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Expression of bovine lactoferrin N-lobe by the green alga, *Chlorella vulgaris*

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The purpose of this study was to express bovine lactoferrin N-lobe in *Chlorella vulgaris*, a green microalga, using the pCAMBIA1304 vector. *Chlorella*-codon-optimized bovine lactoferrin N-lobe (*Lfb-N* gene) was cloned in the expression vector pCAMBIA1304, creating the plasmid pCAMLfb-N. pCAMLfb-N was then introduced into *C. vulgaris* by electrotransformation. Transformants were separated from BG-11 plates containing 20 μg mL^{-1} hygromycin. Polymerase chain reaction was used to screen transformants harboring *Lfb-N* gene. Finally, total soluble protein was extracted from the transformants, and the expression of Lfb-N protein was detected using western blotting. Using this method, we successfully expressed bovine lactoferrin in *C. vulgaris*. Therefore, our results suggested that recombinant lactoferrin N-lobe, which has many uses in the biomedical and pharmaceutical industries, can be produced economically.

Key Words: *Chlorella vulgaris*; electrotransfomation; expression; hygromycin; lactoferrin

INTRODUCTION

Lactoferrin (also called lactotransferrin) is a member of the transferrin family of proteins (Aisen and Listowski 1980, Brock 1985, Rose et al. 1986) and is found in milk, saliva, and tears. Lactoferrin is secreted from mammary glands, mucous, and secondary granules of neutrophils, which function in the nonimmunological defense system. Moreover, bovine colostrum contains 5 mg mL^{-1} lactoferrin, while normal milk contains only about 0.02 mg mL^{-1} lactoferrin. Human lactoferrin consists of 691 amino acids, which associate with 2 glycan chains of glycoproteins (Metz-Boutigue et al. 1984), and bovine lactoferrin contains 689 amino acids (Picerce et al. 1991), with a predicted molecular weight of about 76 kDa, or 83 kDa when accounting for its glycoconjugates. The lactoferrin glycoprotein consists of 2 lobes, with each lobe having an Fe^{3+} binding site. The antibacterial core of lactoferrin, called lactoferricin, can be produced through pepsin-mediated cleavage. This peptide has antimicrobial properties and is therefore a potential therapeutic agent in various clinical settings.

Currently, most commercially obtainable recombinant protein is produced using bacteria, yeast fungi, or bioreactors designed for animal cell culture. Bacterial protein expression systems lack critical post-transcriptional and post-translational regulatory mechanisms, such as splicing, glycosylation, and protein assembly. Moreover, there is a high possibility that purified proteins may contain bacterial endotoxin and protease contaminants. Similarly, yeast expression systems, while eukaryotic in nature, do not have machinery for the glycosylation. Currently, most proteins generated for medical use are cultured and produced in animal cells. However, it is difficult to mass produce such proteins, the cost of culture medium is high, and cultures are easily contaminated. Moreover, low

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yields have high costs, making this type of expression system unsustainable for clinical applications. Additionally, while plant-based expression systems have many advantages for mass production of stable, functional proteins, challenges to these methods include environmental pollution by genetically modified plants, allergic reactions to plant components, contamination of protein, and regulation of medical protein permission (Choi and Sim 2009).

Genetic engineering technology has been under development for more than ten years now, and eukaryotic microalga represent an alternative expression system to overcome issues of other expression systems based in animals, fungi, bacteria, and plants. Recently, many studies have investigated the production of antibodies, medical proteins, and vaccines in microalgae (Yang et al. 2006, Tran et al. 2009, Dreesen et al. 2010). Moreover, microalgae have the potential for mass culture in growth conditions similar to that required for general crops. For these reasons, microalgae have become the focus of novel gene manipulation methods to increase the productivity of natural components and novel compound. However, while studies are currently being conducting to investigate the potential for genetic transformation in microalga models, such as *Chlamydomonas reinhardtii*, gene manipulation technology in microalgae is still in its infancy, and recombinant products from microalgae have not been commercialized.

In the present study, to the best of our knowledge, we report the first successful expression of bovine lactoferrin N-lobe gene in *Chlorella vulgaris* via electrotransformation.

MATERIALS AND METHODS

Codon optimization and the expression vector

In this study, bovine lactoferrin N-lobe (including a lactoferricin) was used for codon optimization and determined to be *Lfb-N*. In order to optimize the *Lfb-N*, *Chlorella*-codon usage database was chosen to reflect online at http://www.kazusa.or.jp/codon/ (Nakamura et al. 2000). *Chlorella*-codon optimized Lfb-N DNA sequence (including an *Spe*I restriction site on the 5′ end and a 6× His-tag sequence and *BstE*II restriction site on the 3′ end) was synthesized (Bioneer, Daejeon, Korea), and the correct sequence was confirmed by DNA sequence analysis (Bioneer). The codon-optimized *Lfb-N* gene was inserted into the pGEM T easy vector, and the obtained construct (pLfb-N) was transformed in *Escherichia coli* XL1-blue strain using the heat shock method. The algal expression vector pCAMBIA1304 containing a GUS-*mgfp5* fusion reporter and a selectable marker for hygromycin driven by the CaMV35s promoter was used for transformation.

