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Research article

EXPRESSION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) CAPSID PROTEIN VP1 IN *CHLAMYDOMONAS REINHARDTII* AS A POSSIBLE SOURCE OF RECOMBINANT VACCINE

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ABSTRACT: A chimeric construct of foot and mouth disease virus capsid protein VP1 was expressed in *Chlamydomonas reinhardtii* via *Agrobacterium*-mediated genetic transformation. Integration of the foreign gene in nuclear genome of the algae was confirmed by PCR and expression of the recombinant protein was confirmed at translational level. Results showed that the foreign gene was integrated in nucleus and the recombinant protein was produced in the microalgal cells. Transformation process together with detection of recombinant protein are discussed.
Keywords: genetic transformation, Foot and Mouth Disease, *chlamydomonas*, recombinant vaccine, VP1

INTRODUCTION

During recent years, green microalgae have emerged as highly suitable production platforms for a wide range of industrial and therapeutic proteins. A number of therapeutic proteins such as human antibodies, vascular endothelial growth factor and structural proteins like fibronectin have been successfully expressed in fresh water microalgae *Chlamydomonas reinhardtii* [1, 2]. Application of microalgae as a novel system for production of recombinant vaccines is a potentially valuable option possessing some advantages over microbial and plant systems [3]. Microalgae have tick cell walls protecting the expressed antigen from digestion by hydrolyzing enzymes in stomach medium, they can be grown without so much expense and their biomass accumulation can be achieved in a short time. Moreover, microalgae such as *C. reinhardtii* are generally recognized as safe (GRAS), so they can be eaten fresh and this enhances the immunogenic potential of the expressed vaccine within algal cell [4].
Foot-and-Mouth Disease Virus (FMDV) is the causative agent of foot and mouth disease, highly contagious disease adversely affecting milk and meat-producing animals around the world [5]. In nearly all investigations studying recombinant vaccine production against FMD, the G–H loop of the VP1 capsid protein of FMDV, spanning residues 134 to 158, has been reported as the main immunogenic site for neutralizing antibodies [6]. However, this region lacks T helper (Th) cell epitopes; so is not a suitable candidate for practical development of recombinant vaccine [7]. It has been reported that inclusion of flanking regions of G-H loop, covering 129-169 amino acids, enhances the immunogenicity of recombinant vaccine [7]. In this paper the DNA sequence encoding 129-169 amino acids of VP1 capsid (FMDV, serotype O) protein was expressed in green microalgae *C. reinhardtii* via *Agrobacterium*-mediated genetic transformation. To enhance expression level of the foreign gene, some modifications such as inclusion of ribosome binding site and signal peptide were performed. A eukaryotic ribosome binding site called Kozak sequence was added to 5' end of the gene. Kozak sequence has been reported to elevate gene expression in many eukaryotic systems [8]. Furthermore, an endoplasmic reticulum signal peptide (SEKDEL) was included in 3' end of the foreign gene just before stop codon. This signal peptide has been reported to enhance recombinant protein production by targeting the protein to endoplasmic reticulum where enzymes for correct folding of proteins are present [8]. Integration and expression of the foreign gene in transformed cells were evaluated by molecular methods.

MATERIALS AND METHODS

A 120 bp long fragment of VP1 coding region including G-H loop and its flanking region was adopted as the base of our new construct. Some modifications were performed to enhance expression of the construct in microalgal cells. A eukaryotic ribosome binding site called Kozak sequence, GCCACC, was introduced prior to the start codon and an endoplasmic reticulum signal peptide called SEKDEL consisting of six amino acids was attached to 3' end before stop codon. Start codon (AUG) and stop codon (UAA) were also added in to the 5' and 3' ends of the construct, respectively. Moreover, to facilitate cloning procedure, restriction sites of *Bam*HI and *Sac*I were introduced into the 5' and 3' ends of the synthetic gene, respectively (Figure 1). The construct was synthesized and cloned into the pGem T-Easy vector (Bioneer, South Korea).

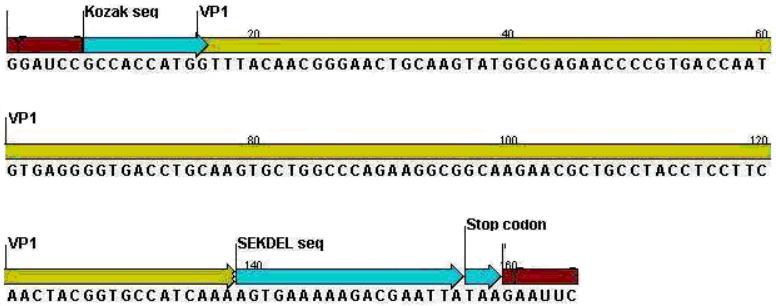

Fig. 1 Schematic presentation of VP1 synthetic construct.

