryogenesis and then using them to recover Algal-CAM from protein extracts. Algal-CAM exhibits three primary structural domains: an N-terminal extensin-like domain and two C-terminal domains with homology to Fasciclin I. The N-terminal extensin-like domain exhibits characteristic Ser-Pro(3–7) repeat motifs that are heavily glycosylated and hydroxylated. Based on concanavalin A staining, informatic sequence analysis, guanidine hydrochloride denaturation/SDS-PAGE analysis, and tryptic peptide sequencing, Huber and Sumper (1994) concluded that the N-terminal extensin-like domain of Algal-CAM is *N*-glycosylated at multiple sites and *O*-glycosylated at its hydroxyproline and serine residues. The fasciclin I glycoprotein, to which the two C terminal domains of Algal-CAM show homology, was discovered in *Drosophila* neurons, where it acts in growth cone extension and guidance. Zinn et al. (1988) observed *Drosophila* fasciclin I to reside on the cell surface of embryonic and sensory axons and concluded it was a homophilic cell adhesion molecule. Domains of other proteins homologous to *Drosophila* fasciclin I mediate cell-cell adhesion in bacteria, plants, and algae. For this reason, they are thought to represent an ancient line of cell adhesion molecules (Huber and Sumper 1994; Ulstrup et al. 1995; Zinn et al. 1988). The combination of a wall-anchoring, extensin-like domain and two homophilic fasciclin I-like domains in Algal-CAM allow it to bind adjacent *V. carteri* cells together during the colony inversion stage of embryonic development (Huber and Sumper 1994). Algal-CAM also contains an N-terminal signal peptide with a characteristic Ala-Cys-Ala cleavage site for secretory pathway targeting and secretion into the ECM (Huber and Sumper 1994).

Interestingly, homologues of Algal-CAM appear to be absent from *C. reinhardtii*. A BLAST search for Algal-CAM

gene and protein sequences in *C. reinhardtii* showed few homologous sequence hits. The most homologous sequence to Algal-CAM in the *C. reinhardtii* genome is an unknown predicted protein sequence with 27 % identity and 19 % coverage (NCBI Accession ID: XP_001703613.1).

The close evolutionary relationship between *C. reinhardtii* and *V. carteri* allows a streamlined approach to expression of *Volvox* cell adhesion molecules in *Chlamydomonas*. The two species share codon usage, protein secretion mechanisms, post-transcriptional modification processes, transcriptional regulatory factors, and codon related gene expression stability (Cerutti et al. 1997; Fuhrmann et al. 1999, 2004; Prochnik et al. 2010). Furthermore, although *C. reinhardtii* and *V. carteri* are very similar at the genomic level, Prochnik and colleagues (2010) report that *V. carteri* differs by having expanded families of ECM glycoproteins that mediate cell–cell adhesion and multicellularity throughout colony development. For these reasons, *V. carteri* represents an ideal source of genes that can be exploited for design of artificial cell–cell adhesion in *C. reinhardtii* via heterologous expression of cell adhesion proteins.

In this report, we show that Algal-CAM from *V. carteri* can be used to genetically engineer flocculation in *C. reinhardtii*. Transformed lines of *C. reinhardtii* expressing Algal-CAM consistently exhibited flocculation while controls did not. Immunoblots confirmed that heterologous Algal-CAM was secreted into the ECM of *C. reinhardtii* transformants and likely glycosylated and cross-linked there, with itself or with other ECM components. From this latter finding, we propose that, in addition to causing flocculation, the presence of heterologous Algal-CAM in the *C. reinhardtii* ECM may have altered its physical properties.

Materials and methods

Algal strains for this study were acquired from the UTEX culture collection (http://web.biosci.utexas.edu/utex/) and the Chlamydomonas Center culture collection (http://chlamycollection.org/). *Volvox carteri* f. *nagariensis* (UTEX 1886) was cultured in 0.5× Bolds Modified medium (Sigma-Aldrich, product #B-5282) with addition of 0.5× supplemental soil water (Carolina Biological Supply, product #15-3790). Lighting was continuous warm-white fluorescent light at 60 μmol photons m^{-2} s^{-1}. Temperature was 23 °C. *C. reinhardtii* strains were cultured in liquid TAP or TAP + yeast extract agar medium (Gorman and Levine 1965) as described by the Chlamy Center (http://www.chlamy.org/). Liquid cultures were grown in 125 mL culture flasks with rotary shaking at 150 rpm. Lighting was continuous warm-white fluorescent light at 60 μmol photons m^{-2} s^{-1}. Temperature was 23 °C. Wall-less mutants of *C. reinhardtii* (CC-400) were used for all genetic engineering and flocculation experiments. High efficiency mating strains of *C. reinhardtii* (CC-620 and CC-621) were used to generate crude gamete lytic enzyme (GLE). GLE preparation is described below.

RNA extraction and cDNA synthesis

Qiagen plant RNeasy RNA extraction kits with "Shredder" columns (Qiagen Inc. USA, #74903) were used for RNA extraction per manufacturer's recommendation starting with 3-5x10^6 total cells. *V. carteri* cells were lysed using liquid N_2 and RNase free disposable pestles in 1.7 mL tubes. *C. reinhardtii* (CC-400) cells were lysed without liquid N_2 using RLT buffer and Shredder columns included in the RNeasy kit. DNase treatment of RNA extracts was carried out with NEB DNase I (New England BioLabs, #M0303s), as recommended by NEB, to eliminate genomic DNA contamination. cDNA synthesis was carried out directly after DNase treatment of RNA extracts with the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen/Life Technologies, #18080-400), as recommended by the manufacturer, using oligo$(dT)_{20}$ primers.