Construction of the expression vector

The vector construction was carried out as described by Ebrahimi et al. (2012), with some modifications. For construction of the expression vector, the purified pLfb-N vector and the pCAMBIA1304 vector were digested with *Spe*I and *BstE*II (Fermentas, Pittsburgh, PA, USA). The digested pLfb-N and pCAMBIA1304 vector were electrophoresed on a 1.0% agarose gel and purified from the gel using a gel extraction kit (Qiagen, Valencia, CA, USA). The purified *Lfb-N* gene and the pCAMBIA1304 vector were ligated using T4 DNA ligase (Takara Bio, Shiga, Japan) at 16°C overnight, and the obtained construct (pCAMLfb-N) was transformed in *Escherichia coli* strain XL1-blue using the heat shock method (Fig. 1). Transformed colonies were selected on LB agar plates containing 50 mg L^{-1} kanamycin. The pCAMLfb-N recombinant plasmid was verified by colony polymerase chain reaction (PCR), digestion, and sequencing. PCR analysis was performed with the primer pair 1-F (5′-CTAGTGTTCGATGGTGCACCATTTCCC-3′) and 1-R (5′-CTCACACGTGGTGGTGGTGGTG-3′) specific to the *Lfb-N* gene to amplify a 1.0 kb fragment. A thermocycler (Takara Bio) was programmed for 1 cycle at 95°C for 2 min; 30 cycles of 1 min at 94°C for denaturation, 2 min at 58°C for primer template annealing, 3 min at 72°C for extension; and 1 cycle at 72°C for 7 min as a final extension. The *Lfb-N* gene was then inserted into the reporter gene region of pCAMBIA1304 after the CaMV35s promoter and before the *NOS* terminator by ligation.

Electrotransformation

Electrotransformation was carried out as described by Wang et al. (2007*a*), with some modifications. Fifteen mililiters of *C. vulgaris* culture grown to a cell density of OD_{600} = 0.3-0.5 was harvested by centrifugation at 6,000 rpm for 10 min at room temperature, then treated by osmosis (0.2 M mannitol and 0.2 M sorbitol) for 1 h, followed again by centrifugation. The resulting pellets were re-suspended in 1 mL of electroporation buffer (500 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 20 mM Hepes, 200 mM mannitol, and 200 mM sorbitol; pH 7.2). The 5 μg of pCAMLfb-N was added into a 1 mL cell suspension and kept on ice for 5-10 min. The 1 mL of DNA-cell mixture was transferred into 0.2 cm electroporation cuvette, then

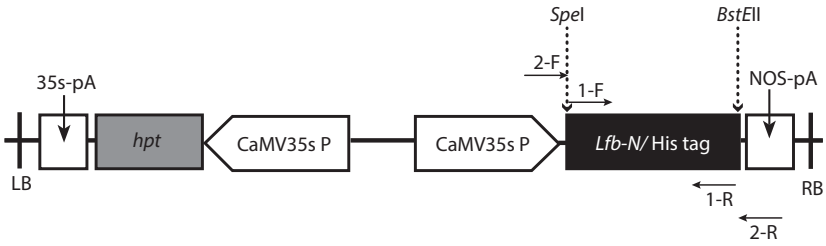

Fig. 1. T-DNA region of pCAMLfb-N and primers designed for detecting the transgene. Primers 1-F and 1-R were used to amplify a 1.0-kb DNA fragment from the transcript of recombinant bovine lactoferrin N-lobe, whereas primers 2-F and 2-R were used to amplify a 1.1-kb polymerase chain reaction fragment from the contaminated plasmid DNA. LB and RB, left and right borders; 35s-pA, CaMV35s-poly A terminator; *hpt*, hygromycin-selectable marker; CaMV35s P, cauliflower mosaic virus promoter; *Spe*I and *BstE*II, restriction sites; NOS-pA, NOS-poly A terminator.

electrotransformation was performed by electroporator ECM 2001 (BTX, Holliston, MA, USA) under voltage 1,000 V at 25 μF and 200 ohm to generate the transgenic *C. vulgaris*. After electroporation, *C. vulgaris* cells were transferred to 6-well plate containing 5 mL of BG-11 medium and cultured in the dark at 25°C for 24 h. The cultured cells were harvested by centrifugation at 6,000 rpm for 10 min at room temperature and discard the supernatant. The cells were resuspended in 200 μL of BG-11 medium at room temperature and plated onto selection agar plate (BG-11 agar plates containing 20 mg L^{-1} hygromycin). The selection agar plate cultures were kept in the incubation under continuous cool fluorescent light with 50 μmol m^{-2} s^{-1} at 25°C. Transformed colonies appeared in a 5 days, and independent colonies were maintained on selection medium. Hygromycin-resistant colonies were grown in BG-11 medium containing hygromycin and used for molecular analysis.