The synthetic VP1 gene fragment was removed from pGem T-Easy vector by digestion with *Bam*HI and *Sac*I and was inserted into the plant expression vector pBI121 downstream of the CaMV 35S promoter and upstream of the nopaline synthase (NOS) terminator, yielding pBI121-VP1vector. The resulted vector was used to transform *E. coli* strain DH5-α and kanamycin-resistant colonies were selected after overnight incubation at 37°C. The plasmid was extracted from bacterial cells using alkaline lysis method and introduced into *Agrobacterium tumefaciens* strain GV3101 by heat shock method. Transformed cells were selected by kanamycin-resistance and PCR.

C. reinhardtii cells were transformed using the method reported by Kumar et al [9] with some modifications. Briefly, 150µl of microalgal suspension in log phase was transferred to solid Tris Acetate Phosphate (TAP) medium and incubated in light for 2 days to form algal lawn. A fresh *Agrobacterium* culture (A600 = 0.5) grown in liquid LB medium containing appropriate antibiotics (20 mg/l rifampicin and 50 mg/l kanamycin) was centrifuged in 6000g for 5 min, the supernatant was discarded and the bacterial pellet was resuspended in 200 µl TAP broth. The bacterial suspension was spread to the thin layer of *Chlamydomonas* lawn growing on agar plate. The co-cultivation plates were incubated for 2 days at 25°C. The cells were then harvested and washed twice with liquid TAP medium containing 500 mg/l cefotaxime to eliminate bacterial residues. The washed *Chlamydomonas* cells were cultured in solid selection medium containing 50 mg/l Kanamycin and 500 mg/l cefotaxime.

Genomic DNA was extracted from the colonies which were formed in the selection medium. The presence of the VP1 synthetic gene was confirmed by PCR analysis using specific primers. The sequence of forward and reverse primers were 5' ATGGAAATTGTAAGTATGGAGA 3' and 5' GAAGAAAGCGAAAGGAGC 3' respectively. Forward primer matches inside the VP1 and reverse primer matches a part in NOS terminator sequence. Genomic DNA of wild type microalgae was used as negative control. PCR was carried out as follows: 94 ˚C 1 min, 58 ˚c1 min, 72 ˚c 2 min, 30 cycles. Expression of the foreign gene in *C. reinhardtii* was evaluated by protein dot blot. Small samples of the protein (about 5µl) were dotted on nitrocellulose membrane and allowed to dry under room temperature. BSA (Bovine Serum Albumin) was used to block non-specific antibody reactions. The membrane was then incubated for 60min in 37◦c with primary antibody (1:2000 dilution), washed by PBS (Phosphate buffer Saline) and PBST for three times and finally incubated with secondary conjugated antibody (1:1500). Color was developed by addition of OPD (Ortho-Phenylenediamine). Protein sample of non-transformed plant was used as negative control. Expression of the synthetic gene was quantitatively measured using Enzyme-Linked Immunosorbent Assay (ELISA) method. ELISA plate was coated with total soluble proteins from the wild type and the transformed microalgae cells and known FMDV VP1 antigen at 37^{o}C for one hour; followed by incubation with 1% bovine serum albumin (BSA) in PBS for 2 h at 37◦ C to prevent non-specific binding. The well was washed by PBST/PBS and incubated with antiserum reactive against FMDV (1:2000 dilutions) and then alkaline phosphatase conjugated with anti rabbit IgG (1:1500). Wells were developed with TMB substrate; the color reaction was stopped by 2N H_2SO_4 and read at 405 nm of wavelength.

