Iran J Biotech. 2014 September;12(3): e1015 DOI:10.15171/ijb.1015

Published online 2014 November 25 Research Article

Transient Expression of Foot and Mouth Disease Virus (FMDV) Coat Protein in Tobacco (*Nicotiana tabacom*) via Agroinfiltration

Maziar Habibi-Pirkoohi [1,*], Saeid Malekzadeh-Shafaroudi [1], Hasan Marashi [1], Nasrin Moshtaghi [1], Mohammadreza Nassiri [2], Saeid Zibaee [3]

[1]Department of Biotechnology and Plant Breeding, Ferdowsi University of Mashhad, Mashhad, I.R. IRAN
[2] Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, I.R. IRAN
[3] Razi Vaccine and Serum Research Institute, Mashhad, I.R. IRAN

**Corresponding author:* Maziar Habibi-Pirkoohi, Department of Biotechnology and Plant Breeding, Ferdowsi University of Mashhad, Mashhad, I.R. IRAN. Tel: +98-1912167172, E-mail: Maziar.habibi.p@gmail.com

Received: March 16, 2014; **Revised:** April 24, 2014

Background: Transient and stable transformation of host plants are the common techniques to produce transgenic plants. However, the main drawback of stable transformation is the fact that it takes quite a long time to produce a transgenic line. While, transient gene expression is a quick method to produce recombinant proteins in plants.
Objective: The main goal of the present study was to evaluate efficient agroinfiltration as an efficient and rapid method for production of recombinant antigen of FMDV.
Materials and Methods: Tobacco leaves were transformed via agroinfiltration using a needle-free syringe. Presence of the gene cassette was verified by polymerase chain reaction (PCR). Expression of the foreign gene was evaluated using Real Time PCR, protein dot blot and enzyme-linked immunosorbent assay (ELISA).
Results: PCR analysis confirmed successful transformation of plant leaves. Expression of foreign protein was confirmed at both transcription and translation levels. Results of Real Time PCR assay indicated that the foreign gene was transcribed in transformed leaves. ELISA results showed that the foreign gene was expressed in the transformed leaves in high level.
Conclusion: Here, the efficacy of agroinfiltration for transient expression of FMDV coat protein in tobacco was illustrated. Accordingly, transient agroinfilteration expedites the process of recombinant antigens expression in plant tissues.

keywords: Recombinant vaccine; Agroinfiltration; Tobacco; Transient gene expression

1. Background

Foot-and-Mouth Disease Virus (FMDV) is a causative agent of foot and mouth disease, a highly contagious disease adversely affecting milk and meat-producing animals around the world, particularly in developing countries (1). The G-H loop of VP1 capsid protein of FMDV, covering residues 134-158, has been reported by many researchers as the main immunogenic site for neutralizing antibodies (2). Moreover, it is well known that flanking regions of G-H loop enhances its potential as recombinant vaccine by virtue that they contain B cell and T helper cell epitopes (3). Current vaccines against FMD are primarily based on the application of attenuated virus. This method, although proved to be effective for prevention of the disease, is costly and risky by the virtue that manipulation of massive amounts of virulent virus could result in virus dissemination. Furthermore, cold chain requirements and need for specialized medical personnel for inoculation put more limitations on this method (2). Therefore, production of plant-based recombinant vaccines against FMDV has been attempted by many investigators during recent years (1, 2, 4).

Application of green plants as bioreactors for production of recombinant proteins has emerged as a promising biotechnological tool over the past two decades. Many investigators have conducted extensive experi-

ments for the production of recombinant plant-based vaccine against a wide range of bacterial and viral disease (5). However, the technique suffers from major drawbacks such as low expression level and long time required for the production of the recombinant protein in plant tissues (6). Many strategies have been proposed for the enhancement of recombinant protein expression including; chloroplast transformation (7), use of strong promoters (8), untranslated leader sequences (9), signal peptide (10), codon optimization (6). The Long time required for the generation of transformed plants expressing foreign antigens is another limitation for the production of recombinant proteins (11). Transient gene expression methods are appropriate alternatives to stable transformation because they allow for a rapid and inexpensive expression of foreign gene(s) in plant tissues (12). This method can be carried out in many ways including protoplast transformation (13), vacuum infiltration (13), agroinfiltration (14) and particle bombardment (15).