Genomic DNA extraction and screening of putative transgenic *Chlorella vulgaris* DNA by PCR

Extraction of *C. vulgaris* genomic DNA was performed as described by Dawson et al. (1997), with some modifications. Microalgae were harvested from 5 mL culture medium (approximately 1 × 10^7 cells mL^{-1}) and resuspended in 500 μL buffer solution (54 mM hexadecyltrimethylammonium bromide, 0.25 mM Tris [pH 8.0], 1.4 M NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], and 2% β-mercaptoethanol). The mixture was incubated at 65°C for 2 h and shaken every 15 min. After incubation, an equal volume of phenol-chloroform was added, and the aqueous phase was recovered after 5 min of centrifugation at 8,000 ×*g* and 25°C for 10 min. We extracted DNA several times until the aqueous layer was no longer cloudy. Genomic DNA was then precipitated with 2 volumes of 100% ethanol, centrifuged at 8,000 ×*g* for 15 min, washed with 70% ethanol, dried, and resuspended in 30 μL Tris hydrochloride (TE) buffer.

Transformed colonies was randomly selected and cultured for 7 days in 5 mL BG-11 broth (containing 20 mg L^{-1} hygromycin) at 25°C, with shaking at 180 rpm. Light conditions were set at 50 μmol m^{-2} s^{-1} using a fluorescent light for 14 h, with a 10 h night cycles. Cultured transformants were recovered after processing with a centrifugal separator, and extracted genomic DNA was used as a temple for PCR. Two oligonucleotide primers were synthesized for detection of the transferred Lfb-N DNA fragment by PCR analysis. PCR analysis was performed with the primer pair 2-F (5′-GAGAACACGGGGGACTCTTG-3′) and 2-R (5′-GGGGAAATTCGAGCTGGTCA-3′) specific to the *Lfb-N* gene to amplify a 1.1 kb fragment containing 3′ end of CaMV35s and 5′ end of *NOS* terminator. A thermocycler (Takara Bio) was programmed for 1 cycle at 95°C for 2 min; 30 cycles of 1 min at 94°C for denaturation, 2 min at 60°C for primer template annealing, and 3 min at 72°C for extension; and 1 cycle at 72°C for 7 min as a final extension.

Protein extraction and western blot analysis

A 50-mL sample of cell suspension (10^8 cells mL^{-1}) was centrifuged for 5 min at 2,000 ×*g* and resuspended in 2 mL lysis buffer (50 mM Hepes, pH 7.9, 250 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The cells were disrupted by sonicating 3 times for 10 s with a micro tip sonicator using a power setting of 30 W (Cannons and Shiflett 2001). The lysate was centrifuged at 12,000 ×*g* for 5 min, and the supernatant was concentrated using a 10 kDa filter membrane. The total protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA, USA) (Bradford 1976) using bovine serum albumin (BSA) as a standard. Equal amounts of total soluble protein were subjected

to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the separation, proteins were electroblotted on to nitrocellulose membranes, blocked with BSA, incubated with mouse anti-6× HIS monoclonal antibodies (Abcam, Cambrige, MA, USA) raised in goats, and detected by an anti-mouse IgG-alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) following standard procedures (Lorimer et al. 2009). In addition, we also predicted the 3D structure of recombinant bovine lactoferrin N-lobe using ESyPred3D.

RESULTS

Codon optimization and construction of the expression vector pCAMLfb-N

In this study, we optimized the *Lfb-N* gene to resemble *Chlorella* nuclear gene codons, i.e., to have a GC content of 61% and a gene size of 1,014 bps. In order to express heterologous genes in microalgae, a codon bias that is similar to the GC-bias of nuclear genes and the AT-bias of chromosomal genes should be expressed or optimized for codon usage (Choi and Sim 2009). In order to optimize for codon usage, we used the codon adaptation index (CAI), a quantitative tool to predict heterologous gene expression levels based on their codon usage. Since the chloroplast, mitochondrial, and nuclear genomes of microalgae may exhibit different codon biases, as is the case in *C. vulgaris*, genome-specific CAI values should be used for optimal translation.

The digested Lfb-N was cloned between 3′ end of CaMV35s and 5′ end of the *NOS* terminator in the pCAMBIA1304 vector between *BstE*II and *Spe*I restriction sites (Fig. 1). The constructed plasmid was named pCAMLfb-N. Transformed colonies were confirmed by colony PCR (Fig. 2) and restriction enzyme digestion with *BstE*II and *Spe*I (Fig. 3). Colony containing of the codon-optimized *Lfb-N* gene was confirmed in transformants T2, T3, T4, T5, T6, T9, and T10. The plasmid sequence of each transformant was confirmed by DNA sequencing, and T10 was finally selected from sequencing analysis.

Electrotransformation and screening of putative *Chlorella vulgaris* by PCR analysis

The pCAMLfb-N was used to transform *C. vulgaris* by electrotransformation. Transformed cells were selected on BG-11 plates containing 20 mg L^{-1} hygromycin. A total of 153 transformants were screened. A total of 12 putative

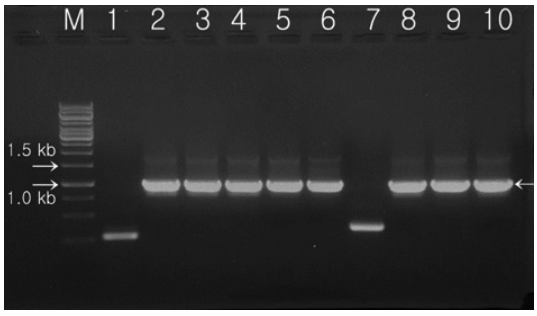

Fig. 2. Proof of the presence of the *Lfb-N* gene in pCAMLfb-N by colony polymerase chain reaction. The *Lfb-N* gene was confirmed in all selected colonies except T1 and T7. M, 1-kb DNA ladder; lanes 1-10, randomly selected colonies (T1-T10).

