



Biolistic transformation of classical swine fever virus E2 gene and its expression in rice (*Oryza sativa*, L.)

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Abstract

Classical Swine Fever (CSF) is a disease caused by a Classical Swine Fever Virus (CSFV). The disease is highly contagious and affects both the domestic pigs and wild boars. The disease poses worldwide problem with large economic losses. The envelope protein E2 is highly immunogenic and therefore represents an important target. Expression of a wide range of foreign proteins in plant systems have gained interest in the recent years. The aim of the present study was to transfer the E2 gene into rice calli via biolistic bombardment method and to investigate the expression of E2 gene of classical swine fever virus in the Transformed rice plants. Confirmation for the presence of E2 gene was done by PCR and Southern blotting. The relative gene expression of the plant-produced E2 was assayed by Real Time PCR. Expression of recombinant E2 protein was immunogenically detected by ELISA and Western blotting. A protein band of approximately 60k Da detected by western blot showed good antigenicity of target E2 protein.

Keywords: NEH megha rice 1 (*Oryza sativa* L.), transformation, biolistic

Introduction

Classical Swine Fever Virus (CSFV) is a small, single stranded RNA virus of the genus *Pestivirus* and family *Flaviviridae* (Moennig, 2000) [20]. Classical Swine Fever (CSF) caused by CSFV is a disease of swine which occurs worldwide leading to severe economic losses. The World Organisation of Animal Health (OIE, 2014) [23] has considered CSFV as one of the most notifiable pathogen of swine. The disease is highly contagious leading to acute CSF infections resulting in high morbidity and mortality or prolonged chronic disease depending on the type of CSF strain virulence (Van Oirschot, 1986; Blome *et al.*, 2017) [31, 4]. The disease affects both domestic pigs as well as wild boars (Martinez *et al.*, 2009) [16] and spreads mainly by contact between healthy pigs with the infected animals (Moennig *et al.*, 2003; Zhang *et al.*, 2010; Blome *et al.*, 2017) [21, 33, 4]. CSF poses a global problem because of its large economic impact associated with its outbreak on animal health. The major characteristic of CSF is marked by immunosuppression that is induced on infected animals (Li *et al.*, 2009) [12]. The CSFV genome consists of a single open reading frame (ORF) of approximately 12.3 kb (Meyers and Thiel, 1996; Meyers *et al.*, 1989) [18, 19] which encodes a single polyprotein that is post-translationally processed into four structural proteins (i.e., C, Erns, E1 and E2) and eight non-structural proteins (i.e., Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Meyers and Thiel, 1996; Zhang *et al.*, 2010; Lowings *et al.*, 1996) [18, 33, 14]. Among the structural proteins, the envelope protein E2, of size 51-55 KDa serve as an important target. The immunogenic nature of E2 induce neutralizing antibodies at times of infection and provides protective immunity in the natural host (Bouma *et al.*, 2000; Van Rijn *et al.*, 1996;

Mauser *et al.*, 2005; Qi *et al.*, 2009) [6, 32, 17, 27]. The E2 glycoprotein is often used for designing DNA vaccines against CSF as it contains neutralizing epitopes responsible for eliciting an immune response (Qi *et al.*, 2008; Bouma *et al.*, 1999) [26, 5]. In India, with approximately 11 million pigs, 40% are present in the north eastern states. Majority of households in the north eastern region rely on pig farming as the major source of livelihood (Bett *et al.*, 2012) [3]. CSFV has emerged as a serious obstacle affecting the growth and profitable pig farming in India, particularly in the north eastern states. The prevalence of CSFV among the pig herds in Assam and in other parts of India have been reported (Ahuja *et al.*, 2015; Nandi *et al.*, 2011; Sarma *et al.*, 2011) [1, 22, 28] and several CSF outbreaks have also been reported suggesting its endemic nature (Barman *et al.*, 2010; Chakraborty *et al.*, 2011) [2, 7]. The present study was carried out with the aim of generating transgenic rice plants capable of expressing the CSFV E2 glycoprotein in Megha Rice 1 for which no previous reports are available. This study is based on transferring a single copy (low copy) or multiple copy (high copy) of CSFV E2 gene by exploiting the DNA delivery method using Biolistic and to produce transformed rice plants with proper level of expression of the CSFV E2 glycoprotein.

