

## Expression of an epitope-based recombinant vaccine against Foot and Mouth Disease (FMDV) in tobacco plant (*Nicotiana tabacum*)

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**ABSTRACT:** Regarding high potential of green plants for development of recombinant vaccines, this research was conducted to evaluate expression of a novel recombinant vaccines against Foot and Mouth Disease (FMDV) in tobacco plant. For this purpose, a synthetic gene encoding 129-169 amino acids of foot and mouth disease virus capsid protein VP1 was transferred to tobacco plant via *Agrobacterium*-mediated genetic transformation. Considering codon usage optimized for tobacco, ribosome binding site and endoplasmic reticulum signal peptide were included in the synthetic gene to enhance expression level. Expression of the synthetic gene in tobacco seedlings was analyzed at transcription and translation levels and production of recombinant protein was quantified. Moreover, an *in vivo* immunization assay was carried out to verify immunogenicity of the expressed peptide in model animals. Results showed that expression of the recombinant protein in two lines of transgenic plants was a high as 0.65% and 0.72% of total soluble protein. The recombinant protein was able to induce immunogenic response when parenterally administered in rabbit.

**KEYWORDS:** FMDV, transgenic plants, recombinant vaccine, mucosal immunity

### INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the causative agent of Foot and Mouth Disease, highly contagious disease adversely affecting milk and meat-producing animals around the world [1]. Current vaccines against FMD are primarily based on 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 [2]. During recent years, there have been many attempts to produce FMD subunit vaccines in green plants as an alternative for traditional vaccines [3]. These techniques are based on expression of immunogenic sites located on coat protein

of the causative virus (FMDV). Since the majority of FMDV immunogenic sites are located on VP1 capsid protein, the protein has been widely used in production of recombinant subunit vaccines against FMD. The prominent G–H loop of the VP1 capsid protein of FMDV, spanning residues 134 to 158, has been identified as the major immunogenic site for neutralizing antibodies. This region lacks T helper (Th) cell epitopes for recognition by Major Histocompatibility Complex (MHC) alleles and B cell for inducing high affinity neutralizing antibodies [1, 4]. However, it has been reported that flanking regions of G-H loop contain epitopes for inducing both B and Th cells [5].

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Received: 24 August 2019/ Revised: 22 December 2019

Accepted: 29 December 2019

Oral delivery of plant-based recombinant vaccines is a potential alternative to injection, owing to the low cost and ease of administration. Plant-based vaccines are free of human and animal diseases and, therefore, can reduce the costs of screening for viral and microbial contamination. In addition, the chances of acquiring mucosal immunity against infectious agents that enter the body across a mucosal surface are increased with oral vaccines [6]. Despite numerous advantages of green plants over other heterologous expression systems such as bacteria, yeast and insect cell culture, low expression level of the antigen in plant hosts is still a main challenge for commercialization of plant-based recombinant vaccines [3]. To overcome this problem, various strategies such as host-specific codon optimization, inclusion of 5' untranslated leader sequences, insertion of introns within coding region, application of signal peptide have been proposed and applied in genetic transformation experiments; with varying degrees of success [5].

In the present investigation, we report the expression of a synthetic gene corresponding to 129<sup>th</sup>-169<sup>th</sup> amino acids of VP1 protein (including G-H loop and its flanking regions) in tobacco plants. Because low level of gene expression is a major hurdle in efficiency of recombinant vaccines, a combination of factors enhancing gene expression was exploited to ensure that the expression level in transgenic plants is high enough to induce strong immune response in animal host. First, a ribosome binding site in eukaryotic organisms called Kozak sequence was included just before start codon. The initial AUG codon was immediately followed by a G nucleotide. This gene arrangement (Kozak sequence before start codon and placement of G immediately after the start codon) has been reported to enhance translation efficiency up to 10-fold [7].

Endoplasmic reticulum (ER) signal peptides transfer the translated peptides to ER, where necessary enzymes for correct folding and assembly are present [8]. It has been reported that fusion of an ER retention peptide called SEKDEL resulted in significant increase in recombinant protein in potato tubers [9]. Therefore, we fused coding region of SEKDEL to 3' terminus of our gene construct just before stop codon. The last strategy we applied to enhance accumulation of the recombinant protein was optimization of codon usage in tobacco. Optimizing gene construct based on codon bias of the host plant is a critical issue for achieving high level of expression [10, 11].