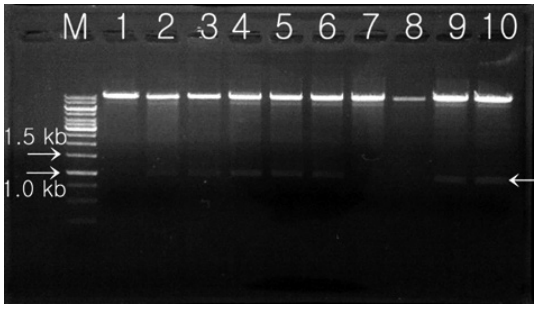

Fig. 3. Proof of the presence of the *Lfb-N* gene in pCAMLfb-N by digestion reaction. The *Lfb-N* gene was confirmed in all selected colonies except T1, T7, and T8. M, 1-kb DNA ladder; lanes 1-10, randomly selected colonies (T1-T10).

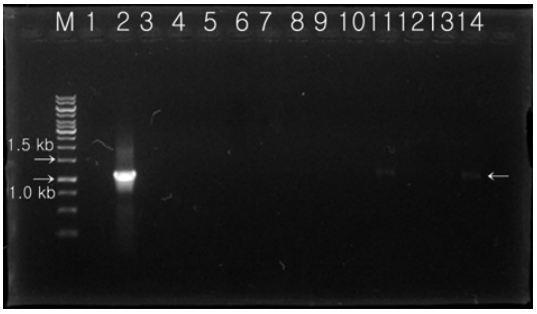

Fig. 4. Polymerase chain reaction analysis of *Lfb-N* gene expression in *Chlorella vulgaris*. Expression of the *Lfb-N* gene was confirmed in CT-9 and CT-12. M, 1-kb DNA ladder; lane 1, *C. vulgaris* (nontransformant); lane 2, pCAMLfb-N; lanes 3-14, transformant colonies (CT-1 to CT-12).

hygromycin-resistant single colonies, confirmed within 7 days on selection media, were randomly selected, grown in liquid media before the DNA was extracted, and used in PCR analysis. The primers 2-F and 2-R were used to carry out the PCR amplification for screening of *C. vul-*

garis harboring the *Lfb-N* gene (Fig. 4). Nontransformed *C. vulgaris* was used as a negative control, and pCAMLfb-N was used as a positive control. Amplification with *Lfb-N*-specific primers (2-F and 2-R) detected the 1.1 kb DNA fragment from 2 putative transgenic lines (CT-9 and CT-12), but not from an additional 10 putative transgenic lines. The 1.1 kb DNA fragment was detected in the positive control but not in the negative control. Finally, results showed that the *Lfb-N* genes were integrated into the *C. vulgaris* genome.

Protein expression and western blot analysis

Expression of the protein product from the introduced *Lfb-N* gene was tested by SDS-PAGE and western blot analysis. Results showed that a protein band with a molecular weight of about 35 kDa was expressed in transformed *C. vulgaris*; this band was absent in the proteins extracted from nontransformed *C. vulgaris* (Fig. 5A). Western blot analysis of protein extracts from the transformed lines revealed the presence of the Lfb-N protein where the protein was not detected in untransformed cells, indicating the stability of the transgene expression (Fig. 5B). The sequence of this protein was 99% identical to that of lactoferrin from 2 different species (*Bos taurus* seq. ID: gb|ABF69106.1| and *Bos grunniens* seq. ID: gb|ACB29795.1|). This 3D structure demonstrated the presence of lactoferricin, the antibacterial core, within Phe_{18} to Phe_{42} and the existence of a α-helix and β-sheet (Fig. 6). Our experimental data is probably the first documentation of expression of the bovine lactoferrin N-lobe, in viral promoter CaMV35s in an algal system using electrotransformation.

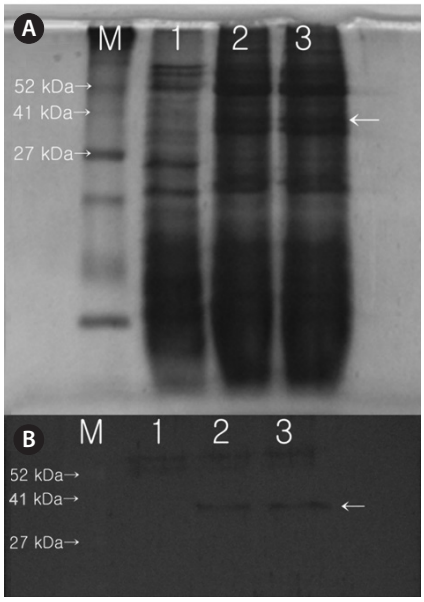

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of the total soluble protein extract from transformed *Chlorella vulgaris*. (A) Total soluble proteins were extracted and subjected to SDS-PAGE (50 μg per lane). (B) Western blot analysis confirmed the expression of this 35-kDa protein in transformed but not nontransformed *C. vulgaris*. Lane 1, nontransformed *C. vulgaris*; lanes 2-4, transformed *C. vulgaris* (lane 2, CT-9; lane 3, CT-12).

