Electronic Journal of Biotechnology 22 (2016) 38–43

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Expression of Hemagglutinin–Neuraminidase and fusion epitopes of Newcastle Disease Virus in transgenic tobacco

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ARTICLE INFO

Article history:
Received 11 January 2016
Accepted 12 May 2016
Available online 07 June 2016

Keywords:
Agrobacterium tumefaciens
ELISA
Recombinant vaccine
Transgenic plants

ABSTRACT

Background: Newcastle disease is an important avian infectious disease that brings about vast economic damage for poultry industry. Transgenic plants represent a cost-effective system for the production of therapeutic proteins and are widely used for the production of poultry vaccines. In an attempt to develop a recombinant vaccine, a plant expression binary vector pBI121, containing the genes encoding Hemagglutinin–Neuraminidase (HN) and Fusion (F) epitopes of Newcastle Disease Virus (NDV) under the control of CaMV35S promoter and NOS terminator was constructed and introduced into the tobacco (*Nicotiana tabacum*) plant by *Agrobacterium*-mediated transformation.
Results: Putative transgenic plants were screened in a selection medium containing 50 mg/L kanamycin and 30 mg/L meropenem. Integration of the foreign gene in plant genome was confirmed by PCR. Expression of foreign gene was analyzed at transcription level by RT-PCR and at translation level by means of dot blotting and ELISA. All analyses confirmed the expression of recombinant protein.
Conclusion: Developments in genetic engineering have led to plant-based systems for recombinant vaccine production. In this research, tobacco plant was used to express F and HN epitopes of NDV. Our results indicate that for the production of recombinant vaccine, it is a novel strategy to use concatenated epitopes without their genetic fusion onto larger scaffold structure such as viral coat protein.

1. Introduction

Newcastle Disease Virus (NDV) is an economically important pathogen that infects both wild and domesticated birds [1,2]. NDV belongs to the *Rubulavirus* genus and Paramyxoviridae family and is a negative-sense, single-stranded RNA virus with 15 kb genome. The genome encodes six major structural and non-structural proteins including nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), Hemagglutinin–Neuraminidase (HN) and RNA-dependent RNA polymerase (L) [3]. F and HN are glycoproteins that are critical for virulence and these two surface proteins are the most important targets for the host immune response and induce neutralizing antibody against NDV [3]. Amino acids 65–81 of F protein and 346–353 of HN have been identified as the most important immunogenic sites for antibody induction [4]. Killed or attenuated viruses are currently used as anti–NDV vaccine [4]. Although these vaccines are effective, high cost of vaccination, side effects such as egg decrease in chickens, high labor cost and stress that may lead to a reduction in egg-laying, or to an increased susceptibility to microorganisms infections call for a new method of production of NDV vaccines [4]. The best route of vaccination against NDV is oral administration as vaccines can be incorporated in poultry diet [5]. Production of recombinant vaccines based on capsid subunits and their application as oral vaccines is an effective alternative for conventional attenuated virus-based vaccines [6].

Plants represent an ideal platform for the production of recombinant vaccines [7]. Transgenic plants expressing foreign proteins of industrial and therapeutic value are good alternatives for fermentation systems. Various vaccines expressed transiently or permanently in green plants showed accurate conformation for the induction of protective and neutralizing immune responses in human, animal and poultry [7]. A major advantage of plant-based recombinant vaccines — in addition to ease of production and administration — is the induction of mucosal immunity which subsequently results in high immunity for the host. Considering that oral or nasal vaccine — delivery is more effective at

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.05.003

stimulating mucosal immunity, it would be quite fitting to express antigens in plants to be delivered as edible vaccines [7]. The successful use of transgenic plants for the expression of a number of pathogen antigens has revealed new prospects for vaccine production [8,9].

Due to numerous advantages, tobacco is widely used in genetic transformation as a plant host. Advantages include ease of transformation, availability of optimized tissue culture systems and high level of transgene expression [10]. Thus, this plant species is commonly used in scientific research as a plant host and when successful, the gene of interest can be transferred into other plants [10,11].

