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The transient expression of coat protein of Foot and Mouth Disease Virus (FMDV) in spinach (*Spinacia oleracea*) using Agroinfiltration

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Abstract
Foot and Mouth Disease (FMD) is a very dangerous livestock disease which causes a serious loss in the production of milk and meat. Therefore, producing an effective recombinant subunit vaccine virus this disease is of great importance. Transient gene expression is a valuable tool to reach rapid and acceptable recombinant vaccine. An Agrobacterium-mediated transient gene expression assay was carried out in spinach (*Spinacia oleracea*) leaves for expression of a chimeric gene encoding a part of capsid protein of Foot and Mouth Disease virus called VP1. The plant leaves were transformed via agroinfiltration procedure. The presence of foreign gene and its expression in transformed plants were confirmed through polymerase chain reaction (PCR), real time PCR, protein Dot blot and ELISA. The results obtained in this examination showed quite a high level of gene expression in spinach leaves, showing that transient gene expression can be applied as an effective and time-saving procedure for the production of recombinant proteins. The procedures for transformation, detection of recombinant protein and its application for molecular experiments are described in the study.

Keywords: Agroinfiltration, FMDV, Recombinant Vaccine, Spinach, VP1.

Introduction

Green plants are appropriate systems for the expression of foreign pharmaceutical proteins including recombinant vaccines; however, the long time required for producing transgenic plants together with the high cost and low protein yield are the major obstacles to the comercialization of plant-based molecular farming (Doran 2006). An ideal solution for this shortcoming is application of transient gene expression using *Agrobacterium tumefaciens*. In this method, the suspension of *A.tumefaciens* containing the gene of interest is transferred to plant leaves either with a needle-free syringe or a vacuum infiltr-ation and the expression of foreign genes on TDNA usually reaches to its maxim-um at 2−3 days post-infiltration (Sohn *et al*. 2011). Transient gene expression has been reported as a simple, cost-effective, fast

and reliable method for a wide range of experiments including gene function (Wroblewski *et al*. 2005), protein production (Vaquero *et al*. 1999), host–pathogen interaction (Tang *et al*. 1996), protein–protein interaction (Ihara *et al*. 2007) and protein localization (Bhat *et al*. 2006) .

So far many plant species have been used for the production of recombinant vaccine (Walmsley *et al*. 2000); the most notably examples are tobacco, potato, tomato, banana, corn, lupine and lettuce (Carter and Langridge 2002). Choosing the plant species for expression of recombinant vaccine is an important task which is mainly determined by considering how the vaccine is going to be used. Edible plant species such as vegetables are appropriate candidates if the vaccine is planned for raw consumption (Sala *et al*. 2003).

Foot and Mouth Disease (FMD) is a highly contiguous animal disease with harmful effects on milk- and meat-producing animals (Wang *et al*. 2002). There have been many efforts to produce recombinant vaccines against this disease in plant systems (Habibi and Zibaee 2013). However, to the best of our knowledge, no investigation has been reported on the production of FMD recombinant vaccine via transient gene expression in plant host has been reported. The capsid of Foot and Mouth Disease virus (FMDV) is composed of four structural polypeptides designated VP1, VP2, VP3 and VP4 (Bachrach *et al*. 1975). The prominent G–H loop of the VP1 capsid protein of FMDV, spanning residues 134–158, has been identified as the major immunogenic site for neutralizing antibodies (Rodriguez and Grubman 2009). Moreover, G-H loop flanking regions have been shown to boost its immunogenicity by inducing B cells and T-helper cells (Wang *et al*. 2002).

This paper reports on the production of a novel recombinant vaccine against FMD in spinach leaves through *Agrobacterium*-mediated transient gene expression. The synthetic gene designed for this study included a DNA fragment encoding 129 to 169 amino acids of VP1 capsid protein. This involved both G-H loop and its flanking regions, so was expected to be an effective tool for inducing immune response in animal host. The gene construct was further elaborated by the inclusion of eukaryotic ribosome binding site (Kozak sequence) and an endoplasmic reticulum signal peptide (SEKDEL) as was described in materials and methods section. Spinach was adopted as a host plant in this study since it is an edible vegetable and a suitable candidate for the production of recombinant vaccines especially those produced for veterinary use.

