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Construction and Validation of a Chloroplast Expression Vector for the Production of Recombinant Proteins in *Chlorella vulgaris*

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Microalgae constitute a diverse group of photosynthetic unicellular microorganisms that have gained immense attention for biotechnological applications. They are promising platforms for the production of high-value metabolites and biopharmaceuticals for commercial and therapeutic applications because of their physiological properties and capability to grow easily in natural and artificial environments. Although the proof-of-concept for some applications have been achieved for model species, such as *Chlamydomonas reinhardtii*, the genetic engineering methods for microalgae are still in their infancy. Thus, an expansion of this field is required. *Chlorella vulgaris* is an important algal species with a high protein content and requires focus for the development of an efficient nuclear and chloroplast transformation process. This research aimed to establish a chloroplast transformation method for the freshwater green-algae species *C. vulgaris* based on a specific expression vector (pCMCC, which was named after Chula Mexico *Chlorella* chloroplast) constructed with endogenous recombination regions, namely, *16S–trn I* (left) and *trn A–23S* (right), and the *Prrn* promoter. Human basic fibroblast growth factor (bFGF) was adopted as a target biopharmaceutical to establish the chloroplast expression approach. The plasmid pCMCC:bFGF was transformed into *C. vulgaris via* electroporation using simple carbohydrate-based buffers, which aided in the transfer of the transgene into the chloroplast genome. Cells transformed with the pCMCC:bFGF vector were selected using kanamycin, and resistant colonies were analyzed by polymerase chain reaction and Western blotting to confirm the presence of the transgene and the recombinant bFGF, respectively. The bFGF that accumulated in the transplastomic *C. vulgaris* clones ranged from 0.26 to 1.42 ng/g fresh weight of biomass, and it was quantified by enzyme-linked immunosorbent assay. Therefore, the designed expression vector, in combination with an optimized electroporation protocol, constitutes a viable approach to successfully develop transplastomic lines of *C. vulgaris* for the potential low-cost production of biopharmaceuticals using this algal species. This study

paves the way for the establishment of chloroplast biotechnology in microalgae other than the model organism *C. reinhardtii*.

Keywords: electroporation, freshwater green algae, homologous recombination, genetic engineering, biopharmaceuticals

INTRODUCTION

The global population is on exponential rise with the drastic depletion of naturally available products, leading to a high requirement of valuable biologicals. Thus, an expansion of such productive systems is required to meet their demand. Hence, the current scenario redirects to the development of alternative strategies for the production of such molecules in high yields using safe and economical technologies (Siddiqui et al., 2020). With the advent of genetic engineering, various platforms, such as those based on bacteria (Huang et al., 2012; Gupta and Shukla, 2016; Demain and Vaishnav, 2009), yeast (Ahmad et al., 2014; Kim et al., 2015; Baghban et al., 2019; Huertas and Michán, 2019), mammalian cells (Hacker and Balasubramanian, 2016; Gupta et al., 2017; Owczarek et al., 2019; O'Flaherty et al., 2020), insect cells (Contreras-Gómez et al., 2014; Felberbaum, 2015; Kost and Kemp, 2016), plants (Shanmugaraj and Ramalingam, 2014; Lomonossoff and D'Aoust, 2016; Shanmugaraj et al., 2020), and transgenic animals (Moura et al., 2011; Maksimenko et al., 2013), have been largely and profoundly studied to explore the potential to develop them as mature platforms for the heterologous expression of proteins that have commercial and industrial importance (Merlin et al., 2014). However, although advances in the use of expression hosts for sustainable bioprocesses for the production of high-value products have been achieved, many challenges still need to be overcome in this field. Such challenges include strategies such as diminishing the risk of contamination (*Mycoplasma*, bacterial, viral, fungal, and endotoxin) (Merten, 2002; Stacey, 2011; Singh et al., 2020), meeting the regulatory requirements, reduction in operational costs, improving the process controls, and robustness in the environmental conditions and testing methods (Tripathi and Shrivastava, 2019; Kumar et al., 2020).

Eukaryotic unicellular microalgae comprise a vast diversified group of organisms that have been adopted as a promising platform for the production of recombinant products with a wide set of applications (Hallmann, 2015). The distinctive features of microalgae, such as habitat, nutrient requirements, and behavioral patterns, make these organisms an attractive platform for biotechnological applications. Apart from having great ecological and economical importance, microalgae are being utilized in the food, feed, fertilizer, cosmetic, and pharmaceutical industries because they are natural bio-factories enriched with valuable molecules, such as proteins, pigments, polysaccharides, lipids, and nutraceuticals (Lordan et al., 2011; Milledge, 2011; Ibañez et al., 2012; Yaakob et al., 2014; Nur and Buma, 2019). Therefore, these ubiquitous (Metting, 1996) and heterogeneous groups of microalgae are promising platforms for the production of a wide range of biological compounds. These microorganisms need to be genetically modified using advanced molecular tools to exploit their full potential for beneficial purposes.

Microalgae are simpler yet more complex organisms for gene manipulation in comparison with higher plants due to the absence of cell differentiation. Although significant efforts have been exerted to establish molecular tools and techniques for the genetic manipulation of microalgae (Potvin and Zhang, 2010; Ashwini et al., 2016; Tanwar et al., 2018; Kumar et al., 2020; Mutanda et al., 2020), they remain a fairly unexplored area to overcome the difficulty in the efficient delivery of exogenous DNA within the cytoplasm, nucleus, or chloroplast in microalgae given that they are single-cell entities with a thick cell wall (Ortiz-Matamoros et al., 2018; Fajardo et al., 2020). The most commonly used techniques to introduce foreign DNA into microalgal cells are cell bombardment with DNA-coated gold or tungsten particles (Sizova et al., 1996; El-Sheekh, 2000; Remacle et al., 2006; Xie et al., 2014; Li et al., 2016; Yuan et al., 2019) and glass bead agitation (Economou et al., 2014; Adachi et al., 2017), followed by *Agrobacterium tumefaciens*-mediated transformation (Kumar et al., 2004; Pratheesh et al., 2014) and electroporation (Ladygin, 2003; Kumar et al., 2004; Herz et al., 2005; Zhang et al., 2014; Kang et al., 2015; Gan et al., 2018). Other methods used include the agitation with foreign plasmid DNA and silicon carbide whiskers (Dunahay, 1993) and polyethylene glycol (PEG) (Maliga, 2004). However, gene manipulation in algae is not easily attained, unlike in bacterial or plant cells, posing various scientific hassles due to the multifarious cell sizes, complex and multitudinous cell wall components, and confining transformation methods to a limited number of species (Doron et al., 2016; Ortiz-Matamoros et al., 2018; Gan et al., 2018).