Among the techniques, agroinfiltration takes advantages of a simple, cost effective and rapid procedure. In agroinfiltration, the suspension of *Agrobacterium tumefaciens* harboring the gene(s) of interest is infiltrated into plant leaves using a needle-free syringe. This technique has been carried out in a variety of plants (16, 17) with different experimental purposes (18). However, there have been few reports on production of recombinant vaccines in plant systems via transient gene expression. The main reason is that transient gene expression assays are not as appropriate as stable transformation for production of recombinant vaccines (4, 5, 16). However, recombinant antigens produced via transient gene expression can be used for the production of specific antibodies, which can then be used in diagnosis and molecular detection processes (19). Moreover, transient expression assay can be carried out as a quick method to investigate efficiency of a candidate antigen for inducing immunogenic response in animal models (19, 20). Time course required for antigen production via agroinfiltration (and other types of transient gene expression) is significantly shorter than that of stable transformation (14).

In this study, we investigated the production of an immunogenic recombinant antigen of FMDV by agroinfiltration of tobacco leaves. The gene construct is composed of a DNA fragment encoding 129-169 amino acids of VP1 capsid protein which includes G-H loop and its flanking regions. Moreover, a ribosome binding site called Kozak and an endoplasmic reticulum (ER) signal peptide called SEKDEL were also included in gene construct.

2. Objectives

Agrobacterium-mediated transient gene expression assay was carried out for expression of Foot and Mouth Disease (FMD) coat protein in tobacco plants. The main goal of this investigation was to evaluate agroinfiltration as an effective and quick method for production of recombinant antigen of FMD. The expressed antigen can be used as a potential recombinant vaccine or, more realistically, as a valuable source for production of specific antibody for application in veterinary diagnosis or molecular detection processes.

3. Materials and Methods

3.1. Construction of Expression Cassette

DNA encoding 129-169 amino acids of VP1 capsid protein was designed as the main part of expression cassette. A ribosome binding site, Kozak sequence GCCACC, was introduced prior to the start codon. This sequence has been reported to enhance translation efficiency (21). An endoplasmic reticulum signal peptide called SEKDEL, which has been reported to increase recombinant protein accumulation in plant tissues (22), 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. The resulted

chimeric gene was optimized based on codon usage pattern of tobacco taken from http://www.kazusa. or.jp/codon/. The optimized chimeric gene was cloned into the pGem-T Easy vector (Bioneer, South Korea). The native sequence of the coding region was GTTTATAATGGAAATTGTAA GTATGGAGAAAATCCAGTTACTAATGTTAGAGGAGATCTTCAAGTTCTTGCTCAAAAGGCTGCTAGAACTCTTCCAACTTCTTTTAATTATGGAGCTATTAAG, which after optimization was changed into GTTTACAACGGGAACTGCAAGTATGGCGAG AACCCCGTGACCAATGTGAGGGGTGACCTGCAAGTGCTGGCCCAGAAGGCGGCAAGAACGCTGCCTACCTCCTTCAACTACGGTGCCATCAAA (the replaced nucleotides are underlined).

3.2. Construction of Binary Vector

The synthetic VP1 gene was removed from pGem-TEasy vector by digestion with *Bam*HI and *Sac*I and was inserted into the binary vector pBI121, yielding pBI121-VP1 vector (Figure 1). 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 tumefaciens* strain GV3101 by heat shock method. Transformed cells were screened by kanamycin-resistance and PCR.

3.3. Plant Transformation via Agroinfiltration

A single colony of *Agrobacterium* containing pBI121-VP1 plasmid was cultured for 48 h in 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 a cell density of OD_{600}=1.5, the culture was centrifuged, 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) , adjusted to OD_{600}=0.5. The suspension was then incubated for 2 h at room temperature. before being transferred to tobacco leaves with a needle-free syringe (Figure 2), as described by Sparkes *et al* (23). Tobacco plants were placed in growth chamber for three days under 25ºC before being analyzed.

3.4. Detection of VP1 Gene in Transformed Leaves

PCR analysis was performed to evaluate presence of foreign gene in leaf tissue of transformed tobacco plants. Genomic DNA was extracted from the leaf tissues of transgenic plants and used as template for PCR analysis, using specific primers. The sequence of forward and reverse primers were 5' ATGGAAATTGTAAGTATGGAGA 3' and