Materials and methods

Construction of synthetic VP1 gene

A 120 bp long fragment of VP1 coding 129-169 amino acids of VP1 capsid protein was designed as the main part of expression construct. 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 just before stop codon. Start codon (AUG) and stop

codon (UAA) were also added into the 5' and 3' ends of the construct, respectively. Recognition sites of *Bam*HI and *Sac*I restriction enzymes 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).

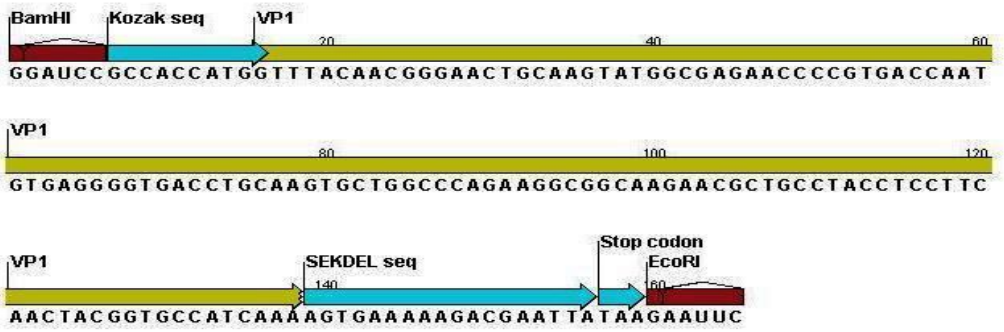

Figure 1. Schematic presentation of the synthetic VP1 gene.

Construction of a Binary Plant Expression Vector

The synthetic VP1 gene fragment was digested from pGem T-Easy vector by *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 ligation reaction mixture was used to transform *E. coli* strain DH5-α and kanamycin-resistant colonies were isolated after overnight incubation at 37°C. After amplification, the plasmid was extracted from bacterial cells using alkaline lysis method. The plasmid was introduced into *Agrobacterium tumef-aciens* strain GV3101 by heat shock method. In summary, a suspension of bacterium with OD=0.6 (600nm) was placed on ice for 15min. 1.5ml of the suspension was centrifuged at 4000g for 10min. Supernatant was removed and 1ml of cold $CaCl_2$ (20 mM) was added to bacterial pellet. The pellet was solved by vortex. One µgr of recombinant plasmid was added and mixed. The reaction tube was frozen in liquid nitrogen for 2min and then placed at 37ºC for 5min. One milliliter of LB medium was added and the solution was placed in shaker incubator at 28ºC. The suspension was again centrifuged; the supernatant was removed so that only 100µl of the suspension along with the bacterial pellet remained in the tube. The pellet was mixed with the culture medium and spread on solid culture containing LB agar medium supplemented with 50mg/l Kanamycin, 50mg/l Rifampicin and 20mg/l gentamicin. The recombinant colonies appeared after 48 hours. The putative transformed cells were further evaluated by PCR assay (Yasmin *et al* 2013).

Plant transformation

Single colony of *Agrobacterium* containing pBI121-VP1 plasmid was cultured for 48h on LB medium (NaCl 10 g/L, yeast extract 5 g/L, tryptone 5 g/L) supplemented with gentamicin 10mg/l, rifampicin 50mg/l and kanamycin 50mg/L. after reaching density of OD_{600}=1.5, the cultures were centrifuged, the supernatant was discarded and the pellet was resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 µM acetosyringone) and density was adjusted to OD_{600}=0.5. The suspension was incubated for 2h at room temperature. Agroinfiltration was then carried out to transform spinach leaves (Wroblewski 2005). The bacteri-al suspension was transferred to spinach leaves with a needle-free syringe as described by Sparkes *et al* [12]. Spinach plants were placed in growth chamber for three days under 25°C, 16h light/8h darkness photoperiod and 75% humidity and then they were analyzed.

Detection of VP1 gene in transgenic plants

Detection of VP1 gene in transgenic lines and other molecular analyses were conducted three days after agroin-filtration. PCR analysis was performed to evaluate presence of the expression cassette in the leaf tissue of transformed spinach plants. Genomic DNA was extracted from leaves of transgenic plants using modified Dellaporta method and used as template for PCR analysis using specific primers. The sequence of forward and reverse primers were 5' ATGGAAATTGTAA-GTATGGAGA 3' and 5' GAAGAAA-GCGAAAGGAGC 3' respectively. The forward primer matches a sequence within VP1 and reverse primers matches NOS termi-nator. Genomic DNA of wild type plants was used as negative control. PCR was carried out by 30 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 45s, followed with a final extension step at 72°C for 10min.