The *Chlorella* genus represents one of the specialized groups of green algae that can produce high levels of proteins with rapid growth rate and large cell densities in controlled conditions, thereby drawing attention toward the genetic manipulation of their genomes (Yang et al., 2016). The *Chlorella* species possess a robust cell wall with a protective function, acting as a strong barrier to extract high-value compounds enclosed in the cell and limiting the insertion process of exogenous DNA. In particular, the *Chlorella vulgaris* cell wall is composed of a single microfibrillar layer at the beginning of the growth phase, followed by the development of a two-layer structure, where the thick outer and thin inner layers constitute the mother and daughter cell walls, respectively. The cell wall of *C. vulgaris* consists of 20–25% neutral sugars, 10–20% uronic acids, 7–17% glucosamine, 6–10% proteins, monosaccharides, such as rhamnose (20 to 34%), arabinose (12 to 20%), and glucose (16 to 46%), and a rigid fraction of polysaccharides, including chitin or chitosan. The cell wall increases from 82 ± 20 nm in the

exponential phase to 114 ± 24 nm in the stationary phase, whereby the nascent cell wall is usually thin and fragile, and the thickness gradually increases in the developed phase (Takeda, 1988; Kapaun and Reisser, 1995; Canelli et al., 2021; Weber et al., 2022). These cell wall characteristics are a major hurdle and should be addressed during the development of a novel genetic transformation approach.

Although the majority of genetically modified organisms are generated by the integration of transgenes in their genome, the case of photosynthetic organisms also offers the opportunity for adopting chloroplast transformation. Such organelles are remnants of endosymbiotic cyanobacteria that have gained great interest for genetic manipulation due to their homologous recombination system, facilitating the site-specific integration of foreign DNA, which avoids position effects and leads to a high copy number for the transgene; however, no silencing processes have been found in these organelles (Daniell et al., 2002; Vafaee et al., 2014). Strong specific promoters [cauliflower mosaic virus 35S (CaMV35S) promoter, α-tubulin, RBCS2, PSAD, Prrn, FCP, NIT1, and Ubi1-Ω] may lead to a higher transgene expression level with protein accumulations (Metting, 1996; Mayfield et al., 2003; Mayfield et al., 2007; Potvin and Zhang, 2010; Rasala et al., 2011; Hallmann, 2015; Doron et al., 2016; Hamed, 2016; Adem et al., 2017; Zhang et al., 2017; Baier et al., 2018; Mutanda et al., 2020). Apart from regulatory elements, the ribosome binding site (RBS) plays a pivotal role in protein translation (Specht and Mayfield, 2013). Therefore, compared with nuclear genome manipulation, the transformation of the chloroplast genome offers various advantages, namely, uniform transgene expression and compartmentalization of the produced recombinant proteins in the chloroplast without affecting other cellular processes (Georgianna and Mayfield, 2012; Doron et al., 2016; Siddiqui et al., 2020). Achievements in chloroplast transformation have been reported in higher plants, such as tobacco, lettuce, rapeseed, potato, tomato, petunia, soybean, and cotton, and some monocots, including rice; cotton, as an efficient system for the expression of functional recombinant proteins, uses specific plastid promoter-driven and other genetic factors (Daniell et al., 2002; Verma and Daniell, 2007; Wani et al., 2015; Daniell et al., 2021). The research and development of plastid microalgal biotechnology are still a major obstacle given that such technology is well established only for the model species *Chlamydomonas reinhardtii* (Bateman and Purton, 2000; Mayfield et al., 2007; Rasala and Mayfield, 2011; Rosales-Mendoza et al., 2012; Almaraz-Delgado et al., 2014; Economou et al., 2014; Munjal et al., 2014; Scranton et al., 2015; Larrea-Alvareza and Purton, 2020). Other species engineered at the chloroplast genome level include *Platymonas subcordiformis* (Cui et al., 2014; Siddiqui et al., 2020), *Nannochloropsis oceanica* (Gan et al., 2018; Siddiqui et al., 2020), *Phaeodactylum tricornatum* (Xie et al., 2014; Serif et al., 2018), *Dunaliella tertiolecta* (Georgianna et al., 2013; Anila et al., 2016), and *Haematococcus pluvialis* (Gutiérrez et al., 2012; Wang et al., 2020).

Chlorella is a genus of considerable interest in biotechnology because its species are extensively applied for commercial use as a food supplement due to their high biomass, nutritive value, and ability to grow easily in mixotrophic conditions (Spolaore et al., 2006; Kent et al., 2015; Cecchin et al., 2019). For *C. vulgaris*, anti-colorectal cancer activity and immunomodulatory effects associated to exopolysaccharides and long-term diet have been described, respectively (Cheng et al., 2017; Zhang et al., 2019). In addition, *C. vulgaris* can be used in medical treatments for non-communicable diseases, such as hypertension, cataract, and dyslipidemia (Panahi et al., 2016; Xie et al., 2018). On the other hand, the development of genetic engineering strategies for their emergent use as bioreactors for the production of recombinant proteins and biopharmaceuticals has attracted scientific interest. In a previous work, protoplasts obtained from *C. vulgaris* C-27 were used for nuclear transformation *via* the PEG method, and a subsequent heterologous expression of human growth hormone (hGH) was achieved. Moreover, the strategy encompassed the addition of an extracellular signal sequence for hGH secretion into the extracellular medium, the use of geneticin as an antibiotic for selection, and the assay of CaMV35s and rbcS2 promoters. The hGH was expressed in the range of 200–600 ng/ml (Hawkins and Nakamura, 1999). Wang et al. (2021) reported a chloroplast transformation-based system in which two codon-optimized antimicrobial peptides (NZ2114 and piscidin-4) were co-expressed; the green microalga *C. vulgaris* was transformed *via* microparticle bombardment, and the homoplastic transformants were screened using spectinomycin as a selectable marker.