Therefore, the synthetic NP1 gene was optimized for expression in tobacco according to *Nicotiana tabacum* codon usage table.

Plant leaf discs were co-cultured with *Agrobacterium tumefaciens* carrying pBI121 plasmid in which the synthetic gene had been inserted. Plantlets were regenerated and expression of the foreign gene was evaluated by different methods. Finally, efficacy of plant crude extract in inducing immunological response in rabbits was investigated.

## MATERIALS AND METHODS

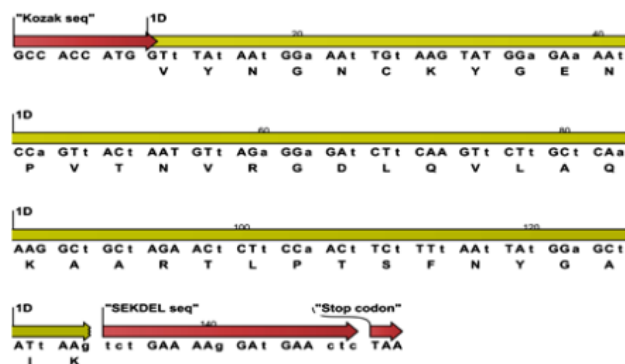
### Construct design

A 120 bp long fragment of VP1 coding region including G-H loop was used as the core part of our new construct. This region encodes 129-169 amino acid sequence of VP1 capsid protein of FMDV serotype O. Using CLC bioinformatics software, some modifications were performed to enhance expression of the construct in plant host. A eukaryotic ribosome binding site called Kozak sequence, GCCACC, was introduced prior to the start codon and coding region of endoplasmic reticulum signal peptide called SEKDEL consisting of six amino acids was attached to 3' end before stop codon. Start codon (AUG) and stop codon (UAA) were also added in to the 5' and 3' ends of the construct, respectively. Moreover, to facilitate cloning procedure, restriction sites of *Bam*HI and *Sac*I were introduced into the 5' and 3' ends of the synthetic gene, respectively. Construct design was based on codon usage pattern of tobacco taken from <http://www.kazusa.or.jp/codon/>. The construct was synthesized and cloned into the pGem T-Easy vector (Bioneer, South Korea).

### Construction of Binary Vector and plant transformation

The synthetic VP1 gene fragment was removed from pGem T-Easy vector by digestion with *Bam*HI (Fermentas company) and *Sac*I (Fermentas company) and was inserted into the plant expression vector pBI121 (donated by Agricultural Biotechnology lab of Ferdowsi university) downstream of the CaMV 35S promoter and upstream of the nopaline synthase (NOS) terminator, yielding pBI121-VP1 vector (Figure 1).

The ligation reaction mixture was used to transform *E. coli* strain DH5- $\alpha$  and kanamycin-resistant colonies were isolated after overnight incubation at 37°C in LB media



**Figure 1.** Schematic presentation of gene construct.

containing 50 mg l<sup>-1</sup> kanamycin (Sigma-Aldrich). After amplification, the plasmid was extracted from bacterial cells using alkaline lysis method. The plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by thaw-melting method [8]. Transformed cells were selected by kanamycin-resistance and PCR.

Leaf disc method was applied to transform tobacco plant as described previously [1] with some modification. Briefly, surface sterilized leaf discs were co-cultivated with *A. tumefaciens* suspension carrying pBI121-VP1 plasmid for 15 minutes, then transferred into MS medium and incubated in darkness. After 48h, the discs were transferred to shoot induction medium containing MS basal salts supplemented with 1.0 mg l<sup>-1</sup> 6-Benzylaminopurine (BAP) (Sigma-Aldrich), 0.1 mg l<sup>-1</sup> Naphthalene acetic acid (NAA) (Sigma-Aldrich), 100 mg l<sup>-1</sup> kanamycin and 300 mg l<sup>-1</sup> cefotaxime (Sigma-Aldrich) for transformants screening.

### PCR analysis for verification of putative transgenic lines

Genomic DNA was extracted from the leaf tissues of transgenic plants according to general extraction procedure [4]. The presence of the VP1 synthetic gene was confirmed by PCR analysis using specific primers (5'atggaaattgtaagtattggaga3', 5'gaagaaagcgaaggagc3').

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 by a final extension step of 72°C for 10 min.