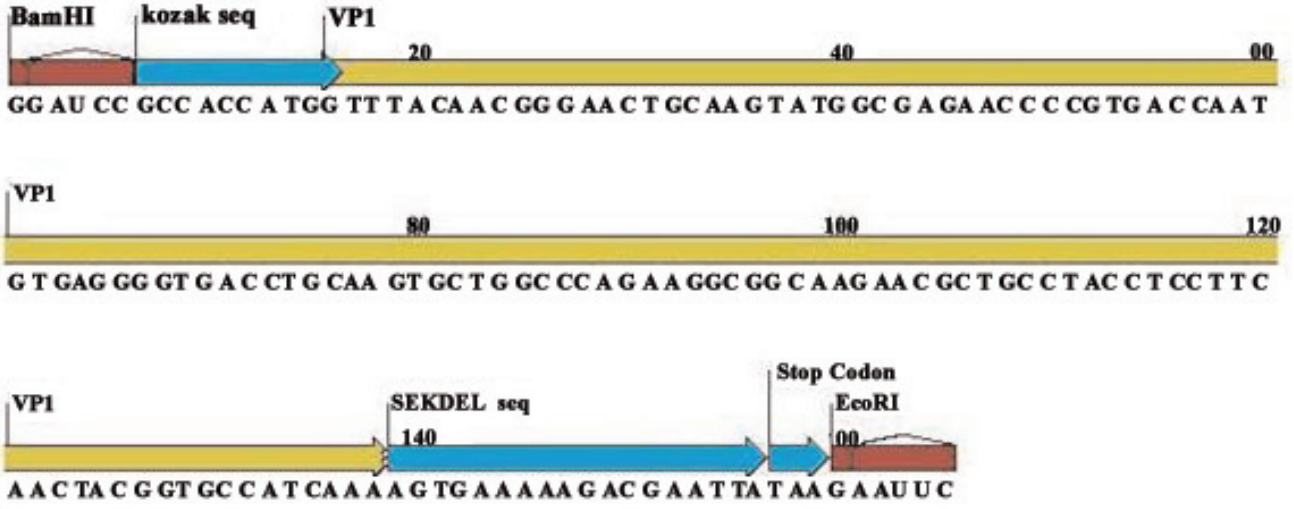

Figure 1. Schematic representation of the synthetic *VP*1 gene

5' GAAGAAAGCGAAAGGAGC 3' respectively. Genomic DNA of wild type plants was used as negative control. PCR was carried out as follow: based on the following teperature profile: 94ºC 1 min, 58ºC 1 min, 72ºC 2 min for 30 cycles.

3.5. Real Time PCR Assay

Real Time PCR assay was performed to analyze gene expression at transcription level. Total RNA was extracted from infiltrated leaf tissue and complementary DNA (cDNA) was synthesized via reverse transcription using oligo(dT) 20 primer. The resulting cDNA mixtures 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 0.5 µM of each primer and 10 µL of SYBR Green Real time PCR master mix. Quantitative Real Time PCR experiments were performed in duplicate for each sample. Forward and reverse primers for Real Time PCR were 5' ATGGAAATTGTAAGTATGGAGA 3' and 5' ATTAAAAGAAGTTGGAAGAGTT 3', respectively.

3.6. Protein dot blot assay

Production of recombinant VP1 protein in transgenic leaves was evaluated by standard protein dot blot assay. Briefly, 5 µL of protein samples from infiltrated leaves was dotted on the membrane and the membrane allowed to get dried. The membrane was incubated with BSA as blocking solution for 1 h. After incubation, the membrane was incubated with primary antibody for 1 h in 37ºC, washed three times with PBST/PBS and again incubated with secondary conjugated antibody for 1 h in 37ºC. The membrane was washed three times with PBST/PBS and incubated with OPD (Ortho-Phenylenediamine) substrate. A small volume of FMDV vaccine serotype O (about 5 µL) was used as positive control and the same volume of protein obtained from wild type plant was used as negative control.

Figure 2. Agroinfiltration of tobacco leaves using needle-free syringe

3.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Expression of the foreign gene was further evaluated by ELISA assay. ELISA plate was coated with total soluble proteins from the wild type and the transformed plants and known FMDV VP1 antigen at 37°C for one h; followed by incubation with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C to prevent non-specific binding. The wells were washed by PBST/PBS and incubated with antiserum reactivated against FMDV (1:1000 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.

4. Results

Tobacco leaves were transformed via agroinfiltration. Presence of the expression cassette was evaluated by PCR analysis. Figure 3 shows the result of PCR test. Excepted band was observed for the DNA sample of transformed leaves, but not for the wild type plant.

Expression of VP1 was evaluated at transcription level using Real Time PCR. Three samples of transformed plants were used for Real Time PCR. Results showed that the foreign gene was transcribed in infiltrated leaves (Figure 4).