Hence, to target the semi-autonomous replicating chloroplast organelle for genetic engineering in *C. vulgaris*, this study focused on the assembly of a species-specific DNA vector to produce high-value proteins. A specific plastid expression vector, namely, pCMCC (named after Chula Mexico *Chlorella* chloroplast), was designed to express various recombinant genes in *C. vulgaris*. The human basic fibroblast growth factor (bFGF) was selected as a representative biopharmaceutical to be expressed using the pCMCC vector. This protein has a significant commercial demand. As an example, the bFGF may be indicated in diverse disciplines of dentistry, such as dental traumatology and periodontics, due to its capability to induce the differentiation and proliferation of cells. Thus, it is a useful tool for the development of natural structures. The production process for this protein can be rapidly scaled up using a transplastomic microalgal strain formerly modified with a simple and efficient transformation method. In the current study, the potential of the pCMCC vector applying the electroporation method for chloroplast transformation of *C. vulgaris* was assessed, and a rapid approach of the method using carbohydrate-based buffers was accomplished.

MATERIALS AND METHODS

Microalgal Growth Conditions

An axenic culture of *C. vulgaris* was obtained from the Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand. The *C. vulgaris* culture was grown in f/2 media without silicates or Bold's basal medium (BBM) from Phyto Tech Labs. The *C. vulgaris* cultures were incubated on a rotary shaker (150 rpm) for

1 week at 28 ± 1°C with cool fluorescent lamps at a photosynthetic photon flux density of 80–100 μmol m^{-2} s^{-1} and a 16:8-h light/dark cycle. Thereafter, a routine subculture was performed by transferring the cultures to a fresh culture medium every 7 days. For chloroplast transformation, *C. vulgaris* was inoculated at 20% (v/v) in fresh f/2 media and cultivated for 4 to 5 days at 150 rpm until the growth approached the log phase. The cell count was performed by using a hemocytometer, and the cell number was adjusted to 5×10^6 cells/ml with f/2 media and grown for 24–48 h before competent cell preparation for chloroplast transformation.

Vector Construction and Transformation into *Escherichia coli*

A species-specific plastid transformation vector called pCMCC was constructed to test the integration and expression of the human bFGF gene (the amino acid sequence was obtained from GenBank with accession number: AAQ73204.1) through homologous recombination in the *C. vulgaris* plastid genome. The homologous recombination elements were designed as R1 (16S-*trnI*) and R2 (*trnA*-23S), where the R1 and R2 regions comprise 2171 and 2000 nt, respectively. The expression cassette was led by the *Prrn* promoter (Klein et al., 1992) from *C. reinhardtii*, untranslated region (UTR) from T7 phage (Herz et al., 2005), *Aph*6 selection marker gene (Bateman and Purton, 2000) flanked by *psbA* terminator (Eibl et al., 1999), and *bFGF* gene optimized for expression in *C. vulgaris* linked with 8X histidine tag. The *bFGF* gene was flanked by *Xho*I and *Sac*I restriction sites to further facilitate the replacement with other genes of interest. The expression cassette possessed a bicistronic arrangement that allowed the expression of the *bFGF* gene and marker *Aph*6, which conferred kanamycin resistance to the transformed cells under the control of the *Prrn* promoter. An RBS (Koop et al., 1996) sequence was placed between the open reading frames to mediate the translation of the second gene (*Aph*6) in the bicistronic arrangement. **Figure 1** shows the schematic representation of pCMCC:bFGF vector. The bFGF gene was subjected to a codon optimization protocol before synthesis, which allowed the achievement of a codon adaptation index of 0.96 with a GC content of 43.8%, which maximized RNA stability and its translation rate. The recombination flanks and expression cassette were fully synthesized by GenScript (New Jersey, USA) and confirmed by sequencing. The pCMCC vector is under patenting process (DIP TH Petition No. 2001006378). The expression vector was transformed into *Escherichia coli* TOP10 cells by heat shock, and the resulting clones were confirmed by polymerase chain reaction (PCR). The pCMCC:bFGF plasmid was isolated from the confirmed *E. coli* clones using a Presto™ mini plasmid kit (Geneaid, Taiwan) and further confirmed by PCR and restriction profiling.

Chloroplast Transformation in *C. vulgaris* by Electroporation

Two methods were employed for the chloroplast transformation in *C. vulgaris* using pulse field electroporation. The first methodology was based on the procedure reported by Ji and Fan (2020). The microalgal cells were centrifuged, and the pellet was washed in ice-cold hypertonic buffer solution containing 0.2 M mannitol and 0.2 M sorbitol for 1 h by immersion in ice. The algal cells were centrifuged and resuspended in 1 ml electroporation buffer (0.2 M mannitol, 0.2 M sorbitol, 0.08 M KCl, 0.005 M $CaCl_2$, and 0.01 M HEPES; pH 7.2). The cell density was adjusted to 5×10^6 cells ml^{-1}. Then, 30 μg/ml circular and linearized pCMCC:bFGF DNA and 50 μg salmon sperm DNA were added to 100 μl cell suspension in an electric-shock cuvette and kept in an ice bath for 5 min. Electroporation was carried out using a Bio-Rad Gene-Pulser X cell apparatus at a