RT-PCR

cDNA preparations served as template for primers amplifying a 519-bp cDNA amplicon. The same primers amplified a 664-bp gDNA amplicon from genomic DNA of *V. carteri*. The forward RT-PCR primer was 5′-AATGGCCAGGAGATTCGAGGTAC-3. It spans the Intron I splice site and thus touches down on the 3′ end of the Hsp70A-RbcS2 promoter and the 5′ end of the KpnI restriction site of the pChlamy_ACAM vector (2 base pairs upstream from the Algal-CAM start codon). The underlined primer sequence denotes annealing on the 5′ end of the KpnI restriction site. The reverse RT-PCR primer sequence was 5′-TCCGACAACTTGTCAAAAGCCG-3. Standard PCR reaction conditions were used with annealing temp at 58 °C for 35 cycles. PCR amplicons were run on 2 % TAE agarose gels stained with ethidium bromide. The NEB 50 bp Ladder (New England BioLabs, N3236s) was used for size comparison.

Cloning Algal-CAM and construction of the pChlamy_ACAM vector

Standard recombinant DNA techniques were used to generate all constructs (Sambrook and Russell 2001). The 1,323-bp Algal-CAM mRNA coding sequence (CDS, GenBank: X80416.1) was amplified from *V. carteri* cDNA preparations (Bio Rad MJ mini thermal cycler #PTC-1148) using Phusion HF DNA polymerase (Finnzymes product #F-530s New England Biolabs) per the manufacturer's recommendations with oligonucleotides ACAMCDS2fwd (5′-ATGCGGATGGCAATCGCTGCCTT-3′) and ACAMCDS2rev (5′-TCACGC

TGCGCCACCGGCT-3′). This CDS corresponds to the open reading frame encoded by the *cam1* mRNA splice variant 2 of Algal-CAM (NCBI accession #XM_002958317.1) which incorporates the C-terminal exon VIII and not the GPI associated exon IX (Huber and Sumper 1994). The Algal-CAM CDS was cloned into pCR-TOPO-2.1 (Invitrogen/Life Technologies, #45-0641) as recommended by the manufacturer and confirmed by sequencing using the M13 primer pair. A codon optimized (Fuhrmann et al. 1999; Fuhrmann et al. 2004) 3′ flexible oligopeptide (GGAGG), FLAG tag (DYKDDDDK) and stop codon were then fused to the Algal-CAM CDS via an overhanging reverse PCR primer (ACAM-3′ flex/FLAG/stop: 5′-**TCActtGTCgtc GTCgtcCTTgtaGTCGCCgccGGCgcc-GCC**CGCTGCGC CACCGGCT-3′). Bold uppercase and lowercase letters within the primer sequence signify optimized codon nucleotide triplets. This amplicon was TOPO cloned as above and amplified again adding 5′-KpnI and 3′-NotI restriction sites via overhanging primers (ACAM-5′-KpnI Fwd: 5′-GGTA CCAA**TG**CGGATGGCAATCGCTG-3′ and ACAM-3′-NotI Rev: 5′-GCGGCCG-C**TCA**CTTGTCGTCGTCGTCCTTG-3′). This Algal-CAM-FLAG tag fusion sequence was ligated into pChlamy_1 (Invitrogen/Life Technologies #A14258) via KpnI and NotI (see Fig. 1). All constructs were confirmed by sequencing. Invitrogen's pChlamy_1 drives transcription with a truncated RBCS2 promoter flanked by enhancer elements of HSP70A and RBCS2 intron 1 and contains a hygromycin selectable marker (aph7) (Fuhrmann et al. 1999, 2004; Schroda et al. 2002).

Transformation

Transformation using pChlamy_ACAM or the pChlamy_1 empty vector was carried out by the nuclear glass bead method on cells at 1.2–1.5×10^6 cells mL^{-1} (Debuchy et al. 1989; Kindle 1990). Transformants were selected on TAP agar with 10 μg mL^{-1} hygromycin B as directed by the *Chlamydomonas* engineering kit (Invitrogen/Life Technologies #A14258) and screened for transgene presence via PCR of the transgene. Colonies were further screened for mRNA expression via RT-PCR as described above. Genomic DNA was extracted by incubating cells at 100 °C for 5 min in 10 mM Na EDTA followed by centrifugation, as described in Cao et al. (2009) or by Qiagen Generation Capture (mini)Columns (Qiagen Inc. USA, #159914).

Heterologous protein analysis by immunoblot

Whole cell lysates were prepared by spinning down 500 μL of liquid culture. Media supernatant was removed and 100 μL of BioRad Laemmli sample buffer with β-mercaptoethanol and 1× end concentration EDTA-free cOmplete protease inhibitor cocktail (Roche Applied Science, Germany; #11873580001) was added and the cells were homogenized by pipetting/vortexing. Soluble and insoluble fractions of the homogenate were separated by centrifugation at 13,000 rpm in a microcentrifuge for 5 min. Soluble fractions were removed and assayed directly. Insoluble fractions were resuspended in 50 μL Laemmli sample buffer, as above, and resolved on BioRad Any kd precast SDS-PAGE gels (BioRad, USA). Resolved proteins were electro-transferred to PVDF membrane, blocked with 5 % dry milk, and probed with Sigma-Aldrich anti-FLAG polyclonal antibody (Sigma-Aldrich, F7425) produced in rabbit (1:10,000 dilution). Alkaline phosphatase conjugated secondary antibody against rabbit was used to probe primary antibody and detected using NBT/BCIP. PageRuler Plus (Thermo Scientific, USA) was used as marker.