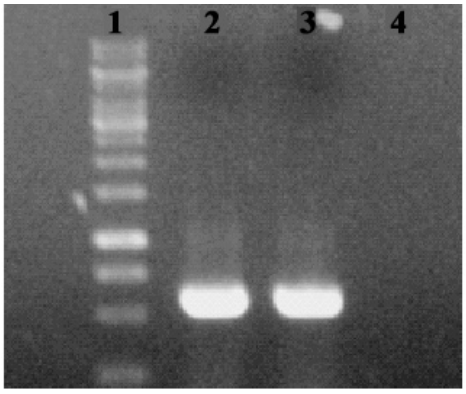

Figure 3. PCR analysis for detection of VP1 gene in transformed leaves of tobacco. 1: 1 Kb Size marker; 2: plasmid pBI121VP1 (positive control); 3: transformed plant; 4: wild type plant (negative control).

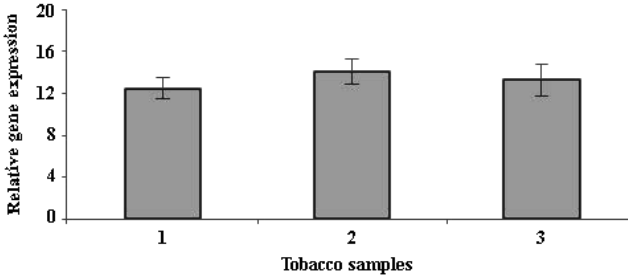

Figure 4. Quantitative measurement of VP1 gene transcription in transformed leaves of tobacco via Real Time PCR. Data presented in this graph are obtained from three samples of transformed plants

Translation of VP1 gene was evaluated by protein dot blot assay. Dot blot results confirmed expression of the foreign gene at translation level, whereas no signal was observed for wild type plants (Figure 5).

Expression of the recombinant protein was further quantitatively measure by ELISA (Figure 6) The production of the recombinant protein was quite high in transgenic leaves. In contrast, no strong signal was observed for non-transformed plants.

Figure 5. Protein dot blot for detection of recombinant protein in transformed leaves of tobacco. (1): positive control, (2): protein sample of non-transformed plant, (3): protein sample of transformed plants.

5. Discussion

Low level of gene expression in stable transformation experiments is a major obstacle for production of recombinant proteins in green plants. The time required for the generation of transgenic lines is another issue in stable transformation (24). An approach to address these limitations is application of transient gene expression assays. Although transient expression is not the preferred method for commercial production of recombinant vaccine in plants, the antigens produced in this procedure can be used for the production of specific antibodies required in molecular diagnosis (19). Moreover, this method makes it possible to evaluate efficacy of a potential recombinant vaccine in a short time (20).

In the present study, tobacco leaves were transiently transformed with a chimeric construct of VP1 gene via agroinfiltration. The method has been reported as an efficient and rapid procedure for transient gene expression in plants (25).

Our data demonstrated that agroinfiltration can be a fast and efficient way to produce FMDV antigens in intact plants. As confirmed by Real Time PCR assay, transient expression level of the transgene was fairly high, similar to earlier reports (14,16, 26). As can be seen from dot blot assay, the protein sample obtained from transformed leaves generated a strong signal which is comparable to that of positive control (a common FMD vaccine); whereas protein of wild type plant was not detectable. Enhanced expression of VP1 in tobacco leaves is more obvious in ELISA assay which quantitatively measures expression of the recombinant vaccine. Slight absorbance observed in wild type plant is probably due to unwanted cross reactions between protein

samples and specific antibodies.

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 (27). This method has proved to be an efficient and rapid way for production of recombinant protein in plants but suffers from the limitation 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 (28). In contrary, genes with large size can be efficiently expressed in plants via *Agrobacterium*-mediated genetic transformation (16).

Our results emphasize the efficiency of *Agrobacterium*-mediated transient gene expression assay as a fast method for production of foreign antigens in plant systems. A major advantage of the method is that the recombinant antigen can be produced within a short period. The produced recombinant antigens can be used for production of specific antibodies, which can be used in molecular detection and diagnosis (19, 29). Furthermore, the method is a fast approach to test efficiency of novel vaccine candidates in inducing immunogenic response in animal models (19, 20, 29, 30).

Here, CaMV 35S promoter, Kozak sequence, ER signal peptide were used and codons were optimized to enhance gene expression, similar to previous reports (8, 10, 28, 31- 36). Although a good level of transgene expression was achieved in our experiment, it should be mentioned that the results cannot be confidently attributed to the presence of these factors. Since, their influences on the foreign gene expression were not evaluated. Approving a connection between these factors and the expression level of the VP1 antigen requires further investigation.