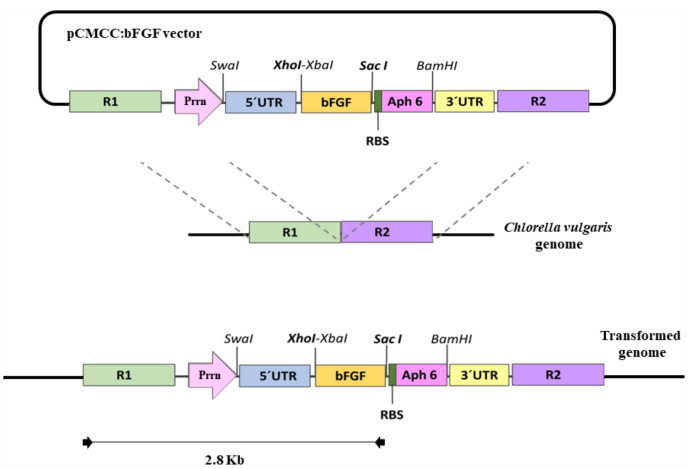

FIGURE 1 | Diagrammatic representation of the pCMCC:bFGF vector. R1, recombination region comprising 16S rRNA gene and *TrnI* genes from *Chlorella vulgaris* genome (NC_ 001865.1); *Prrn*, promoter region from *Prrn* gene from *Chlamydomonas reinhardtii*; *Swa*I, restriction enzyme site; T7 5′ UTR, 5′ untranslated region from gene 10 of T7 phage; *Xho*I/*Xba*I, restriction sites for the cloning gene of interest; bFGF, encodes the human bFGF along with His-tag at the N-terminus end; *Sac*I, restriction site for cloning the gene of interest; *Aph6*, selectable marker gene conferring resistance to kanamycin; *Bam*HI, restriction enzyme site; 3′ psb A, UTR at the 3′ end from *psbA* gene; R2, recombination region from the *C. vulgaris* genome (NC_ 001865.1) comprises the genes for *TrnA*, ORF64, ORF41a, and ORF74 and a part of the 23S rRNA gene. The amplified sequences using specific primers are marked with arrows.

pulse voltage of 643 V and width of 2.3 ms. Later, the cells were recovered after incubation with ice for 10 min, added with 200 µl BBM, and incubated at 30°C for 1 h. The algal cells were collected by centrifugation at 4°C for 2 min at 16,000 rpm, and the pellet was resuspended in 10 ml BBM and kept in the dark overnight at 25°C with continuous agitation. After recovery, the cells were plated on BBM agar plates containing kanamycin (50 mg/L).

Another method employed was based on the use of a sorbitol buffer for chloroplast transformation with modifications as described in the work of Li et al. (2020). *C. vulgaris* was inoculated into fresh f/2 medium to achieve a final cell concentration of 2×10^6 cells in the culture media; the cells were cultivated as per the growth conditions mentioned in the section "Microalgal Growth Conditions". After 4 to 5 days of growth, 200 ml of *Chlorella* cells at the early log phase was harvested by centrifugation at 4,000 rpm for 10 min at 4°C. The harvested cells were washed with ice-cold 6.8% sorbitol for 6 to 7 times. The cells were resuspended in ice-cold sorbitol to attain a final cell count of 2×10^7 cells/ml. Then, 3 µg pCMCC:bFGF DNA and linearized and non-linearized denatured salmon sperm DNA (30–50 µg) were added to 0.2 ml cells. The control was also kept with algal cells and salmon sperm DNA only. The algal cells and plasmid DNA were thoroughly mixed and transferred to a 2-mm cuvette and incubated in ice for 10 min prior to electroporation. Electroporation was carried out with parameters of 2,200 V and 3.5 ms. After electroporation, the cells were transferred to f/2 liquid medium and incubated at 25–28°C with minimal agitation. Following the 24-h dark incubation, the cells were recovered and plated on f/2 agar plates supplemented with 50 mg/L kanamycin. The plates were incubated at room temperature under continuous light intensity until the appearance of independent colonies. Emerging independent colonies with uniform phenotypes from both methods were further grown in media with 20 mg/L antibiotic for four rounds of selection and screened for molecular confirmation.

DNA Isolation and Confirmation by PCR

The independently transformed colonies were selected from the BBM and f/2 selection agar plates and inoculated in fresh f/2 liquid media supplemented with kanamycin. After 5 to 6 days of culture, 50 ml of each culture was centrifuged at 5,000 rpm for 5 min. The supernatant was discarded, and the total biomass was resuspended in the extraction buffer [100 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (pH 8.0), 500 mM NaCl, and 2% cetrimonium bromide]. Then, 1 ml glass beads (acid washed, 425–600 µm; Sigma, St. Louis, MO, USA) was added to the suspensions, and cell disruption was performed in a Mixer Mill MM400 (Retsch®). After grinding, the cell debris were removed by centrifugation at 12,000 rpm for 10 min, and the supernatants were subjected to phenol/chloroform/isoamyl alcohol (25:24:1, v/v) (Sigma, St. Louis, MO, USA) treatment for DNA purification.

PCR was performed in 20-µl reaction mixtures containing Phusion GC Buffer (1X), deoxynucleoside triphosphate (1 mM), Phusion DNA Polymerase (0.02 U/µl), dimethyl sulfoxide (3%), DNA template (50–100 ng), and 0.5 µM of each primer (forward: 5′GCAACTACTTTCCGTTTAGC3′, reverse: 5′CGAGCTCTCAAAGCTCATCCTTTTCAGAAGACTTGGCGCTCATAGG3′) to amplify the sequence comprising the bFGF gene and R1 region (2.8 kb) (**Figure 1**). The temperature cycling conditions were 98°C for 2 min (initial denaturation), 30 cycles at 98°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 1.25 min (elongation), and a final extension at 72°C for 10 min. The negative control consisted of the DNA from an untransformed *C. vulgaris*. This procedure was performed in a Bio-Rad Thermal Cycler. The PCR products were analyzed on 1.5% agarose gel electrophoresis.