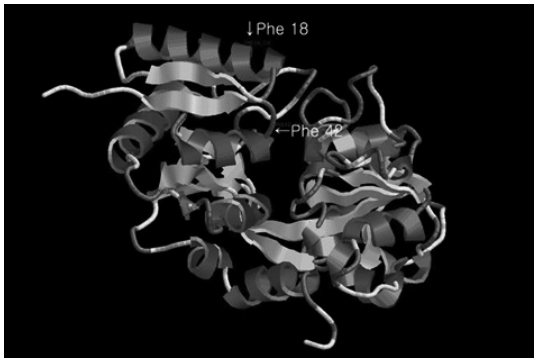

Fig. 6. 3-D structure of recombinant bovine lactoferrin N-lobe protein. The 3-D structure of recombinant bovine lactoferrin N-lobe protein was determined. The existence of 14 α-helix and 16 β-sheet structures was confirmed.

DISCUSSION

Algal transformation

In microalgae, the expression of heterologous proteins presents several difficulties. First, microalgae have an unusual GC-rich codon bias in their nuclear genes; therefore, codon optimization must be performed on any gene for which high levels of protein expression are desired (Heitzer et al. 2007). Additionally, expression levels of optimized foreign genes may vary considerably due to position effect that is driven by random integration of the gene of interest and strong silencing mechanism that drives by epigenetic phenomena similar to those in land plants (Schroda 2006). In *C. vulgaris* and other algae, as in land plants, silenced multiple-copy transgenes exhibit high levels of DNA methylation (Cerutti et al. 1997, Babinger et al. 2001). In contrast, single-copy transgenes are subject to transgene silencing without detectable cytosine methylation (Cerutti et al. 1997).

The nuclear genome and the plastid genome have highly divergent codon usage, with the chloroplast preferring an A or T in the wobble position, while the nuclear

genome prefers a G or C (Nakamura et al. 2000). Using GFP, early work showed that codon optimization to reflect the genome bias could increase transgene protein accumulation 5-fold in the nucleus (Fuhrmann et al. 1999) and up to 80-fold in the chloroplast (Franklin et al. 2002). Today, recombinant genes are universally codon optimized for improved protein expression in almost every system (Puigbò et al. 2007, Xia 2007, Puigbò et al. 2008*b*). For codon optimization, several free software and web applications have recently been developed to estimate CAI values and optimize the codon usage of sequences. E-CAI (http://genomes.urv.es/CAIcal/E-CAI) determines whether differences in CAIs between sequences are significant or arise from biases in G+C or amino-acid composition (Puigbò et al. 2008*b*). The online OPTIMIZER application (http://genomes.urv.es/OPTIMIZER) optimizes the codon usage of provided sequences using preconstructed usage tables based on a 'one amino acid-one codon' basis, Monte-Carlo algorithms, or a novel algorithm for optimization with minimal changes (Puigbò et al. 2007). CAI-cal (http://genomes.urv.es/CAIcal) provides an integrated set of tools for the optimization of codon usage (Puigbò et al. 2008*a*). Some gene design software packages such as Gene Composer include gene optimization functionalities (Lorimer et al. 2009). A database of CAI and codon usage indices for most sequenced species is available online at http://www.kazusa.or.jp/codon/ (Nakamura et al. 2000).

Various genetic transformation methods have been used to express recombinant proteins through introduction of genes into microalgae like *Chlorella* sp. The applied methods include particle bombardment, glass beads method, *Agrobacterium tumefaciens*-mediated transformation, and electrotransformation. Bombardment of target cells with DNA-coated metallic particles is a widespread, simple, effective and highly reproducible transformation method. This method has been successfully employed for the transformation of most standard cellular expression systems, and it is therefore not surprising that it is also useful for the study of microalgae. The main drawback of the particle bombardment method is the cost of the required specialized equipment. Although the number of transformants recovered following particle bombardment can be low, it remains the most effective method for the transformation of chloroplasts, as it allows for the delivery of multiple copies of recombinant DNA through both the cellular and chloroplast membranes, increasing the chance for a successful integration event to occur (Boynton and Gillham 1993). This method has been shown to be effective for the stable nuclear (Mayfield and Kindle 1990) and chloroplast (Boynton et al. 1988, El-Sheekh 2000) transformation of *C. reinhardtii*. A simple and effective transformation method consists of agitating cell wall-deficient microalgal cells with recombinant DNA, polyethylene glycol, which greatly increases transformation efficiency, and glass beads. Despite the drop in cell viability to 25% following agitation with the beads, a nuclear transformation efficiency of 10^3 transformants μg^{-1} DNA was achieved using this method (Kindle 1990) and an efficiency of 50 transformants μg^{-1} DNA was achieved for the transformation of *C. reinhardtii* chloroplasts (Kindle et al. 1991). Compared to the particle bombardment method, the glass beads method is simpler, more efficient for nuclear transformations, and much less expensive as it does not require specialized equipment. A recent study showed that the glass beads method is also more efficient than particle bombardment for the transformation of *Dunaliella salina* (Feng et al. 2009). Transformation by the tumour-inducing *A. tumefaciens* is another efficient means of delivering genetic material, although this method has so far been mainly used to modify plant cells. Transformation results from the stimulation of cell division by products encoded by T-DNA transferred from *Agrobacterium* to the target cell. The T-DNA and virulence (*vir*) regions are located on the tumour inducing plasmid (pTi). The *vir* system processes and transfers any DNA between the short flanking repeats that delimit the T-DNA, making *Agrobacterium* an efficient DNA delivery system (Akhond and Machray 2009). Using the *Agrobacterium*-mediated transformation method, *C. reinhardtii* was successfully transformed with *uidA* (β-glucuronidase), *gfp* (green fluorescent protein) and *hpt* (hygromycin phosphotransferase) reporter genes, with a fifty-fold increase in resulting transformants compared to the glass beads method (Kumar et al. 2004).