The present study aims to transfer and express F and HN epitopes in tobacco. Since tobacco is an ideal model plant for transgene expression, the successful expression of the epitopes in this plant can be the beginning for the commercial production of recombinant anti-NDV vaccines in other plant species.

2. Materials and methods

2.1. Designing gene construct

Four tandem repeats of HN epitope (encoding amino acids 346–353) with 96 bp length followed by three tandem repeats of F epitope (encoding amino acids 65–81) with 153 bp length were used. The sequences were retrieved from NCBI. The gene was codon optimized to ensure high level of expression. The initiation codon (AUG) followed by histidine tag (18 bp) and omega sequence as ribosome binding (67 bp) site were attached in upstream of the gene. The endoplasmic reticulum signal (SEKDEL) was included to 3′ end just before termination codon. Finally, two restriction sites namely *BamHI* and *SacI* were added to 5′ and 3′ ends. The final length of the gene was 376 bp. Gene was designed by CLC software (Fig. 1). The resulting gene construct was synthesized and cloned in pGH vector by Gene ray Company.

2.2. Construction of pBI121 construct containing F and HN antigens

Gene construct was removed from pGH via digestion by *BamHI* and *SacI*; binary vector pBI121 containing CaMV 35S promoter and NOS terminator was digested by the same enzymes and after removing GUS sequence, gene construct of F and HN epitopes was inserted between promoter and terminator (Fig. 2). The resulting construct is called pBI121-NDV epitopes which was transferred to agrobacterium using electroporation. To confirm the presence of the plasmid within bacterial cell after extraction and digestion by *HindIII*, a fragment of about 1096 bp was revealed on electrophoresis gel (Fig. 3).

2.3. Agrobacterium-mediated transformation of tobacco

Agrobacterium tumefaciens strain C58 harboring pBI121-NDV epitopes was cultured in LB medium for 18 h. The bacterial suspension at $OD_{600} = 0.6$ was centrifuged at 3500 × g for 20 min. Bacterial pellet was cultured for 120 min at 28°C in transformation medium (MS salts, 5% glucose and 200 μM acetosyringone, pH = 7.5).

Leaf disc method was applied to transform tobacco plant. Briefly, surface sterilized leaf discs of *Nicotiana tabacum* cv. Turkish were co-cultivated with *A. tumefaciens* suspension carrying pBI121-NDV epitopes plasmid for 15 min, then transferred into MS medium containing 2 mg/L BA and 2 mg/L NAA and incubated in darkness. After 48 h, the discs were washed with 30 mg/L meropenem to eliminate agrobacterium. The explants were then transferred to regeneration medium containing MS salts, 2 mg/L BA, 2 mg/L NAA, 50 mg/L kanamycin and 30 mg/L meropenem at 25°C and photoperiod of 16 h light/8 h darkness. Subculture was done every two weeks and for rooting of the explants, the regenerated plantlets were cultured in root induction medium (MS containing 2 mg/L IBA).

2.4. DNA extraction and PCR

To determine the presence of foreign gene in genome of the regenerated seedlings resistant to kanamycin, genomic DNA was extracted from young leaves of the seedlings and from wild type plants using the method proposed by Dellaporta et al. [12]. PCR was performed by specific primers with sequences of 5′TCATTGCGATAAAG GAAAGGC3′ and 5′AATGTATAATTGCGGGACTC3′. PCR was carried out by 35 cycles of 94°C for 60 s, 54°C for 60 s and 72°C for 60 s, followed by a final extension step of 72°C for 10 min. DNAs of wild type plant and plasmid pBI121-NDV epitopes were used as negative and positive control; respectively. PCR product was segregated on 1% agarose gel.