ECM digestion

ECM digestion and isodityrosine cross-linking inhibition was carried out as described by Waffenschmidt et al. (1993) but with the following modifications: Cells were grown to 2–3×10^6 cells mL^{-1} in TAP and 1.5 mL of culture was gently centrifuged in a microfuge at 3,000 rpm for 5 min. The pellet was resuspended in 1 mL of crude gamete autolysin (GLE) and incubated for 1 h at room temperature. Cells were spun down again as above, rinsed once with TAP, and resuspended in 300 μL TAP with 50 mM ascorbate and 5 mM tyrosine (at pH 7.4) to inhibit formation of iso-dityrosine linkages. After a 2-h incubation, supernatant fractions were separated from cell pellets by a 5-min spin at 13,000 rpm in a microfuge and used directly in immunoblot assays as described above. Crude

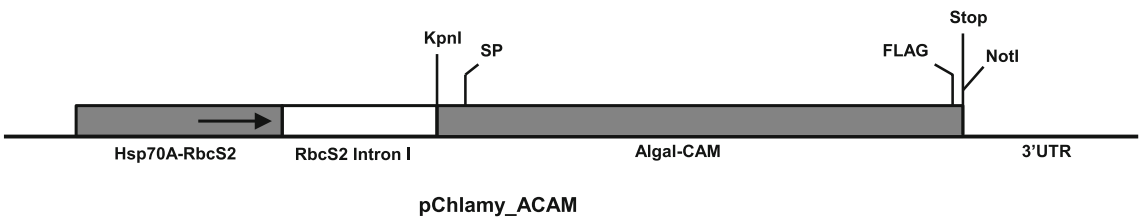

Fig. 1 pChlamy_ACAM expression vector. The Algal-CAM CDS was ligated in frame with Invitrogen's pChlamy_1 start codon using KpnI and NotI. An *Hsp70A-RbcS2* chimeric promoter and *RbcS2 Intron I* drives Algal-CAM transcription in this vector and a *Cop1* 3′ UTR downstream of the Algal-CAM CDS improves expression. pChlamy_1 also contains the *Aph7* hygromycin resistance marker under control of a β2-tubulin promoter. The Algal-CAM sequence includes a 5′ Signal Peptide (SP) for secretion and a 3′ codon-optimized flexible linker and FLAG tag

gamete autolysin was prepared according to the "improved preparation from plates" protocol by Sabine Waffenschmidt posted on the chlamy.org methods page (http://www.chlamy.org/).

Flocculation assay

Three replicates each of control and flocculating cells were cultured side-by-side in liquid until control cells reached an OD_{750} ~ 1.7. Cells were then vortexed, allowed to settle for 5 min, and OD_{750} measurements were taken using an Ocean Optics Chem2000 spectrometer (Ocean Optics, USA). After OD_{750} measurements were taken, cells were separated from culture water via centrifugation, dried at 60 °C overnight, and weighed to determine dry mass. Data for OD_{750} and dry weights were statistically analyzed using a one-way analysis of variance set in a completely randomized design. If significant differences (alpha=0.05) were indicated by the *F* test, then post hoc mean separations were performed using Tukey's HSD. Statistical analyses were facilitated by using the GLM procedure of SAS (SAS Institute, version 9.3).

Results

Transformation and heterologous Algal-CAM gene expression in *C. reinhardtii*

Extensive genetic and biochemical characterization of Algal-CAM was carried out previously by Huber and Sumper (1994). We used this information to isolate and clone the mRNA coding sequence (CDS) of the Algal-CAM splice variant 2 from *V. carteri* using RT-PCR. The Algal-CAM CDS was then subcloned into Invitrogen's *Chlamydomonas* expression plasmid, pChlamy_1, forming pChlamy_ACAM (Fig. 1).

The CC-400 cw 15 mt^+ "wall-less" strain of *C. reinhardtii* was used for transformation with pChlamy_ACAM. Hygromycin resistant colonies were screened by PCR for transgene insertion into the nuclear genome. Colonies that tested positive for presence of the nuclear transgene were further screened for expression of Algal-CAM mRNA by RT-PCR using recombinant Algal-CAM-specific primers that targeted a 519-bp amplicon spanning the *HSP70A/RBCS2* promoter region and transgene CDS. Results of the RT-PCR screen are shown in Fig. 2. Colonies C11 and C12 clearly showed Algal-CAM mRNA expression. Colony C8 did not show Algal-CAM mRNA expression, though it tested positive for genomic transgene insertion by the PCR screen (data not shown). C8 was representative of most hygromycin resistant colonies in our study, which tested positive for Algal-CAM genomic insertion but failed to express transgene mRNA due to possible silencing mechanisms, improper positional insertion or other unknown reasons (Cerutti et al. 1997; Fuhrmann et al. 1999; 2004; Schroda et al. 2002; Wu-Scharf et al. 2000). As a negative expression control, transformants were also generated with pChlamy_1 lacking the Algal-CAM CDS. Figure 2 includes one of these empty vector controls, designated (−). In the right panel of Fig. 2, a negative RT-PCR control performed without reverse transcriptase is also shown. No amplification was detected in this control, demonstrating that genomic DNA contamination or spurious amplification of a native *Chlamydomonas* sequence were not responsible for the Algal-CAM mRNA expression exhibited by strains C11 and C12.