Materials and Methods

Plant material and gene construct

The plant material selected for the present study was NEH Meghalaya rice 1 (*Oryza sativa* L.). It is a cold tolerant, semi-dwarf, non-lodging rice lines of Meghalaya which was developed and released in 1992. This variety was derived from a cross between Pusa 33 (*Indica*) x Khonorullo (*Japonica*). The seeds were obtained from the Division of

Plant Breeding, ICAR Research Complex for NEH region, Umiam, Meghalaya. The DNA sequence of E2 glycoprotein was downloaded from GenBank (accession no. KM362426). It was optimized for expression in rice using the OPTIMIZER software (<http://genomes.urv.es/OPTIMIZER>). Plasmid pUC57 was used as a vector. The construct contains the CSFV E2 gene linked to the cauliflower mosaic virus (CaMV) 35S promoter and CaMV 3'UTR terminator (Fig 1). Synthesis, ligation and insertion in vector was outsourced to GCC Biotech (Pvt.) Ltd., Kolkata, India. The bacterial strain used for replicating the vector was *E. coli* strain, DH5 α . Plasmid DNA carrying CSFVE2 gene was isolated from the transformed *E. coli* DH5 α and was transformed into one month old rice calli by biolistic bombardment. Callus induction and regeneration was done as described previously (Gatphoh *et al.*, 2018) [8].

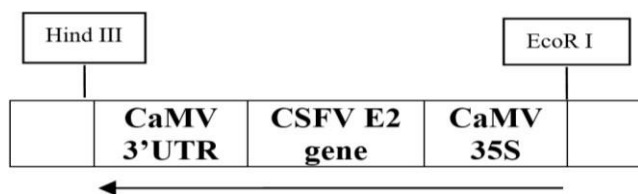


Fig 1: Plasmid pUC 57-CSFV2 containing CSFV E2 gene

PCR identification of transformants

Genomic DNA from leaves of putative transformants was isolated using CTAB method. A preliminary screening to identify the integration of the E2 gene in the transformants was carried out with PCR using DNA from transformed plants as templates. DNA from a non-transformed rice plant as well as plasmid DNA was also run as a negative and positive control respectively. PCR analysis was carried out using gene specific primers for CSFV E2. The CSFV E2 gene was amplified using the following 18 mer oligonucleotide primers.

E2-Fw: AGGGCAAGTACAACACCA

E2-Rv: GCTCGTAGTACTTGTTC

Realtime PCR analysis

A simple quantitative real-time PCR procedure was also carried out to determine the transgene integration. Amplification reactions were carried out on cDNA generated from total RNA extracted from newly emerged leaves of transformed and non-transformed rice plants. The thermal cycling conditions were: initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec and extension at 60°C for 2 min. The following primer sequences were used in the reactions for the amplification of the gene.

E2 F: TCRWCAACCAAYGAGATAGGG

E2 R: CACAGYCCRAAYCCRAAGTCATC

Δ CT for all samples was calculated from CT values obtained after the experiment [Δ CT = Ct (gene of interest) – Ct (housekeeping gene)]. Relative gene expression of the transformed plants was calculated using the delta-delta CT method [$\Delta\Delta$ CT = Δ Ct (treated sample) – Δ Ct (untreated sample)] (Ingham *et al.*, 2001; Livak and Schmittgen, 2001; Rao *et al.*, 2013).

Qualitative elisa assay

Expression of E2 protein in the transgenic rice plants was evaluated by simple qualitative ELISA (Enzyme-Linked

Immunosorbent Assay). The test was conducted following a standardized in-house protocol using known positive and negative sera as identified by IDEXX ELISA kit. The indirect-ELISA utilizes an unlabeled primary antibody (positive swine serum) and an enzyme-labeled secondary antibody (Anti-Pig IgG- Peroxidase antibody produced in rabbit, Israel). A positive antigen (Ag) control of the in-house ELISA containing non-infectious CSFV Ag was also used in the ELISA test. Also, extract from a non-transformed healthy plant was used as a negative control. The plant extracts was diluted to a concentration of 1:100 in coating buffer and 100 μ l of the diluted plant extract was used as coating antigen for coating the ELISA plate. Primary antibody (swine serum) at a concentration of 1:100 and an Anti-pig IgG HRP-conjugated secondary antibody at concentration 1:15000 was used to detect the presence of E2 antigen.