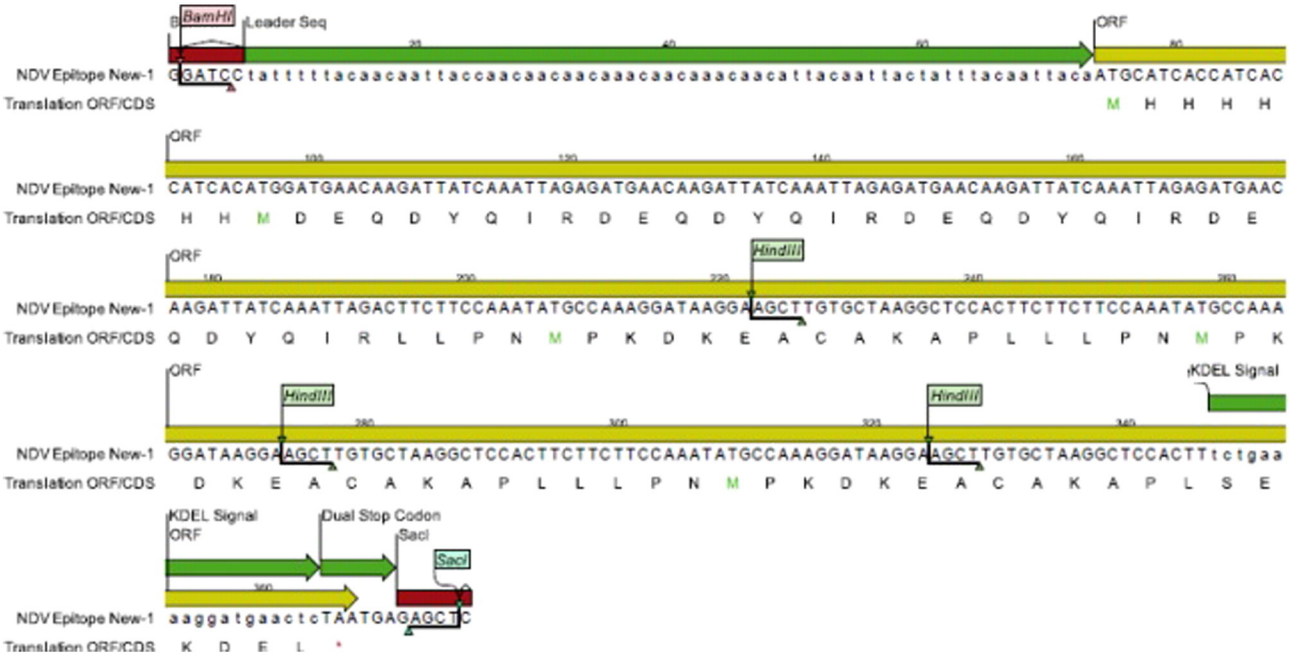

Fig. 1. Gene construct including F and HN epitopes together with needed sequences for expression in tobacco.

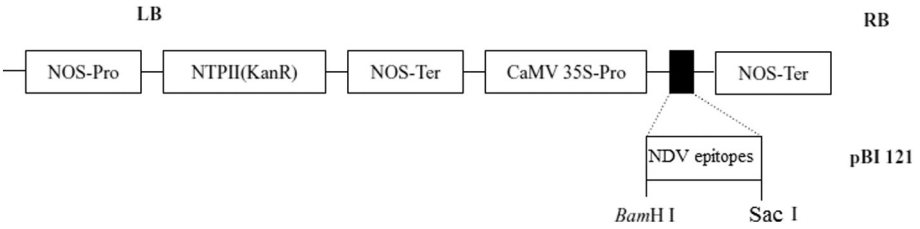

Fig. 2. Schematic structure of the recombinant plasmid pBI121-NDV epitopes for Agrobacterium-mediated transformation of tobacco plant. The DNA sequence encoding NDV epitope was cloned the downstream of the CaMV 35S promoter of a binary plasmid pBI 121.