Acknowledgement

The authors would like to thank staff of biotechnology and plant breeding department, faculty of Agriculture, Ferdowsi University of Mashhad.

Authors' Contribution

Maziar Habibi and the authors conducted the experiment.

Financial Disclosure

There is no financial interest.

Funding/ Support

The research was funded by department of

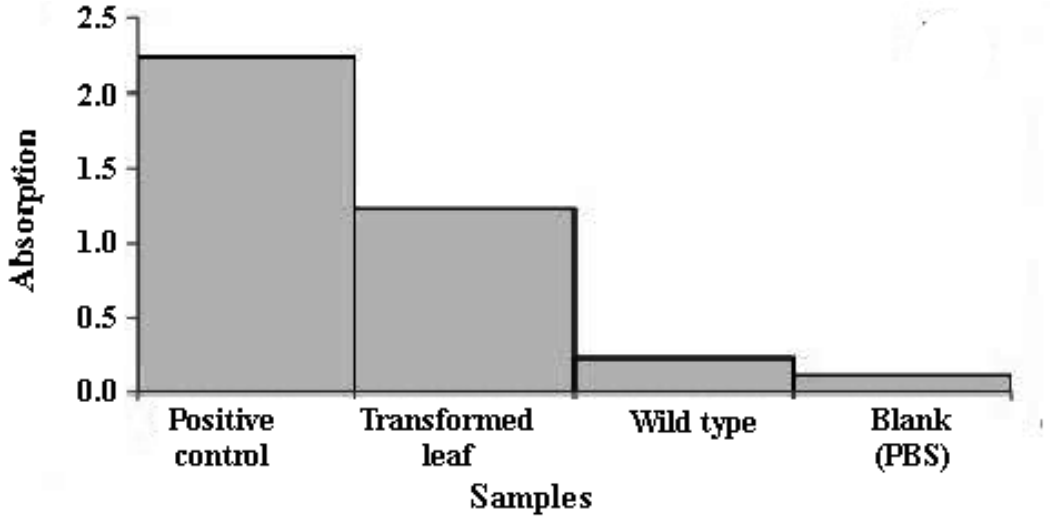

Figure 6. Quantification of recombinant VP1 expression in transgenic plants by ELISA

Biotechnology and Plant Breeding, faculty of Agriculture, Ferdowsi University of Mashhad.