In this study, we used electrotransformation to move recombinant DNA into microalgae, allowing the growth of hygromycin-resistant colonies. The effectiveness of microalgal electroporation, or the induction of macromolecular uptake by exposing cell walls to high intensity electrical field pulses, was first reported by Brown et al. (1991). Electroporation specifically disrupts lipid bilayers, leading to efficient molecular transport across the plasma membrane (Azencott et al. 2007). Efficient electrotransformation was achieved in both wild-type and cell wall-deficient strains (Brown et al. 1991). The transformation efficiency of electroporation is two orders of magnitude higher than the glass beads method, and only requires relatively simple equipment (Shimogawara et al. 1998). Important parameters affecting the effectiveness

of electroporation include field strength, pulse length, medium composition, temperature and membrane characteristics (Brown et al. 1991) as well as the concentration of DNA (Wang et al. 2007*a*). Electroporation was successfully used for the transformation of *C. reinhardtii* (Tang et al. 1995, Shimogawara et al. 1998, Kovar et al. 2002, Ladygin 2003, 2004), *Chlorella* sp. (Chow and Tung 1999, Wang et al. 2007*a*), *D. salina* (Geng et al. 2004, Sun et al. 2005, 2008, Wang et al. 2007*b*), *Dunaliella viridis* (Sun et al. 2006) and *Dunaliella tertiolecta* (Walker et al. 2005) species, and *Nannochloropsis oculata* (Chen et al. 2008).

Expression of recombinant lactoferrin

Studies of recombinant lactoferrin expression have been conducted in several hosts, including *E. coli* (Weickert et al. 1996). However, lactoferrin expressed in *E. coli* has been shown to easily form inclusion bodies. Therefore, many researchers have attempted to use insect or animal cells as hosts, but these hosts are more time consuming to work with and do not produce high yields. Another alternative method to produce recombinant lactoferrin was to adopt plant cells as hosts. In a previous study, human lactoferrin was inserted into the dP35SCaMV vector (enhanced 35S promoter), transformed into *Nicotiana tabacum xanthi* NC through agro-infiltration, and expressed as an 80-kDa recombinant protein (Salmon et al. 1998). Additionally, the potato has been used to express plant microsomal retention signal peptide (SEK-DEL) linked to full-length recombinant human lactoferrin under control of the masP2 and CaMV35s promoters (Chong and Langridge 2000). The antibiotic core of lactoferrin, located in the N-lobe, has also been expressed in *Nicotiana benthmiana* via agro-infection, resulting in the production of a 40 kDa protein (Li et al. 2004), and in edible rice under control of the CaMV35s promoter, resulting in the production of a 45 kDa protein (Takase et al. 2005).

Thus, genetic transformation in a plant-based expression system has some benefits, enabling the production of stable, functional protein. However, mass culture via such plant-based systems is limited due to spatial restrictions and risks of exposure of genetically transformed organisms into the ecosystem. However, as we have shown, recombinant protein production using microalgae can be performed using micro-organism culture within a bioreactor, the volume of the culture is adjustable, and the risk of exposure to the ecosystem is relatively low.

In conclusion, we confirmed that recombinant bovine lactoferrin N-lobe could be expressed from the microalga *C. vulgaris*. This system allowed for more stable expression of recombinant lactoferrin N-lobe than in other expression systems. The bovine lactoferrin N-lobe, which exhibits a 400-fold increase in antibacterial activities compared to full-length lactoferrin, is difficult to separate from the full-length protein using proteases. However, this important peptide, known as lactoferricin, shows antibacterial activities against gram-positive and -negative bacteria, but does not show antibacterial activities against bifidobacteria, a bacterium found in the intestines. Therefore, the production of recombinant bovine lactoferrin N-lobe protein is expected to be beneficial in a variety of clinical applications and fields.

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REFERENCES

Aisen, P. & Listowsky, I. 1980. Iron transport and storage proteins. Annu. Rev. Biochem. 49:357-393.

Akhond, M. A. Y. & Machray, G. C. 2009. Biotech crops: technologies, achievements and prospects. Euphytica 166:47-59.