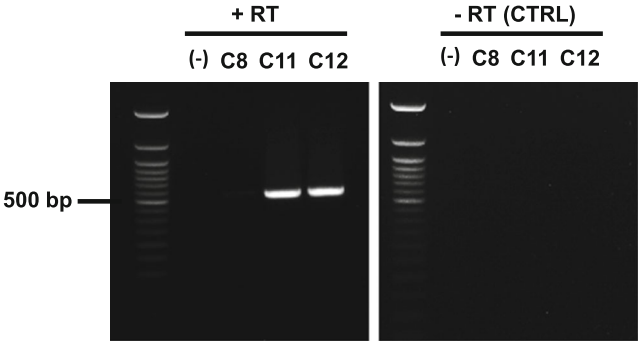

Fig. 2 Two *C. reinhardtii* transformants showing heterologous transcription of the Algal-CAM transgene. RT-PCR results are shown for four different strains. *Left panel* The empty vector control strain, **(−)**, and transformant **C8** tested negative for recombinant Algal-CAM transcript. In PCR screens, transformant **C8** tested positive for genomic insertion of the Algal-CAM CDS (data not shown). Transformants **C11** and **C12** showed high levels of transgene mRNA expression. The Intron-I spanning forward primer and reverse primer position were designed to yield a 519-bp amplicon from cDNA template. *Right panel* Absence of genomic DNA contamination was confirmed by replicating the experiment using no reverse transcriptase (−RT) in the cDNA synthesis step

Heterologous Algal-CAM protein analysis and localization

Huber and Sumper (1994) concluded that native Algal-CAM is secreted to the *V. carteri* cell surface and insolubilized in the ECM by covalent crosslinking of tyrosine residues of the extensin domains. Extensin proteins of *C. reinhardtii* and higher plants are thought to behave similarly (Fry 1982; Waffenschmidt et al. 1993). Furthermore, after removal and analysis of peripheral membrane proteins, Huber and Sumper proposed that Algal-CAM attaches to the *V. carteri* cell surface by protein–protein or protein–carbohydrate interactions. Supporting this conclusion, protein informatics analysis shown in Fig. 3 reveals an N-terminal signal peptide cleavage site, predicted N-linked glycosylation sites and putative tyrosine crosslinking motifs located within the extensin-like domain. Figure 3 also shows extensin-like and fasciclin-like regions, characteristic extensin Ser-Pro(3–7) repeat motifs, and recombinant protein features, such as the C-terminal FLAG tag epitope and flexible linker.

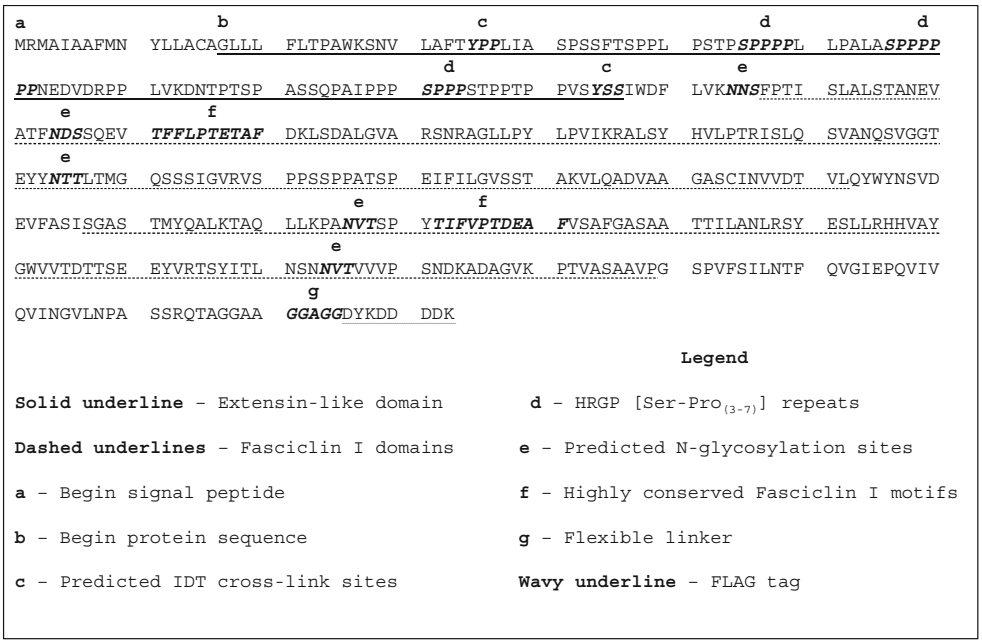

Fig. 3 Amino acid sequence of the recombinant Algal-CAM protein predicted to result from the pChlamy_ACAM insert. Analysis is based on the *V. carteri* Algal-CAM study of Huber and Sumper (1994). Features of the sequence are indicated by *underlining* or ***bold italics*** as described in the *Legend*. The predicted molecular weight of the signal peptide-cleaved, non-modified, non-hydroxylated, and non-glycosylated Algal-CAM polypeptide is 45.8 kDa