### Real Time PCR assay

Expression of the transgene was evaluated at transcription level by Real Time PCR assay. Total RNA

was extracted from 500 mg of leaf tissue according to general process of RNA extraction [2]. Complementary DNA (cDNA) was synthesized via reverse transcription using oligo(dT)<sub>20</sub> primer (Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit). The resulting cDNA mixtures were used as templates for real-time PCR that 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 (Genet Bio, South Korea). Quantitative Real-Time PCR experiments were performed in duplicate for each sample. EF-1alpha gene was used as housekeeping (reference) gene in Real Time PCR.

### Western blot assay

Total soluble protein (TSP) was extracted from transgenic tobacco plants according to Kang et al (2004) [8]. Briefly, 1gr of fresh leaves was ground in liquid nitrogen. One milliliter of extraction buffer (100mM Tris-HCl pH 8; 10 mM EDTA pH 8; 0.5% Beta Mercaptoethanol; 50µM PMSF)

was added, homogenated and then centrifuged at 13,000g for 15 min at 4°C and the supernatant was used for further analysis. Protein samples were segregated using 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane at 130 mA for 2 h. To prevent nonspecific antibody reactions, the membrane was blocked with BSA. After blocking with BSA, proteins were allowed to react with specific antibodies and the membrane was then washed using PBST/PBS, and incubated with an alkaline phosphatase labeled anti-mouse Ig goat antiserum for 1 h at 37°C. After washing three times, the reaction was developed by addition of the substrate.

### 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 hour; followed by incubation with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C to prevent non-specific binding. The well was washed by PBST/PBS and incubated with antiserum reactive against FMDV (1:1000 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<sub>2</sub>SO<sub>4</sub> and read at 405 nm of wavelength. Furthermore, we applied the method proposed by Dus Santos et al [4] to quantify recombinant

protein content in transgenic tobacco plants via ELISA. Concentration of the recombinant protein was quantified by comparison with known amounts of pure protein in ELISA assay and expressed as a percentage of total soluble protein (%TSP).

### Induction of immune response in rabbit

Rabbits were immunized at days 0, 28 and 40 with 150 $\mu$ l of crude protein of wild type and transgenic plants emulsified in Incomplete Freund's adjuvant. Animals were then bled and the sera analyzed for the presence of anti-FMDV antibodies in ELISA assay as described in previous section.

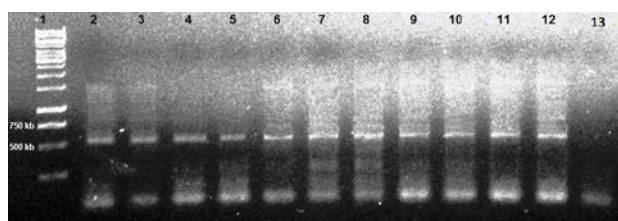
## RESULTS

### PCR detection of foreign DNA in transgenic plants

Tobacco leaf discs were cultured on kanamycin containing MS medium for primary selection. All 41 plantlets regenerated from leaf discs were analyzed by PCR assay to confirm foreign DNA integration in the plant genome. Ten lines showed positive signal in PCR assay demonstrating expected band in gel electrophoresis. No band was amplified from wild type plants (Figure 2).

### Quantification of gene expression by Real Time PCR

The transgenic lines were analyzed for gene expression level via Real Time PCR. Since the transgene has no background in plant host, a transgenic line (line 22) was randomly assigned as standard for quantitative comparison among the transgenic lines. Real Time PCR results confirmed foreign gene expression in all the transgenic lines but no signal was detected for control line.

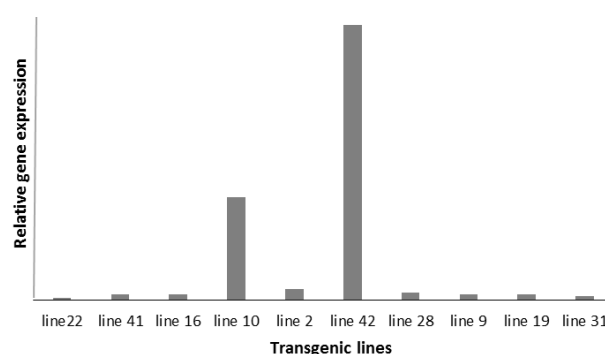


**Figure 2.** Detection of the VP1 synthetic gene in transgenic plants using PCR. Lane 1: 1kb size marker, lane 2: pBI121-VP1 plasmid, lane 3-12: DNA from transgenic plants, lane 13: DNA from wild type plant.