Azencott, H. R., Peter, G. F. & Prausnitz, M. R. 2007. Influence of the cell wall on intracellular delivery to algal cells by electroporation and sonication. Ultrasound Med. Biol. 33:1805-1817.

Babinger, P., Kobl, I., Mages, W. & Schmitt, R. 2001. A link between DNA methylation and epigenetic silencing in transgenic *Volvox carteri*. Nucleic Acids Res. 29:1261-1271.

Boynton, J. E. & Gillham, N. W. 1993. Chloroplast transformation in *Chlamydomonas*. Methods Enzymol. 217:510-536.

Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B. & Sanford, J. C. 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534-1538.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brock, J. H. 1985. Metal proteins with non-redox roles (Part II). Topics in molecular and structural biology. *In* Harrison, P. (Ed.) *Metalloproteins*. Macmillan Press, Lodon,

pp. 183-262.

Brown, L. E., Sprecher, S. L., & Keller, L. R. 1991. Introduction of exogenous DNA into *Chlamydomonas reinhardtii* by electroporation. Mol. Cell Biol. 11:2328-2332.

Cannons, A. C. & Shiflett, S. D. 2001. Transcriptional regulation of the nitrate reductase gene in *Chlorella vulgaris*: indentification of regulatory elements controlling expression. Curr. Genet. 40:128-135.

Cerutti, H., Johnson, A. M., Gillham, N. W. & Boynton, J. E. 1997. Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell 9:925-945.

Chen, H. L., Li, S. S., Huang, R. & Tsai, H. -J. 2008. Conditional production of a functional fish growth hormone in the transgenic line of *Nannochloropsis oculata* (Eustigmatophyceae). J. Phycol. 44:768-776.

Choi, S. P. & Sim, S. J. 2009. Production of CO_2 by biological process using microalgae. KIC News 12:1-11.

Chong, D. K. X. & Langridge, W. H. R. 2000. Expression of full-length bioactive antimicrobial human lactoferrin in potato plants. Transgenic Res. 9:71-78.

Chow, K. -C. & Tung, W. L. 1999. Electrotransformation of *Chlorella vulgaris*. Plant Cell Rep. 18:778-780.

Dawson, H. N., Burlingame, R. & Cannons, A. C. 1997. Stable transformation of *Chlorella*: rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. Curr. Microbiol. 35:356-362.

Dreesen, I. A. J., Charpin-El Hamri, G. & Fussenegger, M. 2010. Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. J. Biotechnol. 145:273-280.

Ebrahimi, N., Memari, H. R., Ebrahimi, M. A. & Ardakani, M. R. 2012. Cloning, transformation and expression of human gamma interferon gene in tomato (*Lycopersicon esculentum* Mill.). Biotechnol. Biotechnol. Equip. 26:2925-2929.

El-Sheekh, M. M. 2000. Stable chloroplast transformation in *Chlamydomonas reinhardtii* using microprojectile bombardment. Folia Microbiol. 45:496-504.

Feng, S., Xue, L., Liu, H. & Lu, P. 2009. Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. Mol. Biol. Rep. 36:1433-1439.

Franklin, S., Ngo, B., Efuet, E. & Mayfield, S. P. 2002. Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. Plant J. 30:733-744.

Fuhrmann, M., Oertel, W. & Hegemann, P. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. Plant J. 19:353-361.

Geng, D. -G., Han, Y., Wang, Y. -Q., Wang, P., Zhang, L. -M., Li, W. -B. & Sun, Y. -R. 2004. Construction of a system for the stable expression of foreign genes in *Dunaliella salina*. Acta. Bot. Sin. 46:342-346.

Heitzer, M., Eckert, A., Fuhrmann, M. & Griesbeck, C. 2007. Influence of codon bias on the expression of foreign genes in microalgae. Adv. Exp. Med. Biol. 616:46-53.

Kindle, K. L. 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. U. S. A. 87:1228-1232.

Kindle, K. L., Richards, K. L. & Stern, D. B. 1991. Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. U. S. A. 88:1721-1725.

Kovar, J. L., Zhang, J., Funke, R. P. & Weeks, D. P. 2002. Molecular analysis of the acetolactate synthase gene of *Chlamydomonas reinhardtii* and development of a genetically engineered gene as a dominant selectable marker for genetic transformation. Plant J. 29:109-117.

Kumar, S. V., Misquitta, R. W., Reddy, V. S., Rao, B. J. & Rajam, M. V. 2004. Genetic transformation of the green alga-*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant Sci. 166:731-738.

Ladygin, V. G. 2003. The transformation of the unicellular alga *Chlamydomonas reinhardtii* by electroporation. Microbiology 72:585-591.

Ladygin, V. G. 2004. Efficient transformation of mutant cells of *Chlamydomonas reinhardtii* by electroporation. Process Biochem. 39:1685-1691.

Li, Y., Geng, Y., Song, H., Zheng, G., Huan, L. & Qiu, B. 2004. Expression of a human lactoferrin N-lobe in *Nicotiana benthmiana* with potato virus X-based agroinfection. Biotechnol. Lett. 26:953-957.