Southern blot analysis

For analysis, 20 μ g DNA extracted from transformed rice plants was digested with *HindIII* and *EcoRI*. The *HindIII* and *EcoRI* digested plasmid DNA containing CSFVE2 gene and digested DNA from untransformed rice plant was used as positive (P) and negative (N) control respectively. The digested DNA fragments were electrophoresed overnight on 0.8 % agarose gel at 20 Volt. The separated DNA fragments were then transferred onto a positively charged Nylon membrane (Hybond Nt, Amersham Pharmacia Biotech, Sweden) using a Trans-Blot semi-dry transfer system (Bio-Rad). A PCR product of the plasmid DNA containing CSFVE2 gene labeled with non-radioactive Biotin Decalabel DNA Labelling Kit (Fermentas) was used as a probe. Detection was done using Biotin Chromogenic Detection Kit (Thermo scientific) following the manufacturer's protocol.

SDS page and western blot analysis

To evaluate the expression of E2 protein, a Western-blot assay was performed. Total protein was extracted from leaf tissues of both transformed rice plants and from control plant. 200 mg fresh/frozen leaves was ground in liquid nitrogen and proteins was extracted in NP-40 extraction buffer. Homogenates was centrifuged and supernatant was collected. Amount of total protein content from crude extract was quantified using Bradford method. Total protein extract (30 μ l) combined with sample buffer containing bromophenol blue was resolved on reducing 12% SDS-PAGE using Mini-Protein II slab cell (Bangalore Genei, India). Two gels were run simultaneously, one for staining protein with Coomassie Brilliant Blue R-250 and the other for immunochemical detection. The separated proteins on SDS-PAGE were transferred onto a PVDF membrane (Immun-Blot, PVDF, BIORAD) using a semi-dry blotting apparatus (Bio-Rad) at 20 V for 1 hour. The membrane was then sequentially treated first by blocking it with 5% skimmed milk prepared in TBST (TBS containing 0.05% Tween-20) for 1 hour at room temperature followed by soaking membrane in primary antibody (positive swine serum) diluted in TBST and was placed overnight at 4°C. Membrane was then soaked in Anti-pig IgG HRP-conjugated diluted in TBST for 1 hour. After the final washes, colour development and detection of E2 protein expression was performed by staining the membrane in NBT-BCIP (Genei) for 10 min in dark. Extract from a non-

transformed plant was used as a negative control in the experiment.

Results

PCR analysis of transformants

Amplifications of a 590 bp fragment was obtained in the transformed rice plants as well as in the positive control. However this band was absent in the negative control (untransformed plant). Thus, PCR results primarily indicated the stable integration of gene under study (Fig 2). Of the total plants regenerated, 38 plants were identified to be PCR positive (Table 1).

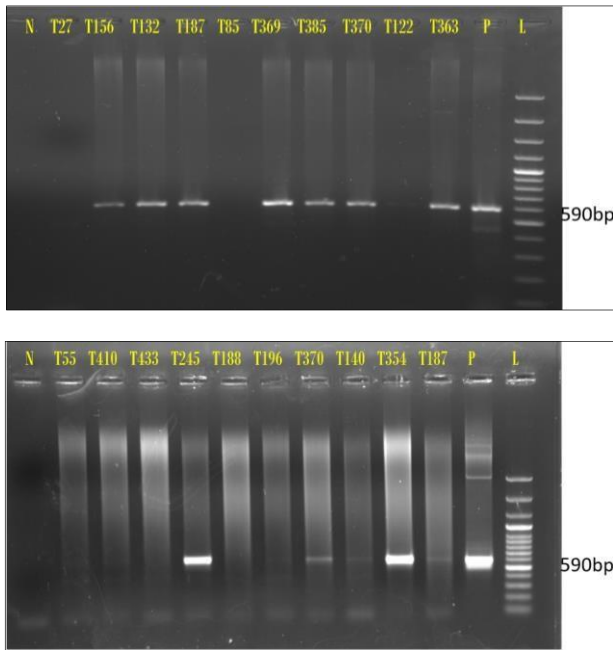


Fig 2: PCR amplification of genomic DNA from putative transgenic plants showing amplification of 590 bp fragment

Table 1: Frequency of plantlet regeneration and transformation

No. of calli transformed	No. of plants regenerated from calli	No. of PCR positive plants	% of plantlet regeneration
270	450	38	166.66

Real time PCR analysis

Real time results revealed variations in the expression of the ten rice plants. A graph was plot according to $\Delta\Delta CT$ values calculated from the data obtained from the experiment performed (Fig 3). It was observed that the $\Delta\Delta CT$ values of T286 and T369 rice plants (9.9 and 9.95 respectively) are significantly higher than the $\Delta\Delta CT$ values of the other rice plants with $\Delta\Delta CT$ value of T156 rice plant showing the least (1.375).