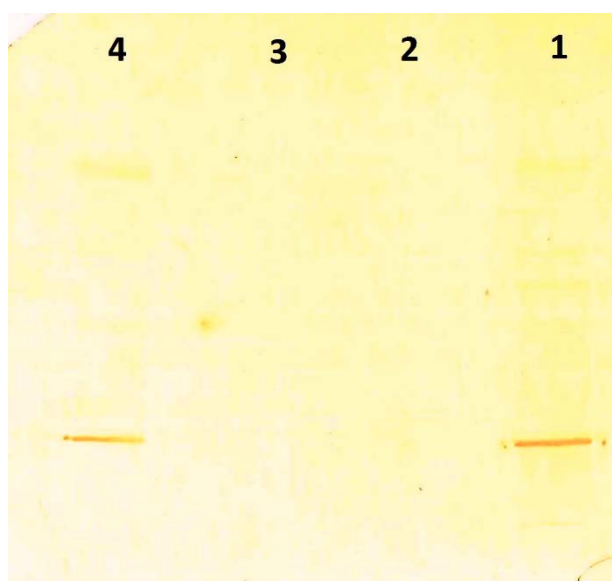
The highest expression level was observed for line 42 which was 1556 fold higher than that of standard (line 22); followed by line 10 which showed expression level 370 times as much as that of standard (Figure 3).

### Western blot assay

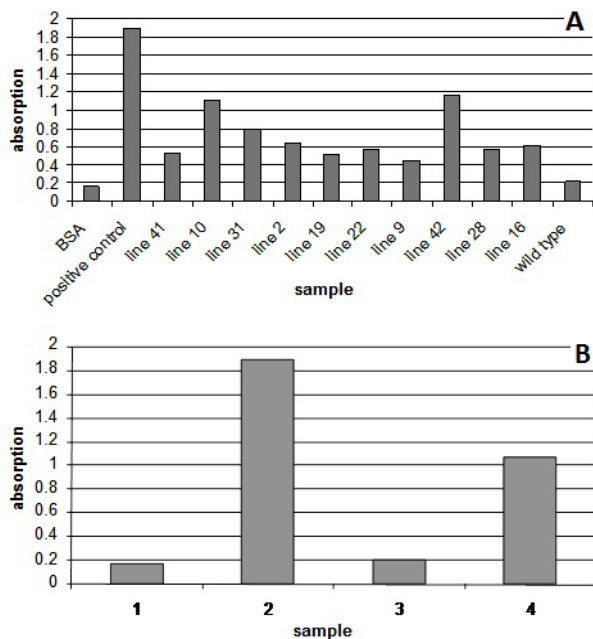
The synthetic gene encodes a recombinant protein with molecular weight about 13.5 kDa (<http://web.expasy.org>). Expression of VP1 synthetic gene in the transgenic lines was evaluated by Western-blot assay (Figure 4). As it is shown in figure 4, the expected protein band appeared obviously in the transformed lines 10 and 42 whereas wild type and other transgenic lines showed no band.



**Figure 3.** Results of Real Time PCR demonstrating expression of foreign gene in different transgenic plants.



**Figure 4.** Western blot analysis of transgenic plants. Lane 1: protein sample of transgenic line 10, lane 2: protein sample of wild type plant, lane 3: protein sample of transgenic line 16, lane 4: protein sample of transgenic line 42.



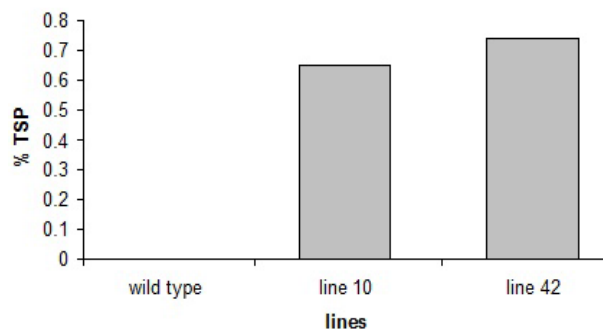
**Figure 5.** ELISA results for quantitative analysis of gene expression. (A) Recombinant protein production in transgenic lines; (B) induction of specific antibody in serum sample of rabbits injected with protein of transgenic plants; 1: BSA, 2: positive control (commercial vaccine), 3: serum of non-immunized rabbit, 4: serum of rabbit immunized by injection of protein of transgenic plant.