Protein Extraction

Chloroplast-transformed algal cultures that were confirmed by PCR were grown in liquid f/2 with kanamycin at 20 mg/L and collected for protein extraction. A total of 50 mg fresh biomass was homogenized in 100 µl extraction buffer containing 750 mM Tris-HCl at pH 8.0 with 15% sucrose, 100 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride as described previously (Bañuelos-Hernández et al., 2017). The extracts were sonicated for 4 pulses for 10 s with an interval of 5 s in between (Malla et al., 2021). The samples were subsequently centrifuged at 8000 rpm for 15 min, and the supernatants were collected for further analysis. The total soluble proteins (TSPs) in the extracts were determined by the Bradford method.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in a Mini-Protean® Electrophoresis System from Bio-Rad under reducing–denaturing conditions. Stacking and separating gels were prepared using 4 and 12% acrylamide, respectively. TSPs (5–10 µg, approximately 50 µl) were mixed with 4X reducing loading buffer, denatured by boiling for 10 min at 95°C, and subsequently loaded onto a stacking gel. Electrophoresis was carried out at 100 V for 2 h and monitored using the molecular weight marker "Precision Plus Protein ™ Dual Color Standards" (Bio-Rad, Vienna, Austria). The gels were stained with Coomassie® Brilliant Blue R-250 (Bio-Rad, Vienna, Austria) at a final formulation of 0.05% (w/v) dye, 10% acetic acid, 50% (v/v) methanol, and 40% distilled water.

Western Blot Analysis

Recombinant protein expression was analyzed by Western blotting. TSPs (5–10 µg, approximately 50 µl) were mixed with 4X reducing loading buffer, denatured by boiling for 10 min at 95°C, and subsequently subjected to SDS-PAGE analysis. The gels were blotted onto 0.45 µm nitrocellulose membranes. Protein transfer was performed using a Bio-Rad electroblotter for 1 h at 100 V in a methanol-based transfer buffer. The nitrocellulose blot was further processed for immunodetection. After blocking with 5% skimmed milk dissolved in 1X phosphate-buffered saline (PBS) for 1 h, the blots were incubated at 4°C overnight with a rabbit anti-His antibody tagged with horseradish peroxidase conjugate (ab1187, Abcam, UK) at 1:5,000 dilution in 3% fat-free milk dissolved in 1X PBS. The blot was washed thrice with PBS containing 0.05% Tween

20. Protein detection was revealed by incubating the blots with SuperSignal West Pico Plus Chemiluminescent Substrate solution, following the manufacturer's instructions (Thermo Scientific, http://www.thermoscientific.com), and exposing the film in the dark.

Quantification by Enzyme-Linked Immunosorbent Assay

The bFGF that accumulated in the transplastomic lines of *C. vulgaris* was quantified using the human FGF basic ELISA Kit (R&D System, Minneapolis, USA). The protocol was performed in accordance with the manufacturer's instructions. All the reagents provided in the kit were brought to room temperature before performing the assay. A total of 100 µl assay diluent was obtained in each microwell, to which 100 µl standard and sample extracts was added and incubated at room temperature for 2 h. The extracts were subjected to twofold dilution in PBS (pH 7.2) and used as samples for ELISA quantification. The plates were washed with a wash buffer, and 200 µl human FGF basic conjugate was added to each well. After 2 h of incubation, the plate was washed, and 200 µl substrate was added, followed by 30 min of incubation in the dark. The reaction was stopped by the addition of the stop solution, and the optical density was measured at 450 nm. For the quantification studies, three biological replicates were obtained, and each sample was subjected to two technical replicates.

RESULTS

To obtain a *C. vulgaris* chloroplast expression vector, we adopted a fully synthetic approach and molecular cloning techniques to lay the recombination regions along with the expression cassette coding for the selective marker and the gene of interest, allowing the assembly of the pCMCC:bFGF vector in a pUC background; the physical map is shown in **Figure 1**. The pCMCC was designed to mediate the integration of an expression cassette for the bFGF gene through the homologous recombination into *C. vulgaris* chloroplasts. The optimized bFGF, which was inserted into the *Xho*I at the 5′ end and the *Sac*I site at the 3′ end, was under the control of the *Prrn* promoter from *C. reinhardtii* and the *psbA* terminator. The *Aph6* gene, which confers kanamycin resistance, was located in a bicistronic arrangement and led by the same transcriptional elements.

The green microalga *C. vulgaris* was grown for 5–7 days in f/2 media without silicates and further used for chloroplast transformation by electroporation using sorbitol–mannitol and sorbitol buffers. Transformed emergent colonies appeared on f/2 agar selection plates after 2 to 3 weeks in both buffer methods, and the wild-type *Chlorella* cells showed no growth in the selective medium. Isolated colonies from sorbitol–mannitol with nonlinearized pCMCC:bFGF plasmid (SM-NL), sorbitol–mannitol with linearized pCMCC:bFGF plasmid (SM-L), and sorbitol with linearized pCMCC:bFGF plasmid (S–L) plates (colonies 1 and 2) were picked and inoculated in f/2 liquid media without silicates and containing 20 mg/L kanamycin to confirm the kanamycin-resistant phenotype of the putative transformed algal cultures. No transformed colonies were observed in the case of sorbitol buffer with nonlinearized plasmid. The grown transformed cultures were further subcultured in the routine f/2 antibiotic media for 4 rounds at 6- to 8-day intervals for molecular screening (**Figure 2**). After four rounds of selection, the clones were screened by PCR using the R1 region/bFGF-specific primer set, which led to the detection of a 2.8-kb PCR product in the samples from the kanamycin-resistant clones obtained by electroporation using sorbitol–mannitol and sorbitol buffers; DNA from the wild-type *C. vulgaris* was used as the negative control. The expected amplicon was observed in the chloroplast-transformed colonies using both buffer methods. **Figure 3** shows the absence of amplification in the wild type.