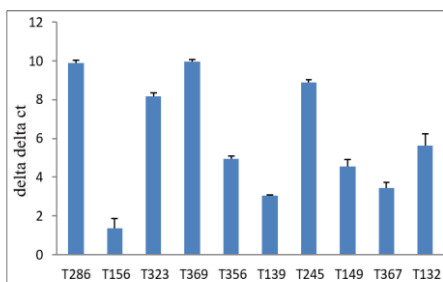
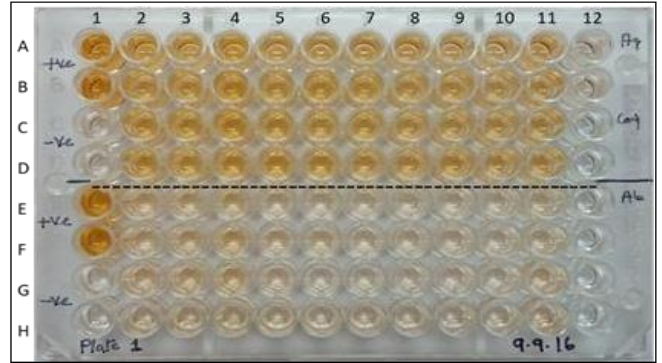


Fig 3: Real Time PCR analysis of ten transgenic rice plants

ELISA assay of the expressed protein

Analysis for the presence of E2 protein was done using a known positive and known negative serum sample as confirmed by IDEXX kit. A simple qualitative in-house indirect ELISA revealed the expression of E2 protein in the ten transgenic rice plants (Fig 4). Thus, ELISA assay showed good antigen-antibody interaction which indirectly confirmed the expression of E2 protein.



A1, B1, E1 & F1 – Positive control
 C1, D1, G1 & H1 – Negative control
 A2 – D11 – Plant extract with positive sera
 E2 – H11 – Plant extract with negative sera
 A12 & B12 - Antigen control
 C12 & D12 – Conjugate control
 E12 & F12 – Antibody control
 G12 & H12 – Blank

Fig 4: ELISA plate showing expression of E2 protein in the transformed rice plants

Southern blot analysis of transformed rice plants

Southern hybridization of genomic DNA of the transgenic rice plants digested with *HindIII* and *EcoRI*, and probed with CSFV E2 gene specific biotin labelled probe confirmed the integration of the target gene into the transgenic rice plants genome (Fig 5). Genomic DNA of non-transformed plant as negative control was run in lane 1 where no hybridization signal was detected. Lane 2 shows the positive control of 2.0 KB fragment and in lane 3-7, a band of approximately 2.0 KB fragment was observed in the transformed rice plants which confirmed the stable integration of the transgene. Thus, southern blot assay indicated that the transformed rice plants contained the foreign gene insert.

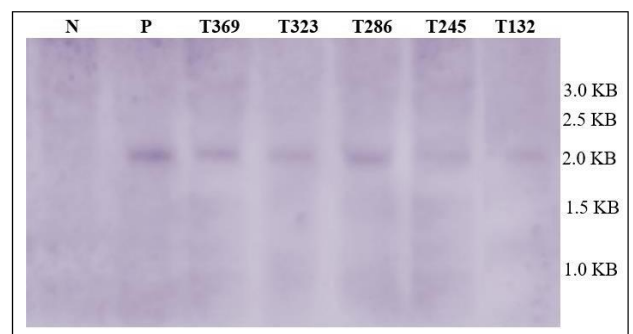


Fig 5: Southern blot analysis of five transgenic rice plants

Western blotting analysis of rice transformants

Protein content in plant extracts was quantified using Bradford assay. The integrity of the protein was first monitored by SDS-PAGE which revealed a protein band of approximately 60k Da in transformed plants which was

absent in control plant. To confirm the products, western blot assay was performed. The target protein was immunogenically detected using primary and secondary antibodies. A prominently stained clear protein band corresponding to an approximate molecular mass of 60 kDa was identified in the five transformants *viz.* T369, T323, T286, T245 and T132, and no band was detected in control plant (Fig 6). Thus, Western blotting showed a good antigenicity of the target E2 protein.