### ELISA assay

ELISA assay was performed to evaluate presence of recombinant VP1 protein in transgenic lines. Protein samples of all the transgenic lines were tested by ELISA. Protein samples of non-transgenic lines together with non-immunized rabbit sera were used as negative control and purified FMDV antigen was used as positive control. The test was performed as duplicate. All transgenic lines were positive in ELISA with the strongest reactions were observed for lines 10 and 42. No reaction occurred in negative control wells. Amount of recombinant protein was further evaluated in lines 10 and 42 which showed better results in both Real Time PCR and ELISA assay. Recombinant protein content was quantified by ELISA through comparing with known amounts of pure protein. As can be seen from Figure 6, results of quantification assay indicated that recombinant VP1 protein level in transgenic lines 10 and 42 were 0.65% and 0.72% of TSP, respectively.

### Induction of immune response in immunized rabbit

Rabbits were intraperitoneally immunized at days 0, 28 and 40 with approximately 150  $\mu$ l of transgenic plants



**Figure 6.** Quantification of recombinant protein in transgenic plants by ELISA assay. Recombinant protein constituted 0.65% and 0.72% of total soluble protein in transgenic lines 10 and 42, respectively. No signal was observed for wild type plants.

crude protein emulsified in Incomplete Freund's. The protein of non-transgenic plants was used in control group. Animals were then bled and the sera analyzed for the presence of anti-FMDV antibodies in ELISA assay. Antibodies raised in immunized mice showed a strong response in ELISA against VP1; while no response was observed in sera of control group (Figure 5B).

### DISCUSSION

Production of therapeutic proteins including recombinant vaccines in plants represents an economical alternative to both traditional inactivation of infectious agents and fermentation-based expression systems, especially for production of high-volume reserves of subunit vaccines [4]. Oral delivery of antigens expressed in plant parts is possible because the plant cell wall provides effective protection against degradation [12] so that much of the vaccine antigen expressed in the cells can reach the gut-associated lymphoid tissue (GALT) in an immunogenic form as stated by Alvarez et al (2006) [13]. Although the traditional inactivated vaccines against FMD are effective in protection of animal against challenge, there are major disadvantages of this vaccine that makes it necessary to develop alternative methods [14]. In recent years, there have been extended efforts to develop new subunit vaccines either through designing synthetic peptides or by production of recombinant antigen in live systems [1, 2, 5].

In this study, we conducted an *Agrobacterium*-mediated transformation and obtained ten transgenic lines, two of which (lines 10 and 42) were positive in both ELISA and western blot assays. Furthermore, recombinant protein produced in transgenic lines induced immune response when intraperitoneally administered to rabbit.

The G–H loop of the VP1 capsid protein of FMDV, spanning residues 134–158, has been reported by many investigators as the major immunogenic site for neutralising antibodies [5, 14, 15, 16].

Though simple and surprising candidate as immunogenic site, G–H loop is not effective in protection of animal against viral challenge when applied alone, because the region lacks T helper (Th) cell epitopes for recognition by MHC alleles and B cell for inducing high affinity neutralising antibodies [2, 4]. This reduces immunogenicity of G–H loop and its feasibility as an effective vaccine. In the current study, we designed a synthetic gene including G–H loop and its flanking region so that both T-cell and B-cell inducing regions were included. The final construct was composed of a DNA fragment encoding 129-169 amino acids of VP1. It has been previously reported that flanking regions of G–H loop enhances its immunogenicity by inducing B and Th cells [5]. Similarly, in the present study a strong immune response was observed when the recombinant protein was intraperitoneally injected to rabbit. Induction of FMD specific immune response by recombinant antigen had been reported by many authors in laboratory trials [2, 4, 18], however, problems such as low expression level of foreign gene have put great limitation toward commercialization of recombinant subunit vaccine production. Therefore, enhancing expression level of recombinant vaccines in plant cells is an important, and even urgent, step toward practical use of recombinant vaccine technology.

Indeed, the low level of the antigen expression in transgenic plants is a major drawback limiting efficiency of plant systems as cell factories for the production of recombinant vaccines. Some of the strategies successfully exploited to increase expression level of foreign gene include the use of specific reticulum retention signals sequences [19, 20, 21], the increase of the translational efficiency using ribosome binding sites [8, 21] or the utilization of plant-optimized synthetic genes [22, 23].