2.5. Evaluation of transgene expression

2.5.1. Assessing expression at transcription level using RT-PCR

Total RNA was extracted using Dena Zist commercial kit. After treatment with DNAse, cDNA was synthesized by cDNA synthesis Kit, Thermo Fisher Scientific. PCR was performed by specific primers (forward: 5′ACTATTTACAATTACAATGCATCAC3′; reverse: 5′GAGTTCAT CCTTTTCAGAAAGTG3′) to verify transgene expression. PCR cycles comprised denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 45 s. cDNA of wild type plant and pBI121-NDV epitopes plasmid were used as negative and positive controls.

2.5.2. Assessing expression at translation level using dot blotting

After extraction of total protein from transgenic and wild type plants, dot blot analysis was conducted. Protein samples were dotted on the membrane and the membrane allowed to get dried at 37°C. The membrane was incubated with BSA as blocking solution for 1 h. the membrane was then incubated with anti-His-tag conjugated with Hrp (diluted in BSA at 1:1000) at 37°C. The membrane was washed three times with PBS/PBST and then incubated with TMB substrate. Three microliters of wild type protein was used as negative control.

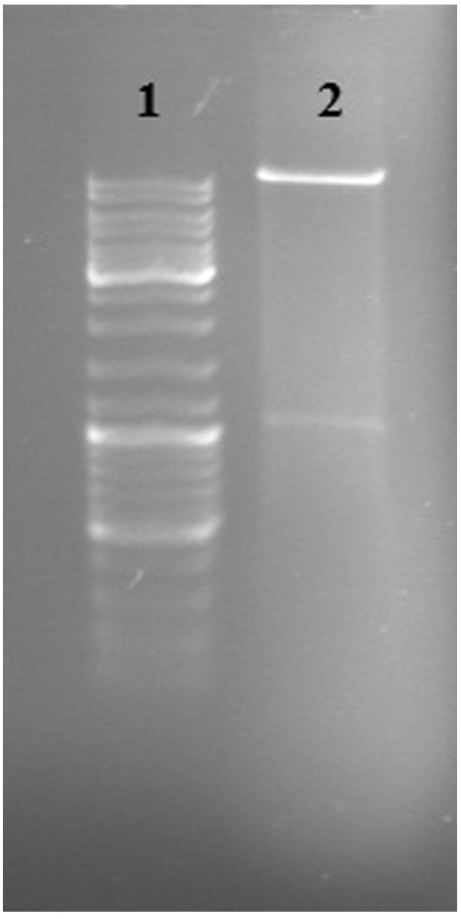

Fig. 3. Digestion of pBI 121-NDV epitopes plasmid extracted from *A. tumefaciens* by *Hind* III, 1: 100 bp ladder; 2: digested plasmid.

2.5.3. Quantification of recombinant protein by indirect ELISA

Total soluble proteins were extracted from fresh leaves using pre-chilled phosphate-buffered saline (PBS, pH 7.2) with 5 mMEDTA and 0.001% PMSF (phenyl methlysulforyl fluoride). Approximately 0.5 g fresh leaves was ground to fine powder in a pre-cooled mortar and two volumes of ice-cold extraction PBS were added. The homogenate was centrifuged at 14,000 × g for 15 min at 4°C. The supernatant obtained was used for ELISA analysis.

The 96-well plate was coated with total soluble proteins (TSP) from the wild type and the transformed plant and purified NDV particles (commercial vaccine) at 37°C for 1 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 anti-NDV serum and then horseradish peroxidase conjugated with anti-rabbit IgG (1:1500). The anti-NDV serum was prepared after three times of immunization in rabbit with the inactivated V4 NDV particles and diluted at 1:1000 in PBST.

Wells were developed with TMB substrate; the color reaction was stopped by 2N H_2SO_4 and read at 450 nm of wavelength. The OD450 nm value of samples was compared with that of known concentration of purified NDV particles to estimate the relative quantity of recombinant protein expressed in transgenic tobacco plant.