Protein sorting, post-translational processing, membrane trafficking, and secretion are predicted to be almost identical between *V. carteri* and *C. reinhardtii* (Prochnik et al. 2010). Because of this homology, we reasoned that Algal-CAM would likely be secreted to and anchored in the *C. reinhardtii* ECM in a manner similar to its function in *V. carteri*. The CC-400 "wall-less" strain of *C. reinhardtii* used for transformation lacks the "central triplet" ECM layers (W2, W4 and W6) present in other *C. reinhardtii* strains but retains perchlorate-insoluble cell wall layers resembling the innermost and outermost (W1 and W7) layers (Goodenough and Heuser 1985; Hyams and Davies 1972; Monk et al. 1983). The insolubility of W1 and W7 layers in chaotropic solutions demonstrates the covalent nature of cross-linking in these layers (Goodenough and Heuser 1985). Based on previously noted observations of Huber and Sumper (1994), the isodityrosine (IDT) cross-linking characteristics of W1 and W7 wall layers of *C. reinhardtii*, protein informatics analysis, and knowledge of the CC-400 strain ECM composition, we predicted that the extensin-like domain of Algal-CAM would covalently anchor into the W1 and W7 ECM layers of *C. reinhardtii*.

Presence of the heterologous Algal-CAM protein in C11 and C12 transformants was verified by immunoblot. Owing to the likelihood of covalent anchoring within the ECM, we concluded that heterologous Algal-CAM would be more readily detectable within the intracellular secretory pathway rather than in the ECM. Thus, we initially probed whole cell lysates by immunoblot against the FLAG tag of the heterologous sequence. Figure 4a shows that a faint FLAG tag presence was detected in mRNA positive expression colonies (C11 and C12) but was absent from negative mRNA expression (C7 and C8) or empty vector (−) negative controls. However, the protein fragments detected in C11 and C12 whole cell lysates were below 35 kDa, while the amino acid sequence of heterologous Algal-CAM predicts a molecular mass of 45.8 kDa for the non-hydroxylated, non-glycosylated polypeptide with signal peptide cleaved. Moreover, we anticipated heterologous Algal-CAM to be heavily glycosylated, giving it a much larger molecular mass than 45.8 kDa. Indeed, Huber and Sumper (1994) observed an apparent molecular weight of 150 kDa for Algal-CAM extracted from the *V. carteri* ECM, which was attributed to glycosylation, covalent cross-linking with other ECM proteins, and a general characteristic of extensin proteins to appear larger in SDS-PAGE gels than predicted. For these reasons, we hypothesize that the <35 kDa protein fragment observed in our whole cell lysates (Fig. 4a) was a partially degraded form of heterologous Algal-CAM resulting from intracellular protease activity during normal cellular function or after cell lysis (Scopes 1994).

Since probing for heterologous Algal-CAM in whole cell lysates did not yield evidence of the intact protein and did not address whether Algal-CAM was present in the ECM, we adopted a strategy of digesting Algal-CAM from the ECM and inhibiting subsequent ECM reformation by oxidative isodityrosine (IDT) cross-linking. Waffenschmidt et al. (1993) devised this strategy previously, digesting cross-linked ECM proteins with *Chlamydomonas* gamete lytic enzyme (GLE)