In the present study, we added Kozak sequence, ribosome binding site in eukaryotic organisms, upstream the gene before initiation codon and an endoplasmic reticulum signal peptide (SEKDEL) just before stop codon. The gene was codon optimized for expression in tobacco. To the best of our knowledge, it was the first time that a combination of these three strategies is used in production of recombinant FMD vaccine. As can be inferred from figure 4, two transgenic lines (lines 10 and

42) showed high level of gene expression compared to positive control. Recombinant protein content in these lines was shown to be 0.65% (line 10) and 0.72% (line 42) of TSP; a percentage significantly higher than 0.01% to 0.2% usually observed in nuclear transformation of plants [6]. This quantitative measurement indicated that application of expression enhancing strategies including codon optimization, Kozak sequence and SEKDEL signal peptide was successful in promotion of gene expression level. This observation is in agreement with the work conducted for expression of B-Subunit of E. coli Heat-Labile enterotoxin in tobacco [8]. These authors reported recombinant protein content to be 2.2% of TSP. Although we didn't reach such a high level of transgene expression, the results obtained in the present investigation was quite promising.

## CONCLUSION

As a conclusion, we describe here an approach for efficient production of recombinant vaccine against FMD by developing a new construct including G-H loop of VP1 protein and its flanking regions. To enhance its expression efficiency and especially translation rate in plant systems, Kozak sequence and endoplasmic reticulum signal (SEKDEL) was added and whole the synthetic gene was adapted to codon usage of tobacco. ELISA and western blot assays confirmed the production of recombinant protein in two transgenic lines 10 and 42. Moreover, a robust immune response was observed when the sera of rabbits immunized by injection of recombinant protein were tested by ELISA.

We believe that our results provide additional support to the feasibility of using transgenic plants as an effective system for production of recombinant vaccines. The present study is promising at least in two aspects. First, we demonstrated that a small part of VP1 capsid protein (129-169 amino acids) rather than whole the protein can be effective in inducing immune response in animal. Second, we applied some strategies such as codon optimization, leader sequence (Kozak sequence in this study) and signal peptide that have been proposed to enhance foreign gene expression in transgenic plants [5]. Application of other immunogenic sites of FMDV capsid protein along with other kinds of promoters, enhancers, ribosome binding sites, signal peptides and any molecular element involving in enhancement of foreign gene expression could be the subject of future studies.

## ACKNOWLEDGEMENTS

The authors wish to thank Razi vaccine and serum institute staff for their assistance.

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## بیان واکسن نو ترکیب اپی توپی تب برفکی (FMDV) در گیاه توتون (*Nicotiana tabacum*)

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### چکیده

با توجه به قابلیت بالای گیاهان سبز جهت توسعه واکسن‌های نو ترکیب، این تحقیق با هدف ارزیابی بیان یک واکسن نو ترکیب جدید علیه بیماری تب برفکی (FMD) انجام شده است. بدین منظور، یک ژن سنتزی کدکننده آمینواسیدهای ۱۲۹-۱۶۹ پوشش پروتئینی ویروس تب برفکی موسوم به VP1 با استفاده از تراریخته‌سازی به کمک اگروباکتريوم، به گیاه توتون انتقال داده شد. در طراحی ژن سنتزی مواردی چون بهینه سازی کدونی برای گیاه توتون، جایگاه اتصال ریبوزومی و پپتید نشانه شبکه اندوپلاسمی لحاظ شده است تا سطح بیان افزایش پیدا کند. بیان ژن مورد نظر در گیاهچه‌های توتون در دو سطح رونویسی و ترجمه بررسی شده و تولید پروتئین نو ترکیب در گیاه توتون به صورت کمی اندازه‌گیری شد. علاوه بر این یک آزمایش این ویوو انجام شده تا ایمنی زایی پپتید بیان شده در حیوان آزمایشگاهی مورد ارزیابی قرار گیرد. نتایج بدست آمده در این تحقیق نشان داد که بیان پروتئین نو ترکیب در دو لاین تراریخته به ترتیب تا ۰/۷۲ درصد و ۰/۴۵ درصد کل محتوای پروتئینی محلول گیاه رسیده است. همچنین مشخص شد که پروتئین نو ترکیب مورد نظر در هنگامی که به صورت تزریق زیر پوستی استفاده شود می‌تواند پاسخ ایمنی را در حیوان آزمایشگاهی القا نماید.

**کلمات کلیدی:** بیماری تب برفکی، گیاهان تراریخته، واکسن نو ترکیب، ایمنی مخاطی