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Research Article

Transient Expression of Coat Protein of Foot and Mouth Disease Virus (FMDV) in Alfalfa (*Medicago sativa*) by Agroinfiltration

Maziar Habibi-Pirkoohi[1*], Saeid Malekzadeh-Shafaroudi[1], Hasan Marashi[1], Saeid Zibaee[2], Afsaneh Mohkami[3], Saba Nejatizadeh[1]

[1] *Department of Biotechnology and plant breeding, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Razi Vaccine and Serum Research Institute, Ministry of Agriculture, Mashhad, Iran*
[3] *Research and Technology Institute of Plant production, Shahid Bahonar University, Kerman, Iran*

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Abstract

An *Agrobacterium*-mediated transient gene expression assay was carried out in alfalfa (*Medicago sativa*) 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 the foreign gene and its expression in transformed plants were evaluated by polymerase chain reaction (PCR), real time PCR, protein Dot blot and ELISA. Moreover, gene expression in the transformed leaves was quantified by ELISA method. The results obtained in this investigation indicated high level of gene expression in alfalfa 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, Alfalfa, VP1

Introduction

In recent years, green plants have been widely applied 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 commercialization of plant-based molecular farming (Wroblewski et al., 2005). An appropriate alternative for conventional genetic engineering procedures 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 infiltration and the expression of foreign genes on TDNA usually reaches to its maximum at 2−3 days post-infiltration (Habibi et al., 2014). Indeed, transient gene expression systems have been extensively used by many authors as a simple, cost-effective, fast and reliable method for a wide range of experiments including gene function (Sohn et al., 2011), protein production (Vaquero et al., 1999), host–pathogen interaction (Tang et al., 1996) protein–protein interaction (Bhat et al., 2006) and protein localization (Doran, 1999). So far many plant species have been used for the production of recombinant vaccine; the most notable examples being tobacco, potato, tomato, banana, corn, lupine and lettuce (Carter et al., 2002). Choosing the plant species for expression of recombinant vaccine is an important issue 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). In the case of veterinary vaccines, forage crops would be the choice of interest (Walmsley et al., 2000).

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 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

Corresponding authors E-mail:
*Maziar.habibi@alumni.um.ac.ir

(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 et al., 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 the production of a novel recombinant vaccine against FMD in alfalfa 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 described in materials and methods section. Alfalfa was adopted as a host plant in this study since it is a forage crop constituting an ordinary portion of livestock diet. This makes alfalfa a suitable candidate for the production of recombinant vaccines against FMD.

Materials and Methods

Construction of synthetic VP1 gene

A 120 bp long fragment of VP1 encoding 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).

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 bacterial growth, the plasmid was extracted from bacterial cells using alkaline lysis method. The plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by so called thaw-melting method. In summary, a suspension of bacterium with OD= 0.6 (600 nm) was placed on ice for 15 min. 1.5 ml of the suspension was centrifuged at 4000 *g* for 10 min. Supernatant was removed and 1 ml of cold $CaCl_2$ (20 mM) was added to bacterial pellet. The pellet was solved by vortex. 1 µgr of recombinant plasmid was added and mixed. The reaction tube was frozen in liquid nitrogen for 2 min and then placed at 37ºC for 5 min. 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 100 µl 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 50 mg/l Kanamycin, 50 mg/l rifampicin and 20 mg/l gentamicin. The recombinant colonies appeared after 48 hours. The putative transformed cells were further evaluated by PCR assay.

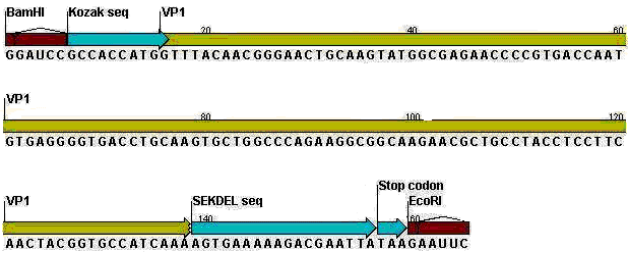

Figure1. Schematic presentation of the synthetic VP1 gene

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 10 mg/l, rifampicin 50 mg/l and kanamycin 50 mg/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 2 h at room temperature. *Agroinfiltration* was then carried out to transform alfalfa leaves. The bacterial suspension was transferred to alfalfa leaves with a needle-free syringe as described by Sparkes et al (2006). Alfalfa plants were placed in growth chamber for three days

under 25°C, 16 h light/8 h 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 agroinfiltration. PCR analysis was performed to evaluate presence of the expression cassette in the leaf tissue of transformed alfalfa 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' ATGGAAATTGTAAGTATGGAGA 3' and 5' GAAGAAAGCGAAAGGAGC 3' respectively. The forward primer matches a sequence within VP1 and reverse primers matches NOS terminator. 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 10 min.