RESULTS AND DISCUSSION

We used an *A. tumefaciens* mediated transformation method [9] for generation of transgenic *C. reinhardtii* cells expressing a synthetic version of VP1 protein. After cocultivation period, a few colonies were appeared on the culture medium supplemented with kanamycin as selection agent. These putative transgenic colonies were evaluated for presence of the foreign gene by PCR analysis, followed by separation on 1% agarose gel. PCR results confirmed the integration of foreign gene in nuclear genome of *C. reinhardtii*, whereas no band was observed for non-transformed microalgae (figure 2). Feasibility of this type of nuclear transformation for expression of chimeric foreign genes in microalgae has been reported by other authors as well [9, 10]. Since the first report of transformation of *C. reinhardtii* in 1989 [11], many protocols have been proposed for this purpose including glass bead agitation [12], electroporation [13], particle bombardment [14], etc. among these, *Agrobacterium* mediated transformation is a fairly simple method with an acceptable frequency of transformation.

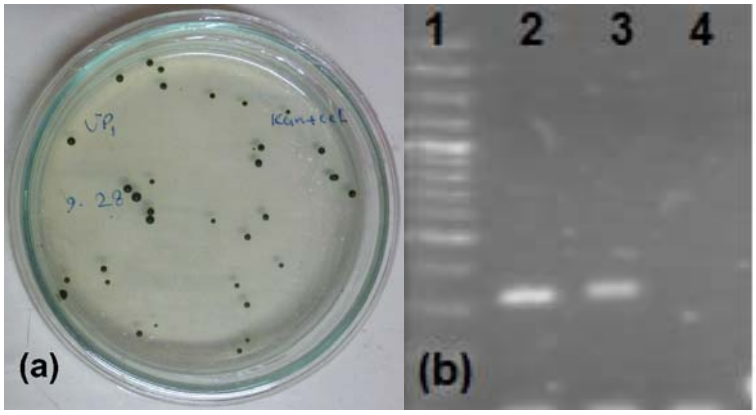

Fig-2: primary screening for transformed cells of *C. reinhardtii*. (a). putative transformed colonies were formed on solid TAP medium supplemented with kanamycin as selectable antibiotic. (b). Detection of VP1 gene in putative transformed colonies of *C. reinhardtii*. Lane 1, size marker; lane 2, purified pBI121vp1 plasmid as positive control; lane 3, transformed cell; lane4, wild type cell.

Expression of the VP1 gene in microalgal cells was evaluated by protein dot blot assay (Figure 3). As can be seen from the figure, positive response was observed for the sample obtained from transgenic colony while a very faint dot was achieved for wild type cells. Although dot blot assay confirmed the expression of foreign gene in transformed cells, color of dot corresponding to transformed cells was not much stronger than that of wild type cells, suggesting low level of gene expression in putative transformed cells.

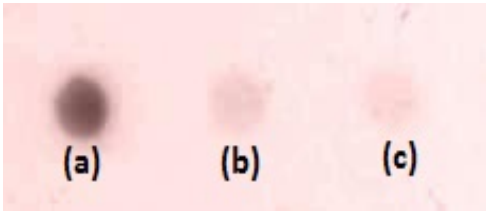

Fig-3: Dot blot analysis of VP1 expression in transformed and wild type cells of *C. reinhardtii*: (a) positive control, (b) transformed colony and (c) wild type colony.

Gene expression was quantitatively assessed by ELISA assay. ELISA results confirmed expression of VP1 gene in transformed cells, although the expression level was not much robust. In our opinion, this rather low expression level can be mainly due to incompatibility between foreign gene construct and translation machinery of *C. reinhardtii*. Generally, Low level of foreign gene expression in microalgae such as *C. reinhardtii* can be due to the lack of adequate regulatory sequences, positional effects, biased codon usage, incorrect polyadenylation, inappropriate nuclear transport, unstability of the mRNA, or gene silencing [15]. For example, CaMV 35S promoter used in this study has been reported not to be much active in algal host compared to higher plant [16]. Moreover, it has been reported that low expression of foreign genes in algal system is often due to the incompatibility of the codon usage in their coding regions which consequently decreases the efficiency of the translation [17].

Expression of VP1 gene in the present study was considerably higher than that of non-transformed cells (Figure 4) showing that the recombinant protein was appropriately assembled in the microalgal cells. This detectable level of the foreign protein in transformed cells can be partly due to inclusion of Kozak sequence and SEKDEL signal peptide whose efficiency in enhancing gene expression has been reported in higher plants [8]. It seems possible to further enhance expression level of foreign genes in *C. reinhardtii* by application of transcriptional and translational elements which are specific for the microalgae as suggested by many investigators [15, 18].