Real Time PCR assay

Real Time PCR assay was performed to analyze gene expression at transcription level. Total RNA was extracted from leaf tissue and complementary DNA (cDNA) was synthesized via reverse transcription using oligo (dT) 20 primer (Wroblewski *et al* 2005). The cDNA mixtur-es were used as templates for real-time PCR. Expression of the synthetic gene was quantitatively analyzed using a Real-Time PCR system (BioRad). Real-Time PCR was carried out in a 20 µL reaction volume containing 0.5µM of each primer and 10µl of SYBR Green Real time PCR master mix (Genet Bio, South Korea). Quantitative Real-Time PCR experime-nts were performed in duplicate for each sample. Forward and reverse primers for Real Time PCR were 5' ATGGAAA-TTGTAAGTATGGAGA 3' and 5' ATT-AAAAGAAGTTGGAAGAGTT 3', res-pectively.

Protein dot blot assay

Expression of VP1 gene in spinach leaves was evaluated using protein dot blot assay. Briefly, total protein was extracted using Tris-HCL method. Small samples of the protein (3 µl) were dotted

on nitrocellulose membrane and allowed to dry. BSA (Bovine Serum Albumin) was used to prevent non-specific antibody reactions. The membrane was then incubated for 60min at 37 °C with primary antibody (1:2000 dilution), washed three times with PBS (Phosphate Buffer Saline) and PBST and finally incubated with secondary conjugated antibody (1:1500). Color was developed by adding OPD (Ortho-Phenylenediamine). Protein sample of non-transformed plant was used as negative control and a small volume of FMDV vaccine serotype O (about 3 µl) was used as positive control.

ELISA assay

Expression of the foreign gene was further evaluated using enzyme-linked immunosorbent assay (ELISA). ELISA plate was coated with total soluble proteins from the wild type and transformed plants and known FMDV VP1 antigen at 37 °C for one hour; followed by incubation with 1% bovine serum albumin (BSA) in PBS for 2h at 37 °C to prevent non-specific binding. The well was washed with PBST/PBS, incubated with antiserum reactive against FMDV (1:1000 dilutions) and then alkaline phosphatase conjugated with anti rabbit IgG (1:1500). Wells were developed with TMB (Tetramethyl benzidine) substrate; the color reaction was stopped by 2N H2SO4 and read at 405 nm of wavelength.

Results

The presence of expression cassette in *A. tumefaciens* and transformed plants was evaluated using PCR analysis. PCR products were separated on 1% agarose gel by electrophoresis. The 587 bp band of foreign gene was observed in transgenic plant and *A. tumefaciens* colony. No band was amplified from non-transformed plant (Figure 2).

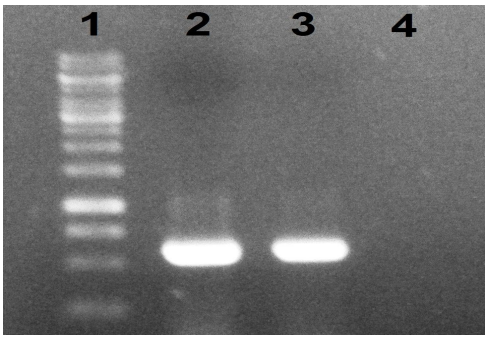

Figure 2. PCR analysis for detection of VP1 gene in transformed leaves of spinach. 1) 1 kb ladder; 2) plasmid pBI121VP1 (positive control); 3) transformed spinach plant; 4) wild type plant (negative control).

Expression of foreign gene was measured at transcription level using Real Time PCR. The results of Real Time PCR confirmed VP1 gene expression in all transformed samples but no signal was detected for control line (Figure 3). As can be inferred from Figure 3, transcription rate was quite high in transformed leaves. Expression of VP1 was further evaluated in translational level by dot blot and ELISA assays.