Reference

1. Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gomez N, et al. Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. *J Virol*. 1998; **72**:1688-90.
2. Wigdorovitz A, Carrillo C, Trono K. 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.* 2001;**14**:49-57.
3. Chang YW, Tseng YC, Walfield AM, Ye J, Shen M, et al. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. *Vaccine* 2002;**20**: 2603-2610.
4. Dus Santos MJ, Wigdorovitz A, Trono K, Rios RD, Franzone PM, Gil F, Javier Moreno J, Carrillo C, Escribano J and Borca M. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine* 2002;**20**:1141-7.
5. Boehm R. Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms. *Ann NY Acad Sci*. 2007;**1102**:121-134.
6. Floss D.M, Falkenburg D, Conrad U. Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. *Transgenic Res.*2007;**16**:315-332.
7. Daniell H, Lee SB, Panchai T, Wiebe PO. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J Molec Biol.* 2011;**311**(5):1001-1009.
8. Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, et al. Plant-based vaccines: unique advantages. *Vaccine* 2001;**19**(17-19):2742-2748.
9. Aziz MA, Singh S, Anand K, Bhatnagar R. Expression of protective antigen in transgenic plants: a step towards edible vaccine against anthrax. Biochem. *Biophys Res Commun.* 2002; **299**:345-351.
10. Kang TJ, Han SC, Jang MO, Kang KH, Jang YS, Yang MS. Enhanced Expression of B-Subunit of Escherichia coli Heat-Labile Enterotoxin in Tobacco by Optimization of Coding Sequence. *Appl Biochem Biotechnology*. 2004;117:175-187.
11. Simmons CW, VanderGheynst JS, Upadhyaya SK. A model of Agrobacterium tumefaciens vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. *Biotechnol Bioeng.* 2009;**102**:965-970.
12. Bhaskar PB, Venkateshwaran M, Wu L, Ane JM, Jiang J. Agrobacterium-Mediated Transient Gene Expression and Silencing: A Rapid Tool for Functional Gene Assay in Potato. *PLoS ONE.* 2009; **4**(6):581-592.
13. Sheen J. Signal Transduction in Maize and Arabidopsis mesophyll protoplasts. *Plant Physiol.* 2011;**127**:1466-1475.
14. Leckie BM, Stewart CN. Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. *Plant Cell Rep.* 2011;**30**:325-334.
15. Schweizer P, Christoffel A, Dudler R. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *The Plant J.* 1999; **20**:541-552.
16. Wroblewski T, Tomczak A, Michelmore R. Optimization of Agrobacterium mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotech J.* 2005;**3**:259-273.
17. Zottini M, Barizza E, Costa A, Formentin E, Ruberti C, Carimi F, *et al*. Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. *Plant Cell Rep.* 2008;**27**:845-853.
18. Zheng L, Liu G, Meng X, Li Y, Wang Y. A Versatile Agrobacterium-Mediated Transient Gene Expression System for Herbaceous Plants and Trees. *Biochem Genet*. 2012;**50**:761-769.
19. Hashemi H, Jourabchi E, Khodabandeh. Transient Expression of Human Growth Hormone in Potato (Solanum tuberosum), Tobacco (Nicotiana tobacum) and Lettuce (Lactuca sativa) Leaves by Agroinfiltration. *I J Biotechnol.* 2005;**3**(2):109-113.
20. Schillberg S,Twyman RM,Fischer R. Opportunities for recombinant antigen and antibody expression in transgenic plants- technology assessment. *Vaccine*. 2005;**23**:1764-1769.
21. Kozak, M. The scanning model for translation: an update. *J Cell Biol.* 1989;**108**:229-241.
22. Haq TA, Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science*. 1995;**268**:714-716.
23. Sparkes IA, Runions J, Kearns A, Hawes C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat Protoc.* 2006;**1**:2019-2025.
24. Bendahmane A, Querci M, Kanyuka K, Baulcombe DC. Agrobacterium transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *Plant J.* 2000; **21**:73-81.

25. Santos-Rosa M, Poutaraud D, Merdinoglu D, Mestre P. Development of a transient expression system in grapevine via agro-infiltration. P*lant Cell Rep*. 2008;**27**:1053-1063.
26. Janssen B, Gardner R. Localized transient expression of GUS in leaf discs following cocultivation with Agrobacterium. *Plant Mol Biol*. 1989; **14**:61-72.
27. Koprowski H, Yusibov V. The green revolution: plants as heterologous expression vectors. *Vaccine*. 2001;**19**:2735-41.
28. Sala F, Rigano M, Barbante A, Basso B, Walmsley AM, Castiglione S. Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. *Vaccine*. 2000;21:803-808.
29. Pourseyedi S, Hashemi Sohi H, Omidi M, Ali-Ghoreshi, S, ShahNejat Boushehri AA, Jourabchi E. Transient expression of VP2 gene of very virulent IBDV in tobacco, Alfalfa and lettuce leaves by agroinfiltration. *Veterin J* (Pajouhesh & Sazandegi). 2009;**83**:18-25.
30. Streatfield SJ. 2005. Plant-based vaccines for animal health. *Rev Sci Tech Off Int Epiz*. 2005;**24** (1):189-199.
31. Gil F, Brun A, Wigdorovitz A, Catala R, Martinez-Torrecuadrada JL, Casal I et al. High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS Lett*. 2001;**488**:13-17.
32. Brun S, Faucon-Biguet N, Mallet J. Optimization of transgene expression at the posttranscriptional level in neural cells: Implications for gene therapy. *Molecular Therapy.* 2003;**7**:782-789.
33. Walmsley AM, Arntzen CJ. Plants for delivery of edible vaccines. *Curr Opin Biotechnol* 2000;**11**:126-9.
34. De Angioletti M, Lacerra G, Sabato V, Carestia C. Beta+45 G --> C: a novel silent beta-thalassaemia mutation, the first in the Kozak sequence. *Br J Haematol.* 2004;**12**(2):224-231.
35. Wydro M, Kozubek E, Lehmann P. Optimization of transient Agrobacterium-mediated gene expression system in leaves of Nicotiana benthamiana. *Acta biochimica polonica.* 2006;**53**(2):289-298.
36. Fischer R, Vaquero C, Sack M, Drossard J, Emans N, Commandeur U. Toward Molecular Farming in the future: transient protein expression in plants. *Biotechnol Appl Biochem.* 1999;**30**:113-116.