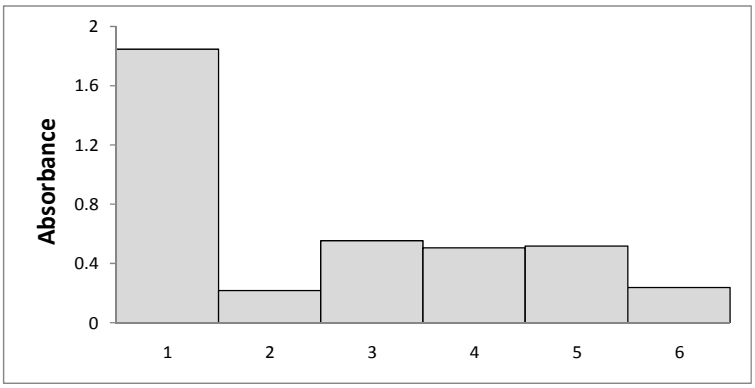

Fig-4: Quantification of VP1 expression in transgenic colonies of *C. reinhardtii*. 1, positive control; 2, blank (PBS); 3-5, transformed cells; 6, non-transformed cells.

Geng et al, [2003] expressed hepatitis B surface antigen gene in nuclear genome of another microalgae *Dunaliella salina*. Similar to our results, low expression level of the foreign gene was observed and the authors maintained that higher level of expression is required to consider this recombinant protein as an effective vaccine [19]. This low level of expression can be addressed by some strategies such as codon optimization. It has been reported that codon optimization can enhance expression level in nuclear transformation as high as 5-fold [20] or up to 80-fold in chloroplast transformation [21]. But if the low expression is due to gene silencing rather than codon optimization, the problem become more complicated because gene silencing is not easy to combat [22]. Further investigation is required for reaching an explicit conclusion on this issue. Another feature of the present study was instability of transformed cells of *C. reinhardtii*. The green colonies of transformed cells gradually turned in to yellow about two weeks after transformation procedure. Furthermore, expression or even presence of the VP1 gene was not detectable in these colonies showing that the transformed cells had lost the foreign construct. This result is in agreement with those reported by some other investigators [18, 23]. Instability of foreign gene expression in *C. reinhardtii* cell specially under nuclear transformation is major drawback of commercialization of recombinant vaccine production in this valuable microalgae. The instability, however, can be overcame by freezing the transformed colonies [24]. As a conclusion, in the present study we showed feasibility of *Agrobacterium*-mediated nuclear transformation of *C. reinhardtii* as a valuable platform for production of recombinant vaccine. Although the expression level was not much high, it is still promising because transformation procedure- as the first step toward production of recombinant vaccine in *C. reinhardtii*- proved to be efficient. However, some modifications seem necessary to reach a reasonable level of antigen expression in this microorganism, among which are codon optimization and application of transcriptional/translational elements specific to *C. reinhardtii*. Although we used Kozak sequence and SEKDEL signal peptide to enhance VP1 expression in *C. reinhardtii*, application of more genetic factors affecting gene transcription and translation seem necessary to achieve an acceptable level of recombinant vaccine production in microalgal cells. This may be the subject of our next study.

CONCLUSION

In this study we investigated production of recombinant FMD vaccine in green microalga *C. reinhardtii*. Results showed that the recombinant protein was well expressed in transgenic microalgal cells. These result reconfirmed microalgal platform as an appropriate system for expression of recombinant vaccine. Low level gene expression can be addressed by application of several strategies as discussed in the text. Enhanced expression of FMD vaccine in microalgae can be the subject of future investigations.