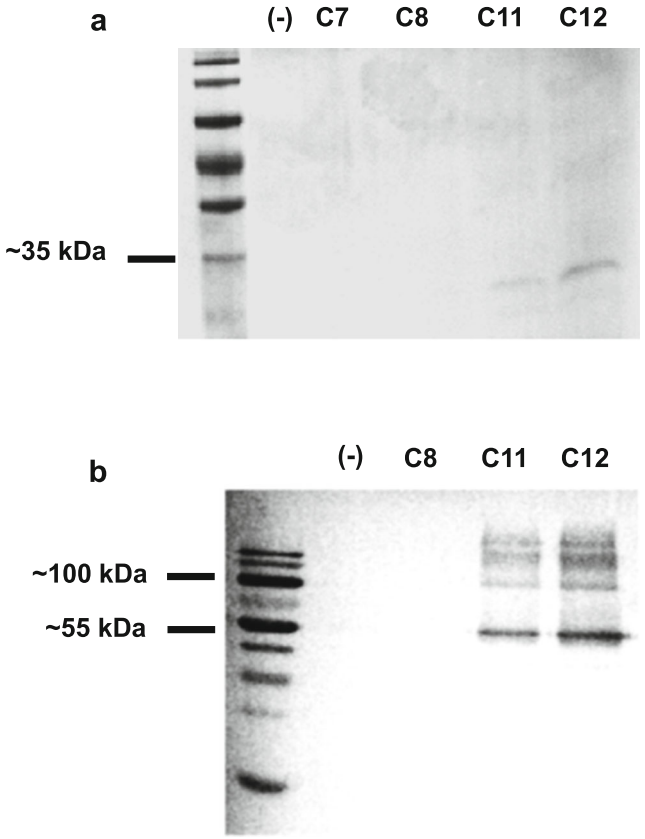

Fig. 4 Expression, secretion, and localization of heterologous Algal-CAM_FLAG tag fusion protein. Following PCR and RT-PCR screening, whole cell lysates (**a**) or ECM-digested/iso-dityrosine cross-linking inhibited supernatant fractions (**b**) were assayed for heterologous protein by immunoblotting with an α-FLAG antibody. Molecular weights were estimated from the standard protein ladder in the *left-most lane* of both gels. The *upper panel* (**a**) shows a <35 kDa FLAG-tagged protein that may be a degradation product present in crude cell lysates of flocculating, transcript-positive transformants **C11** and **C12** but absent from non-flocculating, empty vector control (−) and transcript-negative transformants **C7** and **C8**. The *lower panel* (**b**) shows immunological detection of FLAG tag in supernatant fractions derived from cells treated with crude gamete autolysin (GLE) and iso-dityrosine cross-linking inhibitors. FLAG tag signal was detected in flocculating, transcript-positive transformants **C11** and **C12** but not in non-flocculating, empty vector control (−) or transcript-negative transformant **C8**. The calculated molecular mass of signal peptide-cleaved Algal-CAM fusion protein is 45.8 kDa with no hydroxylation or glycosylation. High molecular weight bands (above 55 kDa) likely indicate glycosylated Algal-CAM and/or Algal-CAM cross-linked with itself or native extensin-like proteins in the *C. reinhardtii* ECM. These high molecular weight bands are consistent with observations by Huber and Sumper (1994) of an apparent molecular weight of 150 kDa for *V. carteri* Algal-CAM with a ladder-like banding pattern explained as variable glycosylation

and inhibiting IDT cross-linking with ascorbate and free tyrosine. We used GLE to digest ECMs of the C11 and C12 transformants and controls. Following digestion, reformation of IDT cross-links was blocked with 50 mM ascorbate and 5 mM tyrosine at pH 7.4 to inhibit peroxidase-mediated IDT cross-linking by an alternative reducing agent and by competitive inhibition, respectively (Waffenschmidt et al. 1993). Following this treatment, supernatants of the digestion were assayed for FLAG tag presence via immunoblot (Fig. 4b). We detected multiple bands of heterologous Algal-CAM via FLAG tag presence in transcript-positive colonies C11 and C12 but not in the transcript-negative control (C8) nor in the empty vector control (−). Combined with the whole cell lysate immunoblot data, these results show that GLE digestion of the ECM and inhibition of IDT cross-link formation liberates heterologous Algal-CAM into the supernatant.

GE flocculating phenotype

When transformant colonies expressing Algal-*CAM* were cultured in liquid, varying degrees of constitutive flocculation were observed in comparison to the empty vector control and the negative expression control (Fig. 5). Transgenic strains expressing heterologous Algal-CAM formed large flocs that tended to loosely adhere to each other (Fig. 5a, C11 and C12) and settle to the bottom of the culture in less than 5 min after shaking. Cells that had been similarly transformed and cultured but did not express Algal-CAM mRNA or protein stayed in solution and did not form flocs (Fig. 5, (−) and C8). Because transformed control strains did not express Algal-CAM and did not flocculate but had been exposed to the same treatments and culture conditions as flocculating Algal-CAM positive strains, we conclude that our observed flocculating phenotype was caused by heterologous Algal-CAM expression and not by transformation treatments or culture conditions. Moreover, earlier transformations with Algal-CAM using preliminary vectors caused flocculation in three additional strains expressing Algal-CAM mRNA while no flocculation was observed in a non-transformed control (Online Resource 1). In sum, we observed five separate transformed strains that expressed Algal-CAM messenger RNA and three separate controls that did not (Fig. 2 and Online Resource 1). All strains expressing mRNA for Algal-CAM flocculated while all controls not expressing mRNA for Algal-CAM did not.

We quantified our GE flocculating phenotype using sedimentation kinetics as described by Salim et al. (2011) and Zheng et al. (2012). This method measures sedimentation rates by simply shaking algal cultures in a spectrophotometer cuvette and measuring the OD_{750} over time at a defined height in the cuvette. Directly after shaking, however, our flocculating strains immediately settled to the bottom of the cuvette before the time 0 measurement could be taken. Therefore we modified the protocol by taking a single OD_{750} measurement 5 min after shaking. Following this modified procedure (Fig. 5b), *C. reinhardtii* cells expressing Algal-CAM (C11 and C12) had significantly lower turbidity (approximately 0.2 OD_{750}) than cells not expressing Algal-CAM (C8 and (−), approximately 1.7 OD_{750}). Lower turbidity could also be due to lower cell numbers or concentration. To show that