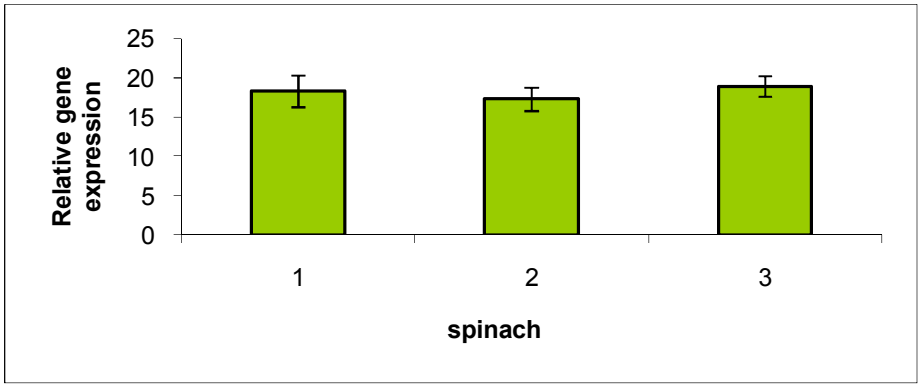

Figure 3. Quantitative measurement of VP1 gene transcription in transformed leaves of spinach via Real Time PCR. Data presented in this graph are obtained from three samples of transformed plants.

The production of recombinant VP1 protein was evaluated by dot blot assay. Positive signal showing specific antigen/antibody reaction was observed for protein samples obtained from transformed spinach plants and for those samples from positive control as well. As expected, no signal was detected for protein sample of non-transformed plant (Figure 4).

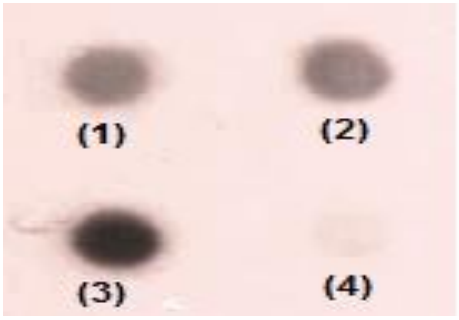

Figure 4. Dot blot assay for detection of recombinant protein in transiently transformed leaves of spinach. (1) and (2): protein samples of transformed plants, (3): commercial FMD vaccine as positive control, (4): protein sample of non-transformed plant.

Expression of VP1 recombinant protein was quantitatively assessed using ELISA assay. ELISA results showed that the recombinant protein was produced in all the three samples obtained from transformed plants, whereas no detectable signal was observed for that of non-transformed plant (Figure 5).

Discussion

In the present study, spinach leaves were transiently transformed with a chimeric construct of VP1 gene via agroinfiltration method. The method has been reported as an efficient and rapid procedure for transient gene expression in plants (Sohn *et al*. 2011).

The results of this study demonstrated that agroinfiltration can be a fast and efficient tool for production of recombinant vaccines in intact plants. As confirmed by Real Time PCR assay, transient expression level of the transgene was fairly high which was in agreement with the results obtained by Leckie and Stewart (2011) who reported high level of gene expression in leaves of *Nicotiana benthamiana* through agroinfiltration. Indeed, some investigators have claimed that transgene expression level in transient expression assays can be up to 1000 fold higher

than that of stable transformation (Janssen and Gardner 1989). Although such a high expression level was not observed in the present study, the expression of VP1 was of great magnitude as shown by both Real Time PCR and ELISA assays. It is quite surprising that in spite of the wide range of experimental purposes of transient gene expression, there have been few studies on the application of this transformation approach for producing recombinant vaccines in plant systems. Most of the works in the field of transient antigen expression in plant hosts have been conducted by means of plant viruses as vehicle for gene delivery and expression, in which the epitope of interest is usually inserted within the coat protein gene (Koprowski and Yusibov 2001). This method has proved to be an efficient and rapid way for the production of recombinant protein in plants but is limited by the fact that construction of viral vector for expression of foreign protein is much laborious and time-consuming. Moreover, when the size of foreign gene exceeds a certain threshold, efficiency of the viral vector is reduced (Sala *et al*. 2003). In contrary, genes with large size can be efficiently expressed in plants via *Agrobacterium*-mediated genetic transformation. In the other word, agroinfiltration (and other types of *Agrobacterium*-mediated transient gene expression) combines advantages of both viral-based transient gene expression, that is the production of recombinant protein in a short time, and *Agrobacterium* mediated transformation, the ability to transfer large foreign genes. This makes agroinfiltration a promising alternative for the production of recombinant vaccines in plant-based systems.