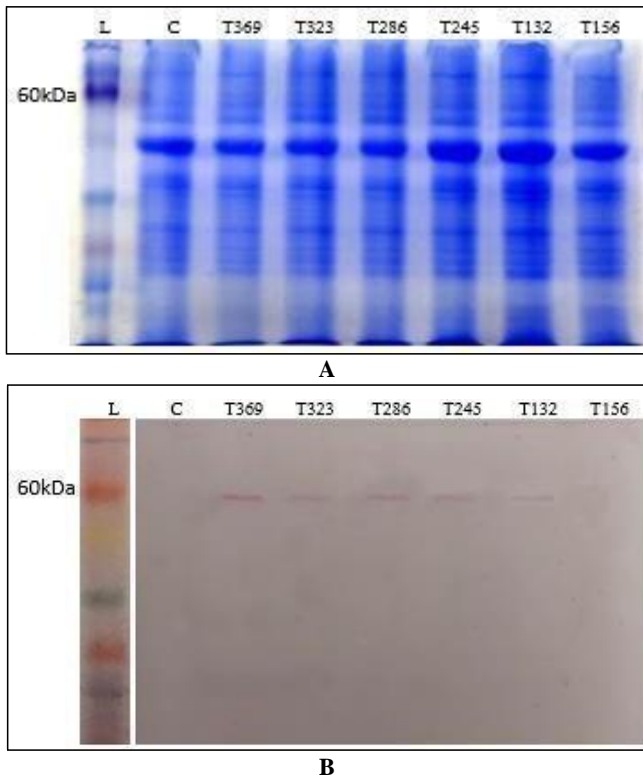


Fig 6: SDS-PAGE and Western blot analysis of six transgenic rice plants. Protein extracts from untransformed rice plant was loaded in lane 2 and from transformed rice plants in lane 3-8 against a 100kDa ladder. The molecular weight of the protein at approximately 60kDa confirmed the stable integration of the CSFV E2 gene in the transformed rice plants.

Discussion

Plants are an interesting tool for the construction of edible oral vaccines as they are capable of producing proteins originating from foreign organisms (Lindh *et al.*, 2009; Ma *et al.*, 2003) [13, 15]. Plant system has widely been used for the expression of various proteins of interest that may be useful for veterinary or for medical applications. Currently, there are few studies which reported the expression of CSFV E2 protein in plants. Legocki *et al.*, (2005) [11] reported the expression of E2 protein in lettuce and alfalfa and oral vaccination with the transgenic plants induced antibody responses in laboratory animals. E2 gene expressed in *Chlamydomonas reinhardtii* chloroplasts had shown to induce antibodies against the E2 protein (He *et al.*, 2007) [9]. Shao *et al.*, (2008) [29] reported the expression of CSFV E2 protein in tobacco chloroplasts showing the induction of protective antibody response against CSFV by immunizing mice with leaf extracts containing the E2 protein. Laughlin *et al.*, (2019) [10] successfully expressed the E2 protein in *Nicotiana benthamiana* for use as a subunit vaccine against CSFV. The E2 protein has also been expressed in *Nicotiana benthamiana* and recombinant E2 protein when injected into mice or piglets generated anti-E2 antibodies against CSFV (Park *et al.*, 2019; Park *et al.*,

2020) [24, 25]. Plant-based expression system offers many advantages in terms of lower capital investment, time efficiency, high yield and low contamination risk (Tonegawa *et al.*, 2003) [30]. The objective of this study was to explore the stable expression of E2 protein from Classical Swine Fever Virus in Megha Rice 1. Efficient regeneration alone does not necessarily lead to efficient transformation. There is a need to develop advanced transformation methods that would not only incorporate the required into plants but also enable generation of transgenic events in a high-throughput manner. One of the noticeable points in this experiment is that no negative or positive selection marker was used so that the negative effect of antibiotic resistance protein is avoided. A post regeneration screening was done to select positive plants. This was made possible by first developing a very high frequency regeneration system. The present work showed that combination of a proper construct and a high frequency regeneration system can be combined to use plants as a vaccine factory. Thus, the work presented here is the expression of CSFV E2 protein in the variety Megha Rice 1 using biolistic mediated transformation method, and the strategy used in this study was successful in achieving E2 gene replication, transcription and expression in rice plants.