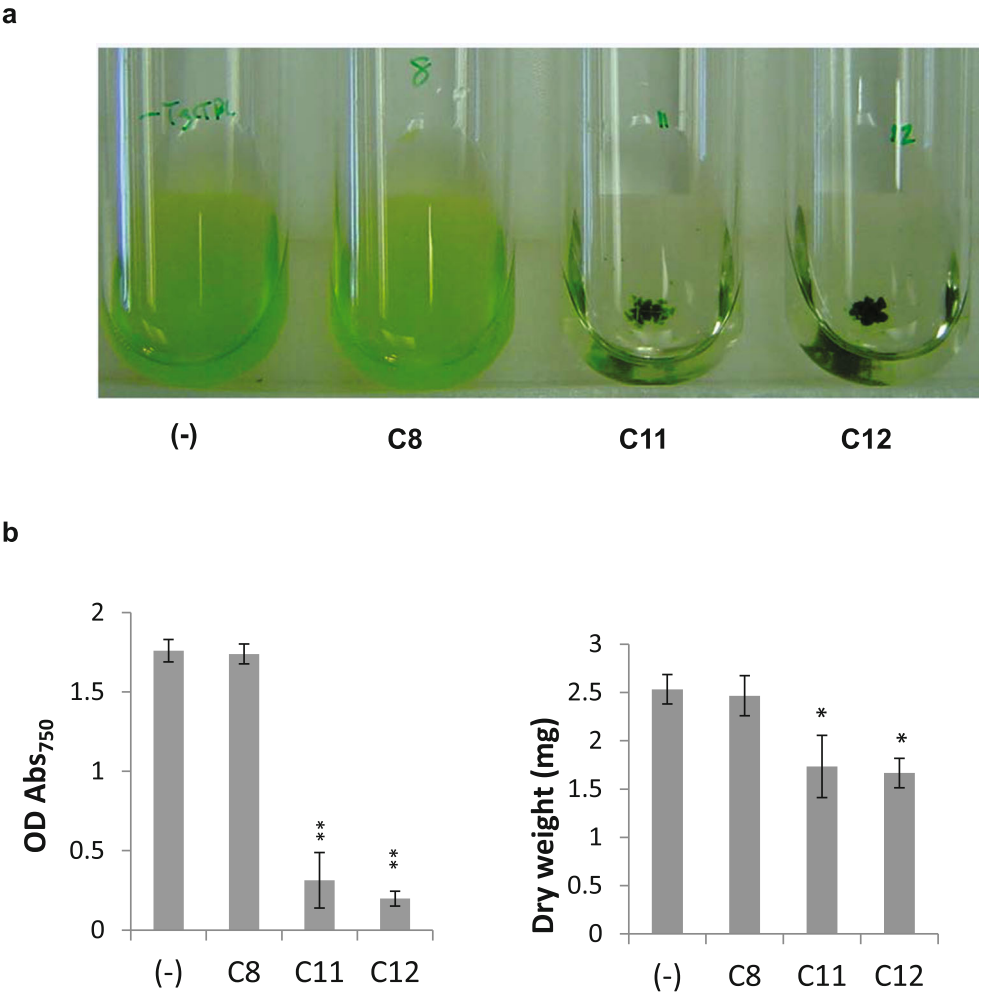

Fig. 5 GE flocculating phenotype. The *upper panel* (**a**) shows GE flocculating cultures. Shortly after shaking, transgenic strains expressing Algal-CAM mRNA and protein (**C11** and **C12**) form loose clumps at the bottom of culture tubes while transgenic control strains not expressing Algal-CAM ((−) and **C8**) stay suspended in solution as unicells. The *lower panel* (**b**) shows quantification of the flocculating phenotype using optical density readings at 750 nm (OD Abs_{750}) in standard 1 cm cuvettes as described in the "Methods and materials" section. Dry weight measurements serve as a biomass reference for OD Abs_{750} readings. *Bars* show mean values +/− standard deviations of the sample. $n=3$ for all mean values. *Asterisks* designate significant difference from (−) and C8 control strains as tested by a one-way analysis of variance and Tukey's post hoc test (**$p<.0001$; * $p<.002$)

the experimental cultures had similar amounts of biomass per sample, we measured the dry weights of samples used for OD measurements (Fig. 5b). These were not identical between experimental groups but were similar, indicating that similar amounts of biomass were settling in the Algal-CAM expressing cuvettes as in controls but much more quickly.

Discussion

For transformation with *Volvox* Algal-CAM, utilized the CC-400 "wall-less" strain of *C. reinhardtii* rather than strains with normal cell walls for several reasons. First, the CC-400 strain was easily transformed using the nuclear glass bead method (Kindle 1990). Second, the decreased bulk of the CC-400 ECM was less likely to provide steric hindrance or interference with intercellular homophilic binding between the Fasciclin I domains of heterologous Algal-CAM molecules. Third, absence of the highly condensed and crystallized W2, W4, and W6 "central triplet" layers of the CC-400 ECM reduced the probability that Algal-CAM transport, assembly, or display on the ECM surface would be restricted. Finally, the W1 and W7 wall layers present in CC-400 were known to be digestible by GLE, allowing for immunoblot analysis of proteins released from the ECM by GLE digestion.

GLE is an HRGP-specific protease that can be isolated from mating *C. reinhardtii* gametes. It rapidly digests the highly cross-linked W2 layer of the wild type *C. reinhardtii* ECM and, with more time, the W6 layer as well. Goodenough and Heuser (1985) concluded that GLE also digested the W1 ECM layer, leading to complete loss of the ECM during gamete fusion. Similarly, Jaenicke and colleagues (1987) observed GLE to digest the perchlorate insoluble layers of the ECM, including the innermost W1 layer, as indicated by peptide fragment release assayed with fluorescamine. These observations supported use of GLE to digest the reduced ECM of the CC-400 strain *of C. reinhardtii*, which are thought to consist of only the W1 and W7 layers (Monk et al. 1983).

Heterologous Algal-CAM in the CC-400 ECM

Immunoblots of GLE-digested cells yielded multiple FLAG-tagged protein bands from the C11 and C12 transformants (Fig. 4b). This pattern likely represents Algal-CAM liberated