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. The cDNA mixtures were used as templates for real-time PCR. Specific forward and reverse primers for Real Time PCR were 5' ATGGAAATTGTAAGTATGGAGA 3' and 5' ATTAAAAGAAGTTGGAAGAGTT 3', respectively. Data were normalized to the expression of Aspartate Aminotransferase (ATT), a housekeeping gene, which has stable expression under different experimental conditions in similar studies. Specific forward and reverse primers for ATT gene were 5'CAATTTCGCATCTCATTAAGATCG3' and 5'ACCACATCCCAAATAAATAAGATTCTAAC 3', respectively.

The efficiency of primer binding was determined by linear regression by plotting the cycle threshold value versus the log of the cDNA dilution.

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 10 µl of SYBR Green Real time PCR master mix (Genet Bio, South Korea). Quantitative Real-Time PCR experiments were performed in duplicate for each sample. Student's t-test was used to evaluate the statistical significance of the data; $p < 0.05$ was considered statistically significant.

Protein dot blot assay

Expression of VP1 gene in alfalfa 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 60 min 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 3 µl of pure synthetic peptide corresponding to VP1-129-169 amino acids (Bioneer, South Korea) 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 2 h 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 2 N H_2SO_4 and read at 405 nm of wavelength.

Quantification of the recombinant protein in transformed leaves

Total soluble protein concentration was determined using Bradford assay. For quantification of recombinant protein content in infiltrated leaves, standard curve for VP1 was drawn using known amounts of a synthetic VP1-129-169 peptide (Bioneer, Sout Korea). For this, serial dilutions of the synthetic VP1-129-169 peptide were used in ELISA assay and the absorbance of each well was measured.

Then, absorbance (Y axis) was plotted against protein concentrations (X axis). The curve was used to determine concentration of recombinant protein in transformed leaves (figure 6). Concentration of the recombinant VP1 protein was calculated by dividing VP1 concentration by total soluble protein, and expressed as the percentage of total soluble protein (%TSP); as follows:

%TSP = [*VP1* concentration/ total protein concentration] × 100

Results

Transgene detection in infiltrated leaves

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 587bp band of foreign gene was observed in transgenic plant and *A. tumefaciens* colony. No band was amplified from non-transformed plant (Figure 2).

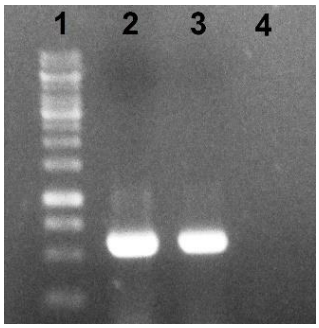

Figure2. PCR analysis for detection of VP1 gene in transformed leaves of alfalfa. 1) 1 kb ladder; 2) plasmid pBI121VP1 (positive control); 3) transformed alfalfa plant; 4) wild type plant (negative control)

Evaluation of transgene expression

Expression of the 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. The difference between infiltrated leaves was not significant ($p<0.05$).

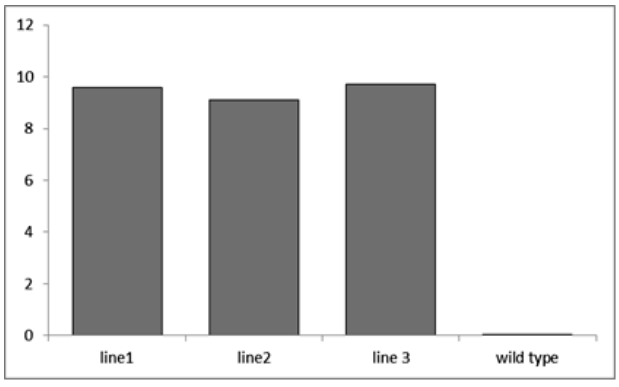

Figure 3. Quantitative measurement of VP1 gene transcription in transformed leaves of alfalfa via Real Time PCR. Data presented in this graph are obtained from three samples of transformed plants.

Expression of VP1 was further evaluated in translational level by dot blot and ELISA assays.

The production of recombinant VP1 protein was measured by dot blot assay. Positive signal showing specific antigen/antibody reaction was observed for protein samples obtained from transformed alfalfa 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).