REFERENCES

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

[2] Rasala, B.A., and Mayfield, S.P. 2011. The microalga Chlamydomonas reinhardtii as a platform for the production of human protein therapeutics, Bioengineered bugs. 2**:** 50-54.
[3] Specht, E., Miyake-Stoner, S., and Mayfield, S. 2010. Micro-algae come of age as a platform for recombinant protein production, Biotechnol Lett. 32: 1373–1383
[4] Dreesen, I.a.J., Charpin-El Hamri, G., and Fussenegger, M. 2010. Heat-stable oral alga-based vaccine protects mice from Staphylococcus aureus infection, Journal of Biotechnology. 145: 273-280.
[5] Carrillo, C., Wigdorovitz, A., Oliveros, C., Zamorano, P., Sadir, AM., G´omez, N. 1998. Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants, J Virol. 72: 1678–1690.
[6] Wigdorovitz, A., Carrillo, C., Trono, K. 2001. Induction of a virus specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants, Virol Immunol. 14: 49–57.
[7] Wang , C.Y., Chang, T.Y, Walfield, A.M, Ye, J, Shen, M,. Chen, S.P. Li, M.C, Lin, Y.L, Jong, M.H, Yang, P.C, Chyr, N, Kramer, E, and Brown, F. 2002. Effective synthetic peptide vaccine for foot-and-mouth disease in swine, Vaccine. 20: 2603–2610
[8] Kang, TJ., Han, SC, Jang, MO, Kang, KH, Jang, YS, Yang MS. 2004. Enhanced Expression of B-Subunit of Escherichia coli Heat-Labile Enterotoxin in Tobacco by Optimization of Coding Sequence, Applied Biochemistry and Biotechnology. 117: 175-187
[9] Kumar, S.V., Misquitta, RW, Reddy, VS, Rao, BJ., Rajam, MV. 2004. Genetic transformation of the green alga—Chlamydomonas reinhardtii by Agrobacterium tumefaciens, Plant Sci. 166: 731–8.
[10] Pratheesh, P.T, Shonima, G.M, Jiji T, Abraham C I, and Muraleedhara, G. 2012. Study on efficacy of different Agrobacterium tumefaciens strains in genetic transformation of microalga Chlamydomonas reinhardtii, Advances in Applied Science Research. 3: 2679-2686.
[11] Fernández, E, Schnell, R, Ranum, L.P. 1989. Isolation and characterization of the nitrate reductase structural gene of Chlamydomonas reinhardtii, Proc Natl Acad Sci USA. 86: 6449-6453.
[12] Kindle, KL. 1990. High-frequency nuclear transformation of Chlamydomonas reinhardtii, Proc Natl Acad Sci USA. 87: 1228-1232.
[13] Shimogawara, K, Fujiwara, S, Grossman, A. 1998. High-efficiency transformation of Chlamydomonas reinhardtii by electroporation. Genetics. 148: 1821-1828.
[14] Sanford, JC, Smith, F.D, Russell, JA. 1993. Optimizing the biolistic process for different biological applications, Methods in Enzymology. 217: 483-509.
[15] Cerutti, H., Johnson, AM, Gillham, NW. 1997. A eubacterial gene conferring spectinomycin resistance on Chlamydomonas reinhardtii: Integration into the nuclear genome and gene expression, Genetics. 145: 97-110.
[16] Fukuda, S., Mikami, K, Uji, T, Park, E. J, Ohba, T., Asada, K., Kitade, Y., Endo, H., Kato, I., and Saga, N. 2008. Factors influencing efficiency of transient gene expression in the red macrophyte Porphyra yezoensis. Plant Science. 174: 329-339.
[17] Mayfield, S. P., and Kindle, K. L. 1990. Stable nuclear transformation of Chlamydomonas reinhardtii by using a C. reinhardtii gene as the selectable marker. Proceedings of the National Academy of Sciences of the United States of America. Vol. 87: 2087- 2091.
[18] Hall, LM., Taylor, KB., and Jones, DD. 1993. Expression of a foreign gene in Chlamydomonas reinhardtii, Gene.124: 75-81.
[19] He, D.M., Qian, K.X, Shen, G.F, Zhang, Z.F, Li, Y.N, Su, Z.L, and Shao, H.B. 2003. Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in Chlamydomonas reinhardtii chroloplasts. Colloids and Surfaces B-Biointerfaces. 55: 26-30.
[20] Geng, D.G., Wang, Y.Q, Wang, P, Li, W.B., and Sun, Y.R. 2003. Stable expression of hepatitis B surface antigen gene in Dunaliella salina (Chlorophyta). Journal of Applied Phycology., 15: 451-456.
[21] 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.
[22] Franklin, S.E., Mayfield, SP. 2005. Prospects for molecular farming in the green alga Chlamydomonas reinhardtii. Curr Opin Plant Biol.7: 159–165
[23] Neupert, J, Karcher, D, Bock, R. 2009. Generation of Chlamydomonas strains that efficiently express nuclear transgenes. Plant J. 57: 1140–1150
[24] Ohresser, M, Matagne, R.F, Loppes, R.1997. Expression of the arylsulphatase reporter gene under the control of the nit1 promoter in Chlamydomonas reinhardtii., Curr Genet., 31: 264–271.
[25] Schroda, M, Blocker, D., Beck, CF. 2000. The HSP70A promoter as a tool for the improved expression of transgenes in Chlamydomonas., Plant J., 21: 121-131.