3. Results

In this research, transgenic tobacco was generated via co-culture with *A. tumefaciens* containing pBI121-NDV epitopes plasmid in a selection medium supplemented with 50 mg/L kanamycin and 30 mg/L meropenem (Fig. 4). PCR primers were designed so that forward attaches CaMV35S promoter and reverse primer attaches NOS sequence producing a 180 bp band. The band was observed in a transgenic plant and positive control and was not seen in wild type plant suggesting lack of transgene in its genome (Fig. 5). Expression of F and HN epitopes were evaluated at transcription level by RT-PCR using three samples of transgenic plant. Results showed that transgene was expressed in all the samples (Fig. 6). Production of recombinant protein was evaluated in the samples that had shown positive results in RT-PCR. Dot blot confirmed production of recombinant proteins in all the transgenic plants which was measured by intensity of blotting signal; whereas it was not expressed in wild type plant (Fig. 7). ELISA analysis was carried out to detect the antigen in total soluble proteins from leaves of transgenic plant and wild types. The results showed that there was immune reactivity with the NDV antibodies in transgenic plant, as the measured OD450 nm value was significantly higher (5% level of significance) than that in the untransformed wild type implying that the transgene was expressed in the transgenic tobacco (Fig. 8).

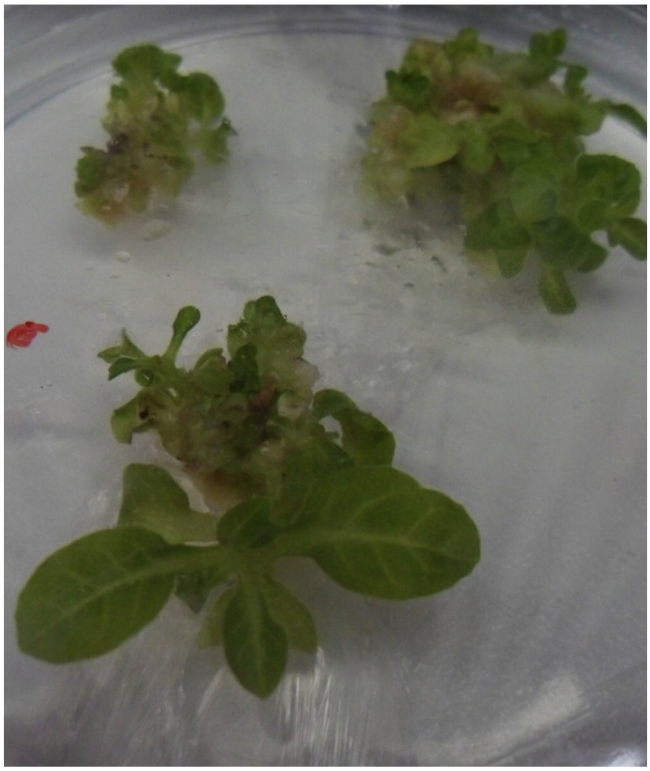

Fig. 4. Regeneration of transgenic tobacco plants in selection medium containing 50 mg/L kanamycin and 30 mg/L meropenem.

From an ELISA titration by using different amounts of purified NDV and specific anti-NDV antibody, the relative quantity of expressed recombinant protein was estimated, and it was observed that concentration of foreign protein in the plant tissue was 0.44% TSP (data not shown). In all, these results demonstrate the specific presence and expression of transgene under the control of CaMV 35S promoter in transformed tobacco plants.

4. Discussion

Application of green plants for the production of recombinant protein is an attractive alternative for the development of new generation vaccines. The 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 in the production of high-volume reserves of subunit vaccines. Genetic engineering of higher plants was a turning point in the field of recombinant vaccine production. The goal is to produce transgenic plants that upon oral or parenteral administration induce an immune response in the body. Many authors have reported that antibody is produced in response to the antigens produced in plant and administered orally or by injection [8,13,14,15]. The results of researchers reporting immunogenicity of viral antigens expressed in transgenic plants encourage the study of expression of other viral antigens in plants. In the studies of plants as bioreactor, tobacco is the most preferred model to express foreign proteins because of its ease of transformation and fast regeneration.