The fresh biomass collected from chloroplast-transformed cultures of *C. vulgaris* with bFGF was lysed and used for protein extraction. The total proteins were visualized on SDS-PAGE gel with Coomassie blue staining (**Figure 4A**). The expression of the recombinant bFGF in transplastomic *C. vulgaris* clones was further evaluated by Western blot analyses. The bFGF protein was observed at the expected molecular weight of approximately 25 kDa in the Western blot for colonies obtained using the sorbitol–mannitol and sorbitol methods (**Figure 4B**). The wild-type extract did not show any immunoreactive band, validating the specificity of the assay (**Figure 4B**, lane 5). Hence, these results confirmed that the constructed pCMCC vector can be used for the chloroplast transformation of *C. vulgaris via* electroporation.

The bFGF yields in the transplastomic clones were determined by sandwich ELISA using a commercial kit. The bFGF protein yields were 0.26, 0.27, 1.42, and 0.56 ng/g in the SM-L colony, SM-NL colony, S-L colony 1, and S-L colony 2, respectively (**Figure 5**). Although the bFGF expression was low, it accounted for 0.005–0.4% of TSPs in the tested transplastomic clones, thereby confirming the functional activity of the expression vector in the chloroplast of *C. vulgaris*. However, the expressed recombinant bFGF protein in the chloroplast genome needs to be further explored using various promoters and regulatory elements to achieve higher protein yields. The purification procedures and functional analysis of the expressed bFGF shall be further studied.

DISCUSSION

Given the limited studies on the chloroplast transformation of green algae other than the model species *C. reinhardtii*, the present research focused on generating a species-specific vector targeting the chloroplast genome of *C. vulgaris* and assessing its functionality *via* electroporation-mediated transformation to achieve the expression of bFGF. The first step in the development of chloroplast transformation methods comprises the construction of species-specific chloroplast expression vector, which requires many PCR-based amplifications of flanking regions intended to mediate the homologous recombination with the target genome and

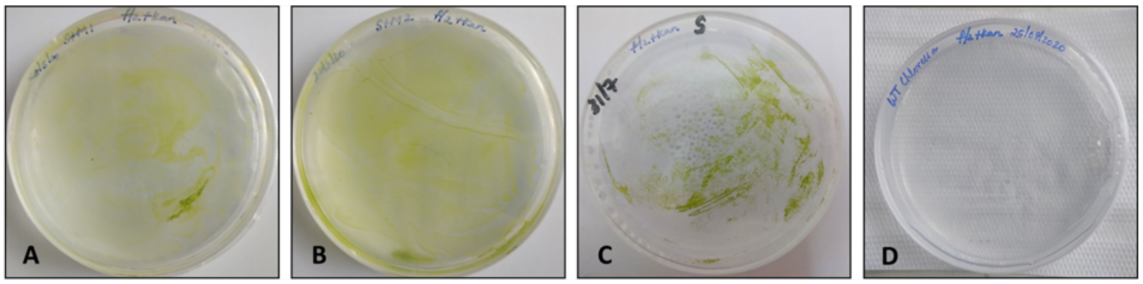

FIGURE 2 | Chloroplast-transformed *Chlorella vulgaris* cells by electroporation with sorbitol–mannitol and sorbitol buffers plated on f/2 agar plates with 50 mg/L kanamycin. **(A)** SM-L transformed cells, **(B)** SM-NL transformed cells, **(C)** S-L transformed cells in colony 1, and **(D)** wild-type *C. vulgaris* cells on antibiotic selection plate.

multiple cloning steps to assemble the expression cassette. A fully synthetic approach was followed as a straightforward way to assemble the *C. vulgaris*-specific vector (pCMCC), which comprised flanking sequences and a bicistronic expression cassette that mediates the transcription of the *Aph6* and bFGF genes. The species-specific chloroplast flanking sequences trnI/trnA were elected to mediate the site-directed insertion of the expression cassette in a manner that avoids the interruption of endogenous genes. Moreover, the trnI gene contains a chloroplast replication origin which mediates vector replication and thus may promote its integration into the target genome (Quesada-Vargas et al., 2005). The other feature of the pCMCC vector is the use of long sequences mediating the homologous recombination with the target genome, where the left side contains partial 16S rRNA and full-length *trnI* and the right targeting region contains *trnA* and partial 23S rRNA (Quesada-Vargas et al., 2005; Verma et al., 2008; Chen et al., 2020).

The expression cassette carried in the pCMCC vector comprises the *Prrn* promoter (Klein et al., 1992) from *C. reinhardtii*, the 3′ UTR from T7 phage (Herz et al., 2005), and the *Aph6* selection marker gene (Bateman and Purton, 2000) flanked by *psbA* terminator (Eibl et al., 1999). The 5′/3′ regulatory sequences were aimed at augmenting the capability of transcription and translation of the foreign gene. Plastid gene expression is regulated at the transcriptional and post-transcriptional levels. Therefore, the protein expression levels depend on the strength of the promoter, mRNA concentration, and its stability, but at a higher extension of translation efficiency (Verma and Daniell, 2007). Hence, in the present study, in addition to selecting a strong promoter, efficient UTRs and codon optimization were adopted to promote the efficient translation of transcripts and ultimately favor the recombinant protein accumulation. Five different restriction sites were included along with these elements to facilitate the further modifications of the vector or the replacement of the gene of interest (**Figure 1**).