from the ECM by GLE as well as Algal-CAM freshly released by the secretory pathway. The major band near 50 kDa may represent Algal-CAM from which the N-terminal, extensin-like domain was digested by GLE, leaving a C-terminal, FLAG-tagged fragment. Alternatively, the band near 50 kDa may represent freshly secreted Algal-CAM that is not extensively glycosylated or otherwise post-translationally modified, since the predicted molecular mass of non-glycosylated, non-hydroxylated Algal-CAM is 45.8 kDa. Higher molecular weight bands in Fig. 4b are consistent with observations by Huber and Sumper (1994) that Algal-CAM exhibits an apparent molecular mass much higher than 45.8 kDa in SDS-PAGE gels owing to cross-linking with other polypeptides, extensive glycosylation, and the inherently slow mobility of extensin proteins in polyacrylamide gels. In control strains of *C. reinhardtii* that did not express mRNA for Algal-CAM ((−) and C8), no FLAG-tagged proteins were detected. Thus, our detection of multiple FLAG tagged protein bands in strains C11 and C12 was specific for presence of heterologous Algal-CAM and not the result of non-specific labeling of native proteins. Furthermore, because whole cell lysates of C11 and C12 that were not treated with GLE did not show high molecular weight protein bands (Fig. 4a), we conclude that the heterologous Algal-CAM in strains C11 and C12 was embedded and cross-linked in the ECMs of these strains. Overall, these results support the conclusion that heterologous Algal-CAM was secreted into the ECM of strains C11 and C12 and cross-linked with itself or with native HRGPs.

Genetically engineered (GE) flocculation

The Algal-CAM-expressing strains of *C. reinhardtii* presented here flocculate on their own power and thus could be described as "autoflocculating." However, the term autoflocculation has been used previously to describe algae that flocculate spontaneously due to changes in pH or ionic strength of their culture water (Spilling et al. 2010; Sukenik and Shelef 1984). Similarly, "bio-flocculation" has been used to describe algae that flocculate with addition of biologically derived flocculants or flocculation-inducing microorganisms (Salim et al. 2011; Salim et al. 2014; Vandamme et al. 2013). To distinguish our approach from these, we refer to it as "genetically engineered" (GE) flocculation.

GE flocculation of microalgae can be extremely challenging because of the complex nature of algal ECMs and their biosynthesis. For this reason, we took a simplified approach by constitutively expressing a well-characterized cell adhesion protein from a close multi-cellular relative in the CC-400 "wall-less" strain of *C. reinhardtii*. The glycoprotein construction of the *Chlamydomonas* ECM allows expression of a single heterologous glycoprotein to change its composition and properties, as we have demonstrated here. In contrast, genetic engineering of algal ECMs composed primarily of carbohydrates, for example, would require coordinated expression of multiple transgenes for altered synthesis of those carbohydrates while running the risk of compromising growth by competition for precursors needed by other metabolism. A further advantage of the *Chlamydomonas* ECM is that its proteins self-assemble by oxidative cross-linking, comparable to self-assembly of lignin molecules in the plant cell wall (Achyuthan et al. 2010). For this reason, manipulation of enzymes resident in the ECM is unnecessary to achieve covalent bonding between native and heterologous ECM glycoproteins. In sum, the glycoprotein ECMs of volvocalean algae offer an exceptionally attractive system for experimental ECM re-engineering.

Our results show that microalgae can be genetically engineered to flocculate by heterologous expression of cell adhesion molecules. An improvement on our results would be to make Algal-CAM expression inducible. For example, the Algal-CAM transgene could be assembled downstream of a nitrate reductase (NIT1) promoter that would induce expression as culture growth depleted ammonium from a high ammonium/low nitrate culture medium (Ohresser et al. 1997). With tuning of the ammonium to nitrate ratio in the medium, N-limitation could simultaneously induce flocculation and accumulation of lipids and starches (Siaut et al. 2011). Govender et al. (2008) demonstrated comparable designs for GE flocculation in yeast using flocculation transgenes that were induced as the cultures entered stationary phase.

Further study of Algal-CAM function in *Chlamydomonas* could lead to Algal-CAM-based flocculation in other taxa of microalgae or use of Algal-CAM for cellular immobilization on artificial substrates. Such immobilization could be accomplished by generating chimeric proteins that contain an N-terminal wall-anchoring domain and a C-terminal, homophilic fasciclin I domain with an optional glycosylphosphatidylinositol (GPI) linkage signal sequence. The variable domain would be the wall-anchoring domain, which could be customized for individual wall compositions of different algal taxa. For immobilization purposes, the extensin domain could be fused to established domains for protein immobilization, such as glutathione S-transferase, SNAP-tag, or others. This strategy could effectively immobilize *C. reinhardtii* or other algal cells on a synthetic substrate for any number of possible applications.

Our results demonstrate genetic engineering of the composition and properties of an algal ECM. To our knowledge, this is a novel advancement. We predict that further manipulation of algal ECMs can lead to new materials and new bioengineering opportunities. For example, photosynthetic unicells could be engineered to adhere together into multicellular sheets or tubes or more complex shapes having novel ECM compositions. Sheet-like and tube-like green algae related to *Chlamydomonas* occur naturally and may offer genetic blueprints for self-assembly that can be emulated in

Chlamydomonas. Ultimately, rational redesign of the ECMs of *Chlamydomonas* and other algae could provide more sustainable replacements for plastics and other petroleum-derived materials, creating new a new field of biomaterial design and innovation.

Acknowledgements The authors gratefully acknowledge funding from a Wyoming NASA Space Grant fellowship to LGL. SKH acknowledges funding from USDA NRI grant 2006-35318-17445, from the School of Energy Resources and Department of Plant Sciences at the University of Wyoming, and from the SciFund Challenge 1, a science crowd-funding experiment held in 2011. We also thank Professor Anne Sylvester and Dr. Anding Luo of the University of Wyoming for graduate training provided to LGL with support from the US National Science Foundation, grant #1027445. Professor David Legg of the University of Wyoming provided statistical consultation.

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