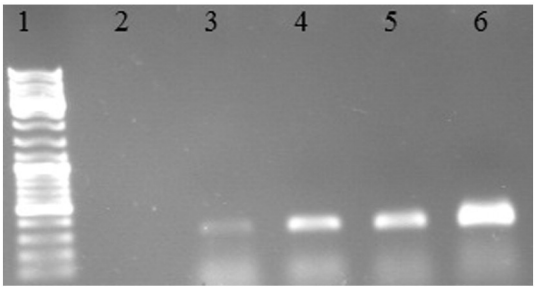

Fig. 6. Evaluation of expression at transcription level using RT-PCR and amplification of 307 bp fragment: 100 bp marker (1); wild type plant as negative control (2); transgenic plant (3–5); NDV epitopes-PBI 121 plasmid as positive control.

In the present paper, a synthetic gene including 4 tandem repeats of HN epitopes and 3F epitopes of NDV was prepared. This construct was optimized based on tobacco preferred codons according to studies reporting that codon optimization can elevate quality and quantity of protein [6,16]. 5′ leader sequence of tobacco mosaic virus (TMV) called omega which is regarded as a translation enhancing element was added to 5′UTR [17]. Regarding the effect of SEKDEL on enhancement of gene expression [18], SEKDEL was attached to 3′ end just before the stop codon. Histidine tag was added to amino terminal of the gene for identification and isolation of target protein using anti-His sequence. The results indicated that the strategies used for enhancing gene expression were successful.

After regeneration of transgenic seedlings, total DNA was extracted to screen for the foreign gene using PCR. PCR primers were designed so that they matched the promoter and terminator of pBI121 yielding a band of 810 kb length. PCR results showed that 62% of the seedlings were

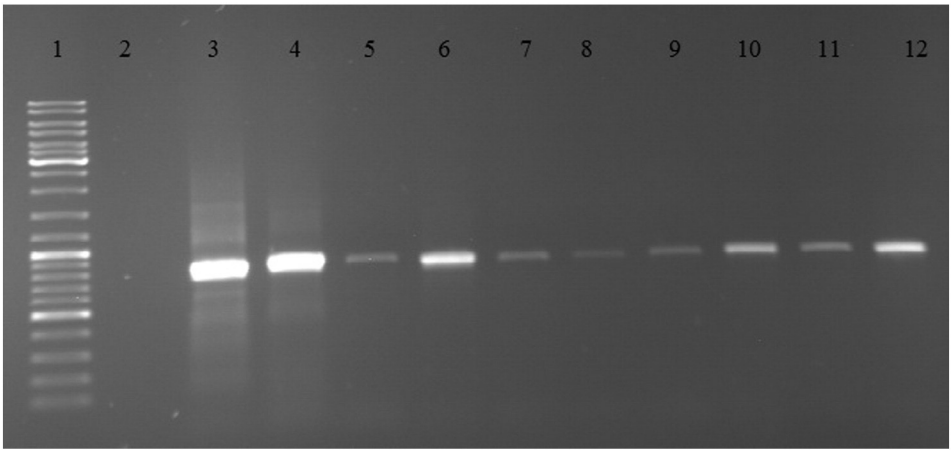

Fig. 5. Evaluation of foreign gene in transgenic tobacco by PCR and amplification of 810 bp fragment: 100 bp marker (1); wild type plant as negative control (2); NDV epitopes-PBI 121 plasmid and *Agrobacterium* containing this plasmid as positive control (3–4); transgenic plants (5–12).