In this work, the plasmid pCMCC was transformed into *C. vulgaris* cells in sugar alcohol buffers by electroporation, and the resultant transformants were selected on a kanamycin-containing medium. Meanwhile, untransformed cells were unable to grow on the selective medium. Certain microalgal cells have the potential to carry episomal plasmids (Xie et al., 2014). Hence, the transformed *C. vulgaris* cells were examined

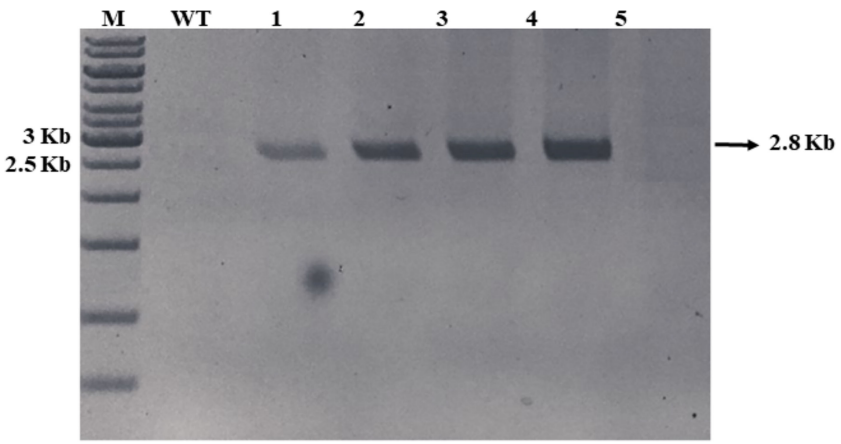

FIGURE 3 | PCR confirmation of chloroplast-transformed *Chlorella vulgaris* with pCMCC:bFGF. M, DNA ladder; WT, wild-type *C. vulgaris* DNA; lane 1, SM-L colony; lane 2, SM-NL colony; lane 3, S-L colony 1; lane 4, S-L colony 2; lane 5, negative reaction control. The 2.8-kb amplicon is indicated by an arrow.

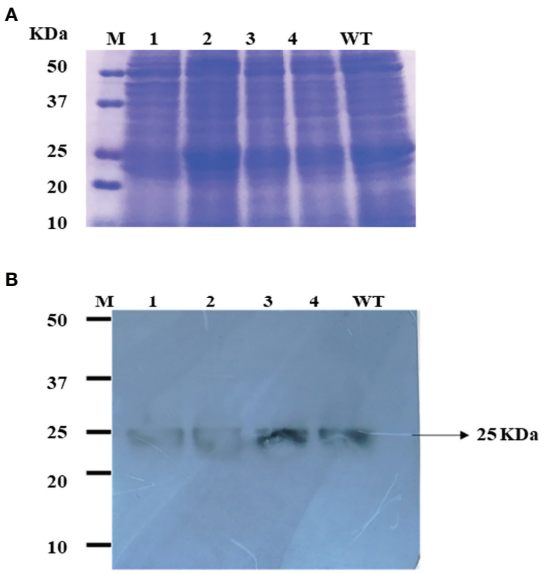

FIGURE 4 | **(A)** SDS-PAGE pattern of the total soluble protein obtained from the transformed and wild-type *Chlorella vulgaris*. Gel stained with Coomassie brilliant blue. M, molecular weight marker; line 1, SM-L colony; lane 2, SM-NL colony; lane 3, S-L colony 1; lane 4, S-L colony 2; WT, wild type (negative control). **(B)** Western blot analysis of bFGF protein produced in the chloroplast of *C. vulgaris*. Crude proteins were extracted from chloroplast-transformed *C. vulgaris* cultures, and the expression of bFGF protein was analyzed on SDS-PAGE, followed by Western blot, and probed with a rabbit anti-his antibody conjugated with HRP. M, molecular weight marker; lane 1, SM-L colony; lane 2, SM-NL colony; lane 3, S-L colony 1; lane 4, S-L colony 2; WT, wild type (negative control). The 25-kDa band corresponding to the bFGF protein is indicated by an arrow.

and confirmed by PCR and Western blotting (**Figures 3**, **4**, respectively). The expressed bFGF was further quantified by ELISA to confirm the FGF expression levels in the transformed lines (**Figure 5**). The sorbitol and sorbitol–mannitol buffer-based transformation resulted in transformed clones. Although the transformation efficiency was not calculated, the linearization of the plasmid affected the number of colonies that appeared on the selection medium. The nonlinearized plasmid that was used in the transformation methods with sorbitol–mannitol showed very few colonies, whereas sorbitol did not show any transformed clones. The amount of bFGF protein expression quantified by ELISA was higher in the case of sorbitol-linearized plasmid-transformed colonies compared with that in the case of sorbitol–mannitol-linearized plasmid. This study is the first report on plastid genetic transformation based on an electroporation gene delivery using sorbitol in *C. vulgaris* without employing cumbersome, time-consuming, and tedious procedures. To date, most plastid transformations rely on the biolistic approach, which is an expensive process with periodical maintenance limiting its use (Xie et al., 2014). In *C. vulgaris*, a

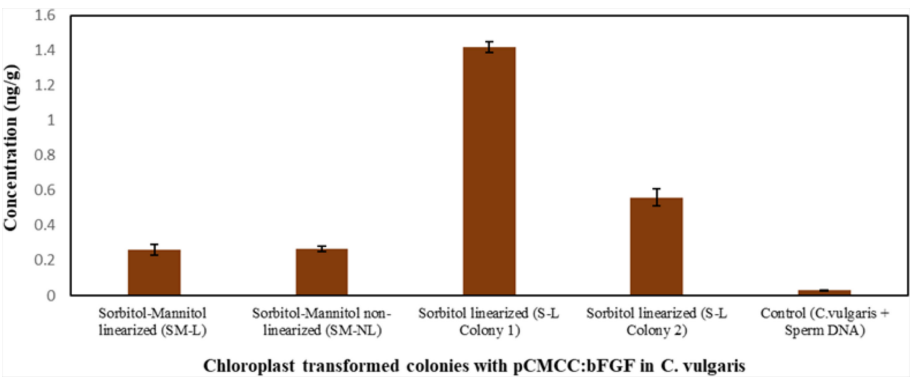

FIGURE 5 | The expression levels of bFGF in chloroplast-transformed *Chlorella vulgaris* cultures were measured by ELISA. *C. vulgaris* was transformed with pCMCC:bFGF plasmid using sorbitol–mannitol and sorbitol buffers by electroporation. Line 1, SM-L colony; lane 2, SM-NL colony; lane 3, S-L colony 1; lane 4, S-L colony 2; control, *C. vulgaris* + sperm DNA. The result was presented on the fresh weight basis. The data are expressed as mean ± standard deviation of three biological replicates with each sample (1:1 dilution) obtained in independent duplicates.