The choice of plant species for production of recombinant vaccine is of great importance at least for two reasons. First, it is well documented that plant genetic background has a significant effect on transient gene expression level (Santos-Rosa *et al*. 2008). Yasmin *et al* (2013), for instance, reported that two out of three genotypes of rose showed higher susceptibility to agro-infection whereas one genotype never allowed for agro-infection. Spinach plants used in the present study showed high level of VP1 expression when transiently transformed with *A. tumefaciens*. This high level of gene expression was evident in both transcription (Figure 3) and translation levels (Figure 5). Although it is more reasonable to perform a stable genetic transformation program for permanent production of recombinant vaccine, transient gene expression can be regarded as a complementary process for achieving large amount of antibody for detection methods such as ELISA, western blotting, etc. The antigen can be quickly expressed in plant system through transient gene expression and the expressed recombinant protein can be parenteraly injected to animal models. This will trigger antibody production in immune system of the recipient animal. Based on the results, spinach is an appropriate platform for production of recombinant antigen of FMDV. The transformed spinach lines can be parenteraly injected or orally

administered to animals, because the crop is a palatable vegetable that can be easily incorporated in animal diet.

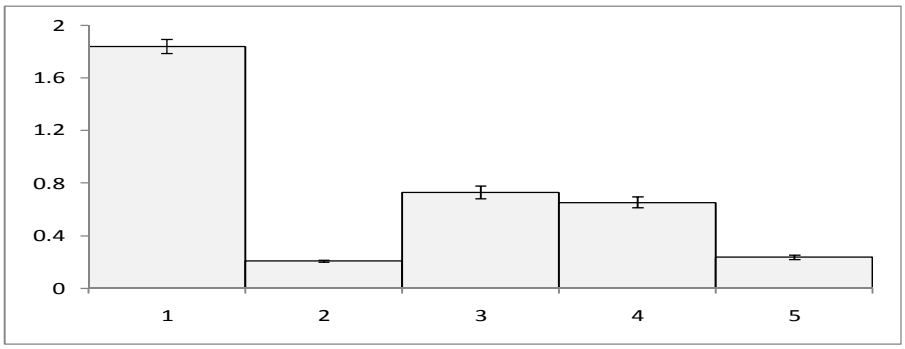

Figure 5. Quantification of recombinant VP1 expression in two transgenic spinach plants by ELISA. (1): positive control, (2) PBS as negative control (blank), (3) and (4): transformed plants, (5): wild type plant.

Conclusion

In this investigation, the efficacy of agroinfiltration for transient expression of VP1 protein in spinach plants was demonstrated. The expression level of the foreign gene was quite high in transformed plants. We believe that this method can be used as an effective and quick way for the production of recombinant antigens. The expressed antigen can be used as a recombinant vaccine or, more realistically, as a valuable source for production of specific antibody in veterinary diagnosis or molecular detection processes.

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بیان موقت پوشش پروتئینی ویروس مولد تب برفکی (FMDV) در اسفناج (Spinacia oleracea) با استفاده از اگروواینفیلتریشن

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چکیده

بیماری تب برفکی یک بیماری خطرناک دامی است که باعث ایجاد خسارات زیادی به تولید شیر و گوشت می‌شود. بنابراین، تولید یک واکسن زیرواحدی علیه این بیماری از اهمیت بالایی برخوردار است. بیان موقت ژن ابزار ارزشمندی جهت تولید سریع و قابل قبول واکسن‌های نوترکیب است. در این مقاله از بیان موقت ژن با استفاده از اگروباکتریوم در گیاه اسفناج جهت بیان یک ژن شیمری استفاده شده است که بخشی از پروتئین پوششی ویروس مولد تب برفکی موسوم به VP1 را کد می‌کند. حضور و بیان ژن خارجی در برگ‌های استفناج با استفاده از آزمون‌های PCR، Real Time PCR، دات بلات و الایزا تایید شد. نتایج بدست آمده نشان داد که ژن مورد نظر در سطح بالایی در برگ‌های اسفناج بیان شده است و این نشان می‌دهد که بیان موقت روشی موثر و سریع برای تولید پروتئین‌های نوترکیب است. مراحل تراریخته‌سازی، تشخیص پروتئین نوترکیب و کاربرد آن در آزمایشات مولکولی توضیح داده می‌شود.

کلمات کلیدی: اگروواینفیلتریشن، اسفناج، تب برفکی، واکسن نوترکیب، VP1.