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Declaration

The authors declare no conflict of interests.

References

1. Ahuja A, Bhattacharjee U, Chakraborty AK, Karam A, Ghatak S, Puro K *et al.* Complete genome sequence of classical swine fever virus sub-genogroup 2.1 from Assam. India. Genome Announcements,2015;3(1):e01437-14.
2. Barman N, Gupt R, Bora D, Kataria R, Tiwari A, Roychoudhury P. Molecular characterization of classical swine fever virus involved in the outbreak in Mizoram. Indian Journal of Virology,2010;21:76-81.
3. Bett B, Deka R, Padmakumar V, Rajasekhar M. Classical swine fever in northeast India: Prevention and control measures. Policy Brief. Nairobi: International Livestock Research Institute, 2012.
4. Blome S, Staubach C, Henke J, Carlson J, Beer M. Classical swine fever-An updated review. Viruses,2017;9(4):86.
5. Bouma A, de Smit AJ, de Kluijver EP, Terpstra C, Moormann RJM. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. Veterinary Microbiology,1999;66:101-114.
6. Bouma A, De Smit AJ, De Jong MCM, De Kluijver EP, Moormann RJM. Determination of the onset of the herd-immunity induced by the E2 sub-unit vaccine against classical swine fever virus. Vaccine,2000;18:1374-1381.
7. Chakraborty SV, Chakraborty S, Veeregoda B, Rathnamma D, Venkatesha M, Leena G *et al.* Molecular characterization and genogrouping of classical swine fever virus isolated from field outbreaks. Indian Journal of Animal Sciences,2011;81:8.