previous work on chloroplast genetic engineering has been reported, with the authors co-expressing two antimicrobial peptides *via* microparticle bombardment using gold particles. The transformant screening employing spectinomycin as a resistance marker was accomplished; subsequent DNA integration and transgene expression by PCR, Southern blotting, and Western blotting were achieved (Wang et al., 2021).

The present research formed a basis to extend the use of sorbitol–mannitol buffers for the electroporation method of other microalgal species at a lower cost and a feasible manner for chloroplast engineering. Although the heterologous gene expression in the chloroplast of *C. vulgaris* has been achieved with relatively low expression levels in terms of percent of TSP, the obtained yields in the present study are higher in comparison with those of other reports on nuclear *C. reinhardtii* expression, such as the nucleocapsid antigen from hepatitis B virus fused with angiotensin II (0.05% TSP), p24 human immunodeficiency virus antigen (0.25% of the total cellular protein), and human epidermal growth factor (0.25% TSP) (Soria-Guerra et al., 2014; Barahimipour et al., 2016; Baier et al., 2018). In addition, the bFGF levels expressed in *C. vulgaris* chloroplast genome surpassed the yields of some proteins produced in *C. reinhardtii* chloroplast, such as bacterial endoglucanase (CelK1, glycohydrolase, family 5) (0.003% TSP), the major birch pollen allergen, Bet v 1 (0.01–0.04% TSP), human glutamic acid decarboxylase 65 (0.25–0.3% TSP), and VapA antigens from the fish bacterial pathogen *Aeromonas salmonicida* (0.3% TSP) (Wang et al., 2008; Michelet et al., 2011; Faè et al., 2017; Hirschl et al., 2017). However, the low expression levels obtained in our study despite the use of a transplastomic approach can be attributed to a mechanism involved at multiple levels, such as transcription, mRNA processing, mRNA stability, and translation of the recombinant gene. Improvements in the expression vector design using different promoter sequences or regulatory elements should be considered.

On the other hand, the electroporation process requires different parameters that can be modified to optimize a protocol for a specific microalgal species. The main adjustments encompass the voltage, resistance, capacitance, pulse duration, temperature, number of pulses, culture medium composition, growth capability, addition of carrier DNA, concentration of exogenous DNA, and solid medium for maximal cloning efficiency (Coll, 2006). In this sense, future investigations focusing on parameter modification will be performed to optimize the electroporation-based chloroplast transformation process.

Genetic modifications are generally performed on the nuclear genome of host organisms. In the last decades, chloroplasts have become attractive hosts, gaining huge interest due to the following advantages with respect to nuclear transformation: high expression levels associated to the high copy number reached for the transgene and low or no position effect due to exogenous DNA integration in particular sites by homologous recombination (Jin and Daniell, 2015; Zhang et al., 2017; Tanwar et al., 2018; Chen et al., 2020; Daniell et al., 2021). Over the past few years, plastid transformation technology has been successfully applied in higher plants to express foreign proteins to achieve agronomic traits or has led to platforms for the production of enzymes, metabolites, and pharmaceuticals (Wani et al., 2015; Jin and Daniell, 2015; Ahmad et al., 2016. Bock, 2015). The chloroplast transformation in model alga *C. reinhardtii* is well established and used routinely for the expression of various heterologous proteins. *C. vulgaris* is another green algal species that has a high nutritive value with economically important characteristics but lacks an efficient stable and transformation method due to its thick cell wall. In this study, the fully synthetic approach adopted for straightforward construction provided a useful model that may allow the redesign of the vector for optimization purposes and expand the transformation of other algal species using a simple and robust transformation method.

CONCLUSION

The pCMCC plasmid constructed using the promoter region *Prrn* from *C. reinhardtii* was successfully applied to transfer the bFGF gene into the plastome of *C. vulgaris via* electroporation using sorbitol and mannitol-based buffers, leading to the expression of the target recombinant protein. To the best of our knowledge, this research is the first study to show evidence on the capacity of electroporation-mediated transformation method using a specific chloroplast vector obtained by a fully synthetic approach in the development of transplastomic technologies for *C. vulgaris*, which is a small and rigid alga. The obtained pCMCC vector will be the basis for the design of next-generation vectors to ultimately optimize the expressions of foreign proteins in the chloroplast of *C. vulgaris*. This study represents a starting point of plastid engineering for green algal species by simple electroporation method where the transformation frequency and expression levels remain to be improved. A routine procedure with insertion of more sites for recombination, selectable markers, and strong promoters in streamline with the specific electroporation conditions should be undertaken for engineering of the plastids, which are recalcitrant by biolistic particle bombardment or PEG-mediated transformation methods in relevant microalgal species. The effort to expand the plastid algal biotechnology will lead to mature platforms for the production of metabolites and recombinant protein expression and holds a great promise for commercialization in nutritional, therapeutic, and industrial applications, making these host systems a competitive production platform.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SV and SR-M conceived, designed, and supervised the study. SV obtained the funding for this work. SR-M wrote, reviewed, and

edited the manuscript. AM performed the experiments, analyzed the data, designed the figures, and wrote the original draft. OCB-M performed the experiments, analyzed the data, designed the figures, and wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.884897/full#supplementary-material

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