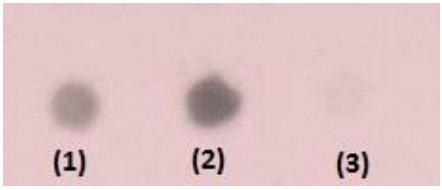

Figure4. Dot blot assay for detection of recombinant protein in transiently transformed leaves of alfalfa. (1): protein sample of transformed leaves, (2) pure VP1-129-169 peptide as positive control, (3): protein sample of wild type plant (negative control)

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

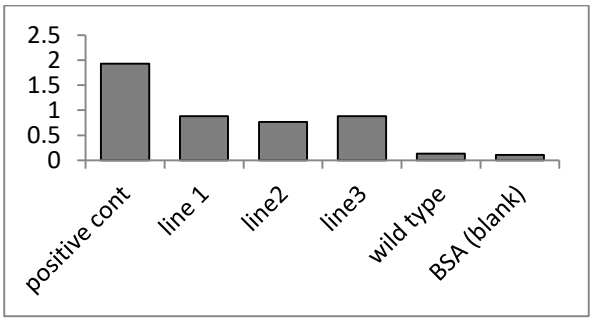

Figure5. Quantification of recombinant VP1 expression in three transgenic alfalfa plants by ELISA.

Finally, concentration of recombinant VP1 was quantified in the transformed leaves. Figure 6 shows the standard curve of VP1 protein. As determined by standard curve of Bradford, total protein concentration of the transformed leaves was 4.4 µg/ µl. Using standard curve of pure synthetic VP1 protein, concentration of recombinant protein was calculated as 0.042 µg/ µl; therefore final concentration of the recombinant VP1 protein was:

0.042/ 4.4 × 100= 0.95%TSP

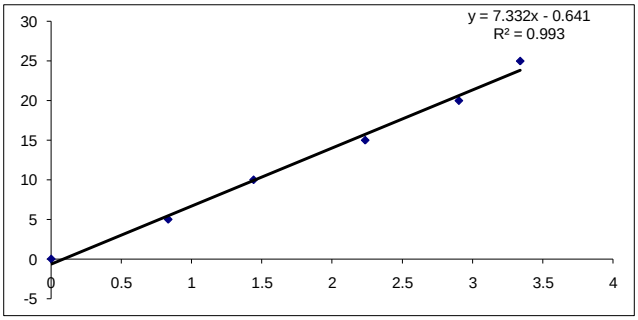

Figure 6. Standard curve of VP1 protein

Discussion

In the present study, alfalfa 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)

PCR assay confirmed presence of the synthetic construct in infiltrated leaves. In transient expression assays, the gene of interest is not integrated in nuclear genome of plant cell. Thus, copy number of the transgene in plant tissue is high and PCR product band in electrophoresis is almost as sharp as that of pure plasmid (Figure 2).

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 (Sparks et al., 2006). Although such a high expression level was not observed in the present study, the expression of VP1 was of great magnitude (0.95%TSP) as quantified by dividing concentration of the recombinant protein by total soluble protein concentration. 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 et al., 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 words, 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.

Plant choice is a critical issue in production of recombinant vaccines and other pharmaceutical proteins (Carter et al., 2002). The leaf biomass produced by alfalfa is somewhat lower than that of the model plant tobacco, but it has several advantageous agronomic characteristics compared to tobacco including the fact that it is a perennial plant (vegetative growth can be maintained for many years), it can be clonally propagated by stem cutting, and its leaves or free of alkaloids. Moreover, since alfalfa is a fodder crop, a major application of this species in molecular farming is the delivery of vaccines to domestic animals (Wigdorovitz et al., 1999). Alfalfa 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. A practical example of transient gene expression of antigens in alfalfa leaves is the case of Medicago Inc., Québec, Canada; the Scientists at this biotechnology company regularly process up to 7500 infiltrated alfalfa leaves per week for diagnostic objectives (Fischer and Schillberg, 2004).

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, alfalfa is an appropriate platform for production of recombinant antigen of FMDV. The transformed alfalfa lines can be parenteraly injected or orally administered to animals, because the crop is a palatable plant that can be easily incorporated in animal diet.

Conclusion

In this investigation, the efficacy of agroinfiltration for transient expression of VP1 protein in alfalfa plants was demonstrated. The expression level of the foreign gene was quite high in transformed plants (0.95% of TSP). 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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