Lorimer, D., Raymond, A., Walchli, J., Mixon, M., Barrow, A., Wallace, E., Grice, R., Burgin, A. & Stewart, L. 2009. Gene composer: database software for protein construct design, codon engineering, and gene synthesis. BMC Biotechnol. 9:36.

Mayfield, S. P. & Kindle, K. L. 1990. Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. Proc. Natl. Acad. Sci. U. S. A. 87:2087-2091.

Metz-Boutigue, M. -H., Jollès, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J. & Jollès, P. 1984. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur. J. Biochem. 145:659-676.

Nakamura, Y., Gojobori, T. & Ikemura, T. 2000. Codon usage tabulated from international DNA sequence databases: status of the year 2000. Nucleic Acids Res. 28:292.

Picerce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J. & Spik, G. 1991. Molecular cloning and se-

quence analysis of bovine lactotransferrin. Eur. J. Biochem. 196:177-184.

Puigbò, P., Bravo, I. G. & Garcia-Vallvé, S. 2008*a*. CAI-cal: a combined set of tools to assess codon usage adaptation. Biol. Direct 3:38.

Puigbò, P., Bravo, I. G. & Garcia-Vallvé, S. 2008*b*. E-CAI: a novel server to estimate an expected value of Codon Adaptation Index (eCAI). BMC Bioinformatics 9:65.

Puigbò, P., Guzmán, E., Romeu, A. & Garcia-Vallvé, S. 2007. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res. 35S:W126-W131.

Rose, T. M., Plowman, G. D., Teplow, D. B., Dreyer, W. J., Hellström, K. E. & Brown, J. P. 1986. Primary structure of the human melanoma-associated antigen p97 (melanotransferrin) deduced from the mRNA sequence. Proc. Natl. Acad. Sci. U. S. A. 83:1261-1265.

Salmon, V., Legrand, M., Slominny, M. -C., El Yazide, I., Spick, G., Gruber, V., Bournat, P., Olagnier, D., Mison, M., Theisen, B. & Mérot, B. 1998. Production of human lactoferrin in transgenic tobacco plants. Protein Expr. Purif. 13:127-135.

Schroda, M. 2006. RNA silencing in *Chlamydomonas*: mechanisms and tools. Curr. Genet. 49:69-84.

Shimogawara, K., Fujiwara, S., Grossman, A. & Usuda, H. 1998. High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. Genetics 148:1821-1828.

Sun, G., Zhang, X., Sui, Z. & Mao, Y. 2008. Inhibition of pds gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). Mar. Biotechnol. 10:219-226.

Sun, Y., Gao, X., Li, Q., Zhang, Q. & Xu, Z. 2006. Functional complementation of a nitrate reductase defective mutant of a green alga *Dunaliella viridis* by introducing the nitrate reductase gene. Gene 377:140-149.

Sun, Y., Yang, Z., Gao, X., Li, Q., Zhang, Q. & Xu, Z. 2005. Expression of foreign genes in *Dunaliella* by electroporation. Mol. Biotechnol. 30:185-192.

Takase, K., Hagiwara, K., Onodera, H., Nishizawa, Y., Ugaki, M., Omura, T., Numata, S., Akutsu, K., Kumura, H. & Shimazaki, K. 2005. Constitutive expression of human lactoferrin and its N-lobe in rice plants to confer disease resistance. Biochem. Cell Biol. 83:239-249.

Tang, D. K. H., Qiao, S. Y. & Wu, M. 1995. Insertion mutagenesis of *Chlamydomonas reinhardtii* by electroporation and heterologous DNA. Biochem. Mol. Biol. Int. 36:1025-1035.

Tran, M., Zhou, B. & Pettersson, P. L., Gonzalez, M. J. & Mayfield, S. P. 2009. Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. Biotechnol. Bioeng. 104:663-673.

Xia, X. 2007. An improved implementation of codon adaptation index. Evol. Bioinform. Online 3:53-58.

Walker, T. L., Becker, D. K., Dale, J. L. & Collet, C. 2005. Towards the development of a nuclear transformation system for *Dunaliella tertiolecta*. J. Appl. Phycol. 17:363-368.

Wang, C., Wang, Y., Su, Q. & Gao, X. 2007*a*. Transient expression of the GUS gene in a unicellular marine green alga, *Chlorella* sp. MACC/C95, via electroporation. Biotechnol. Bioprocess Eng. 12:180-183.

Wang, T., Xue, L., Hou, W., Yang, B., Chai, Y., Ji, X. & Wang, Y. 2007*b*. Increased expression of transgene in stably transformed cells of *Dunaliella salina* by matrix attachment regions. Appl. Microbiol. Biotechnol. 76:651-657.

Weickert, M. J., Doherty, D. H., Best, E. A. & Olins, P. O. 1996. Optimization of heterologous protein production in *Escherichia coli*. Curr. Opin. Biotechnol. 7:494-499.

Yang, Z., Li, Y., Chen, F., Li, D., Zhang, Z., Liu, Y., Zheng, D., Wang, Y. & Shen, G. 2006. Expression of human soluble TRAIL in *Chlamydomonas reinhardtii* chloroplast. Chin. Sci. Bull. 51:1703-1709.