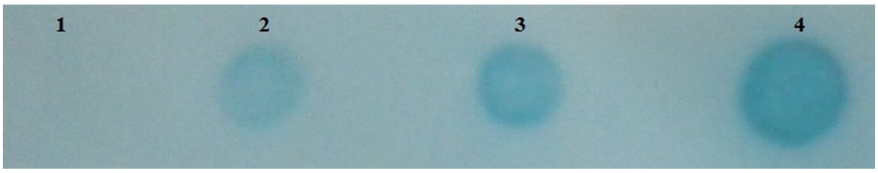

Fig. 7. Assessing expression at translation level using dot blotting: negative control (1); transgenic plant (2–4).

transgenic. Expression of RNA and protein was semi-quantitatively measured in three plants using RT-PCR and Dot blot with the results showing that the sharpness of RNA band is positively related to protein quantity (Fig. 6 and Fig. 7). This finding is in accordance with those reported by Lai et al. [9]. These authors provided scientific evidence on the correlation between RNA band sharpness and protein level. Moreover, the expression of recombinant protein was detected by anti NDV serum in ELISA tests. The levels of recombinant protein obtained were in accordance with those reported by other researchers [3,8].

It was shown in this research that synthetic protein includes numerous repeats of F and HN epitopes in tobacco. The results indicated that it is an interesting and novel strategy to use the concatenated epitopes without their genetic fusion to larger scaffold structure such as viral coat protein.

Application of green plants for the production of recombinant vaccines is of great importance. Considering recent developments in genetic engineering and transformation methods, it is possible to develop a wide range of transgenic plants that can express various recombinant pharmaceutical compounds including viral and bacterial antigens, antibodies, and many other therapeutic proteins. However, the low expression level of foreign antigens represents a major hurdle for commercialization of recombinant subunit vaccine production. This limitation has been reported by many authors as a main barrier to the widespread use of plant-based platforms as bioreactors in commercial production of recombinant vaccines. Therefore, it can be concluded that improving the expression level of recombinant vaccines in plant cells is an important step towards practical use of recombinant vaccine technology. To address this problem, we applied an integrated approach including codon optimization, inclusion of omega leader sequence and an endoplasmic reticulum signal peptide (SEKDEL) to enhance expression of the antigen in transgenic plants.

The results of this research confirm the efficacy of Agrobacterium-mediated transformation for the production of recombinant vaccine. This approach is still the first choice for recombinant protein production compared to other methods. For example, in an attempt to produce anti-NDV vaccine using viral vectors, the authors observed that duplication of the HN epitope rendered the virus non-viable [19]. However, in the present study, insertion of four tandem repeats of HN epitope with 96 bp length followed by three tandem repeats of F epitope with 153 bp did not interfere with foreign gene expression. We believe that our results provide additional support to the feasibility of using transgenic plants as an effective system for the production of recombinant vaccines.

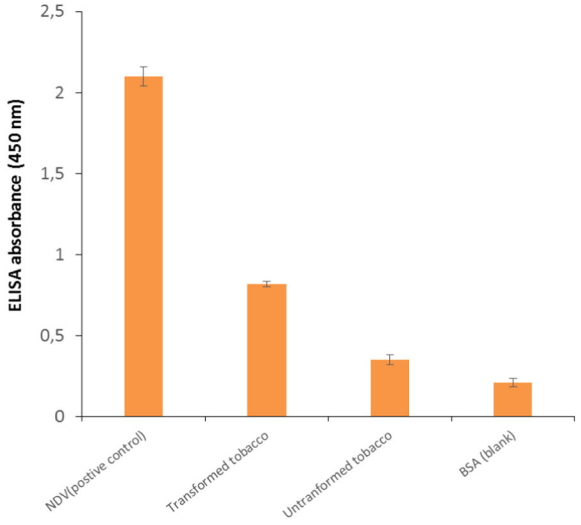

Fig. 8. ELISA analysis of NDV epitope expression in leaves of transgenic plant ($p < 0.05$). Each data represents an average value ± S.D. from three samples.

Financial support

The research was funded by Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad.

Conflict of interest

The authors declare that there are no conflict of interest.