8. Gatphoh EM, Pattanayak A, Iangrai B, Khongwir DEA, Pale G, Kalita MC. Optimizing tissue culture media for efficient callus induction and regeneration from rice seeds. *International Journal of Current Trends in Science and Technology*,2018;8:20201-20210.
9. He DM, Qian KX, Shen GF, Zhang ZF, Li YN, Su ZL et al. Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in *Chlamydomonas reinhardtii* chloroplasts. *Science Direct, Colloids and surfaces B: Biointerfaces*,2007;55(1):26-30.
10. Ingham DJ, Beer S, Money S, Hansen G. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques*,2001;31:132-140.
11. Laughlin RC, Madera R, Peres Y, Berquist BR, Wang L, Buist S et al. Plant-made E2 glycoprotein single-dose vaccine protects pigs against classical swine fever. *Plant Biotechnology Journal*,2019;17:410-420.
12. Legocki AB, Miedzinska K, Czaplinska M, Plucieniczak A, Wedrychowicz H. Immunoprotective properties of transgenic plants expressing E2 glycoprotein from CSFV and cysteine protease from *Fasciola hepatica*. *Vaccine*,2005;23(15):1844-1846.
13. Li M, Wang YF, Wang Y, Gao H, Li N, Sun Y et al. Immune responses induced by a BacMam virus expressing the E2 protein of classical swine fever virus in mice. *Immunology Letters*,2009;125:145-150
14. Lindh I, Wallin A, Kalbina I, SaEVENstrand H, Engström P, Andersson S, et al. Production of the p24 capsid protein from HIV-1 subtype C in *Arabidopsis thaliana* and *Daucus carota* using an endoplasmic reticulum- directing SEKDEL sequence in protein expression constructs. *Protein Expression and Purification*,2009;66(1):46-51.
15. Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Science*. 25: 402-408.
16. Lowings P, Ibata G, Needham J, Paton D. Classical swine fever virus diversity and evolution. *Journal of General Virology*,1996;77(6):1311-21.
17. Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. *Nature Reviews Genetics*,2003; 4(10):794-805.
18. Martinez-Lopez B, Perez AM, Sanchez-Vizcaino JM. A stochastic model to quantify the risk of introduction of classical swine fever virus through import of domestic and wild boars. *Epidemiology and Infection*,2009;137:1505-1515.
19. Mauser R, Stettler P, Ruggli N, Hapmann MA, Tratsch JD. Oronasal vaccination with CSF replicon particles with either partial or complete deletion of the E2 gene induces partial protection against lethal challenge with highly virulent CSFV. *Vaccine*,2005;23:3318-28.
20. Meyers G, Thiel H. Molecular characterization of pestiviruses. *Adv. Virus Research*,1996;47:53-118.
21. Meyers G, Rumenapf T, Thiel HJ. Molecular cloning and nucleotide sequence of the genome of classical swine fever virus. *Virology*,1989;171:555-567.
22. Moennig V. Introduction to Classical Swine Fever: virus, disease and control policy. *Veterinary Microbiology*,2000;73:93-102.
23. Moennig V, Floegel-Niesmann G, Greiser-Wilke I. Clinical signs and epidemiology of classical swine fever: A review of new knowledge. *Veterinary Journal*,2003;165:11-20.
24. Nandi S, Muthuchelvan D, Ahuja A, Bisht S, Chander V, Pandey AB et al. Prevalence of classical swine fever virus in India: a 6-year study (2004-2010). *Transboundary and Emerging Diseases*,2011;58:461-463.
25. OIE M. Classical swine fever (Hog cholera). In *OIE Manual of Diagnostic Tests and Vaccines*. Office International des Epizooties.Paris, France,2014;2:1-25.
26. Park Y, An DJ, Choe S, Lee Y, Park M, Park S et al. Development of recombinant protein-based vaccine against classical swine fever virus in pigs using transgenic *Nicotiana benthamiana*. *Frontiers in Plant Science*,2019;10:624.
27. Park Y, Lee S, Kang H, Park M, Min K, Kim NH et al. A classical swine fever virus E2 fusion protein produced in plants elicits a neutralizing humoral immune response in mice and pigs. *Biotechnology Letters*,2020;42:1247-1261.
28. Qi Y, Liu LC, Zhang BQ, Shen Z, Wang J, Chen YH. Characterization of antibody responses against a neutralizing epitope on the glycoprotein E2 of classical swine fever virus. *Archives of Virology*,2008;153:1593-1598.
29. Qi Y, Zhang BQ, Shen Z, Chen YH. Candidate vaccine focused on a classical swine fever virus epitope induced antibodies with neutralizing activity. *Viral Immunology*,2009;22:205-213.
30. Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2^{- Δ} (-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, Bioinformatics and Biomathematics*,2013;3(3):71-85.
31. Sarma DK, Mishra N, Vilcek S, Rajukumar K, Behera SP, Nema RK et al. Phylogenetic analysis of recent classical swine fever virus (CSFV) isolates from Assam, India. *Comparative Immunology, Microbiology and Infectious Diseases*,2011;34:11-15.
32. Shao HB, He DM, Qian KX, Shen GF, Su ZL. The expression of classical swine fever virus structural protein E2 gene in tobacco chloroplasts for applying chloroplasts as bioreactors. *Science Direct. Comptes Rendus Biologies*,2008;331:179-184.
33. Tonegawa K, Nobusawa E, Nakajima K, Kato T, Kutsuna T, Kuroda K. Analysis of epitope recognition of antibodies induced by DNA immunization against hemagglutinin protein of *Influenza A Virus*. *Vaccine*,2003;21:3118-25.
34. Van Oirschot JT. Hog Cholera. In: *Leman BSAD, Glock RD, Mengeling WL, Penny RHC, Scholl E, editors. Diseases of swine, 6th ed. Ames, Iowa: Iowa State University Press, 1986.*
35. Van Rijn PA, Bessers A, Wenswoort G, Moormann RJM. Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigen unit protects pigs from lethal CSFV challenge. *Journal of General Virology*,1996;77:2737-2745.
36. Zhang H, Wang YH, Cao HW, Cui YD. Phylogenetic analysis of E2 genes of classical swine fever virus in China. *Israel Journal of Veterinary Medicine*,2010;65:151-155.
37. Khan TN, Ansari TH, Zulkifl M. Concept of transformation and incorporation of nutriment in the body: A review. *International Journal of Food Science and Nutrition*. 2019;4(3):93-6.