References

[1] Alexander DJ. Newcastle disease and other avian Paramixoviridae infections. In: Calnek BW, Barnes HJ, Beard CW, Reid WM, Yoder HW, editors. Diseases of poultry Iowa. Iowa: State University Press; 1997. p. 541–70.
[2] Seal BS, King DJ, Sellers HL. The avian response to Newcastle disease virus. Dev Comp Immunol 2000;24:257–68. http://dx.doi.org/10.1016/s0145-305x(99)00077-4.
[3] Berinstein A, Vazquez-Rovere C, Asurmendi S, Gómez E, Zanetti F, Zabal O, et al. Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. Vaccine 2005;23:5583–9. http://dx.doi.org/10.1016/j.vaccine.2005.06.033.
[4] Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect Genet Evol 2016;39:22–34. http://dx.doi.org/10.1016/j.meegid.2016.01.008.
[5] Spradbrow PB, Samuel JL. Oral Newcastle disease vaccination with V4 virus in chickens: Comparison with other routes. Aust Vet J 1991;68:114–5. http://dx.doi.org/10.1111/j.1751-0813.1991.tb00770.x.
[6] Maclean J, Koekemoer M, Olivier AJ, Stewart D, Hitzeroth II, Rademacher T, et al. Optimization of human papillomavirus Type 16 (HPV-16) L1 expression in plants: Comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. J Gen Virol 2007;88:1460–9. http://dx.doi.org/10.1099/vir.0.82718-0.
[7] Sala F, Rigano MM, Barbante A, Basso B, Walmsley AM, Castiglione S. Vaccine antigen production in transgenic plants: Strategies, gene constructs and perspectives. Vaccine 2003;21:803–8. http://dx.doi.org/10.1016/s0264-410x(02)00603-5.
[8] Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci U S A 1996;93:5335–40. http://dx.doi.org/10.1073/pnas.93.11.5335.
[9] Lai K, Yussof K, Mahmood M. Heterologous expression of hemagglutinin–neuraminidase protein from Newcastle disease virus strain AF2240 in *Centella asiatica*. Acta Biol Cracov Bot 2012;54:142–7. http://dx.doi.org/10.2478/v10182-012-0007-x.
[10] Arntzen JC, Mason HS. Oral vaccine production in the edible tissues of transgenic plants. In: Levine MM, Woodrow GC, Kaper JB, Coban GS, editors. New Generation Vaccines. 2nd ed. New York: Dekker; 1995. p. 263–77.
[11] Tripurani SK, Reddy NS, Rao KRS. Green revolution vaccines, edible vaccines. Afr J Biotechnol 2003;2:679–83. http://dx.doi.org/10.5897/ajb2003.000-1124.
[12] Dellaporta SL, Wood J, Hicks JB. A plant DNA minipreparation: Version II. Plant Mol Biol Report 1983;1:19–21. http://dx.doi.org/10.1007/bf02712670.
[13] Wigdorovitz A, Carrillo C, Dus Santos MJ, Trono K, Peralta A, Gomez MC, et al. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 1999;255:347–53. http://dx.doi.org/10.1006/viro.1998.9590.
[14] Thanalava Y, Yang YP, Lyons P, Mason HS, Arntzen CJ. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc Natl Acad Sci U S A 1995;92:3358–61. http://dx.doi.org/10.1073/pnas.92.8.3358.

[15] Sandhu JS, Krasnyanski SF, Domier LL, Korban SS, Osadjan MD, Buetow DE. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. Transgenic Res 2000;9: 127–35. http://dx.doi.org/10.1023/a:1008979525909.

[16] Daniell H, Ruiz G, Denes B, Sandberg L, Langridge W. Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. BMC Biotechnol 2009;9:33. http://dx.doi.org/10.1186/1472-6750-9-33.

[17] Gallie DR. The 5′leader of tobacco mosaic virus promotes translation through enhanced recruitment of eIF4F. Nucleic Acids Res 2002;30:3401–11. http://dx.doi.org/10.1093/nar/gkf457.

[18] Haq T, Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 1995;268:714–6. http://dx.doi.org/10.1126/science.7732379.

[19] Zhao Y, Hammond R. Development of a candidate vaccine for Newcastle disease virus by epitope display in the *Cucumber mosaic* virus capsid protein. Biotechnol Lett 2005;27:375–82. http://dx.doi.org/10.1007/s10529-005-1773-2.