J. Agr. Sci. Tech. (2017) Vol. 19: 439-451

Transient Expression of HA1 Antigen of *H5N1 Influenza Virus* in Tobacco (*Nicotiana tabacum* L.) *via* Agro-infiltration

A. S. Farsad[1], S. Malekzadeh-Shafaroudi[1]*, N. Moshtaghi[1], F. Fotouhi[2], and S. Zibaee[3]

ABSTRACT

The *influenza A virus* is of global concern for the poultry industry, especially the H5 subtype as it has the potential to become highly pathogenic for poultry and mankind. Recently, plant expression systems have gained interest as an alternative for the production of vaccine antigens. The goal of the present study was to investigate the possibility of expressing the HA1 protein in *Nicotiana tabacum* via agroinfiltration. In this study, the Hemagglutinin type 1 (HA1) of a high pathogenic *avian influenza virus* of the H5N1 subtype was synthesized and transiently expressed in *Nicotiana tabacum*. To examine the possibility of expressing the HA1 protein in *N. tabacum*, a cDNA fragment encoding the *HA1* gene was synthesized de novo, modified with a Kozak sequence, a *C*-terminal hexa-Histidine (6His) tag, and an endoplasmic retention signal (KDEL). The construct was cloned into vector and the resulting - HA1 plasmid was agro-infiltrated into *N. tabacum*. The relative gene expression of recombinant plant-produced HA1 was measured by quantitative real-time PCR. Guided by the gene expression profile, HA1 protein was extracted at 3 dpi and subsequently purified utilizing the 6His tag. A recombinant HA1 protein was immunogenically detected by conjugated polyhistidine antibody in western blot, dot blot and ELISA assay. In order to verify the right conformation of HA1 produced in plants, western blot was also done using mouse monoclonal anti-*influenza A virus* (H5N1/HA1) [2B7]. The results of Real Time PCR assay indicated that the foreign gene was transcribed in transfected leaves. Migration size of protein was detected at 45 kD by Western blotting and demonstrated no discrepancy compared to the positive control (HA1). ELISA results showed that the HA1 was expressed in the transfected leaves in high level as the yield of recombinant protein was 8.8 % of TSP and the yield of purified HA1 was 0.16 g purified protein per kg fresh weight of leaves. This is the first research about the transient expression of the tobacco-made HA1 protein where a synthetic sequence was used for its expression. Here, the efficacy of agro-infiltration for expression of HA1 antigen in tobacco was illustrated. Agro-infiltration expedites the process of recombinant antigens expression in plant tissues. Accordingly, our results provide great opportunity for the exploration of transiently plant-manufactured HA1 as vaccine candidate.

Keywords: Avian influenza, Gene expression, Plant-manufactured HA1, Recombinant protein.

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI), H5N1, in poultry has fuelled intense media coverage and health concerns due to the severe outbreaks in Asia, Africa, and Europe. This deadly pandemic is still showing a great potential of spreading. The development of vaccine

candidates for HPAI is utmost crucial and could serve as the best strategy for managing the disease. Two of the currently licensed vaccines against influenza are Conventional Inactivated Virus Vaccine (CIV) and Live-

[1] Department of Crop Biotechnology and Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.
*Corresponding author; e-mail: malekzadeh-s@um.ac.ir
[2] Influenza Research Lab, Pasteur Institute of Iran, Tehran, Islamic Republic of Iran.
[3] Razi Vaccine and Serum Research Institute, Mashhad, Islamic Republic of Iran.

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

Attenuated Vaccine (LAV) with an objective of complete infection inhibition. These vaccines are dominated by the anti-Hemagglutinin (HA) immune response, and hence, the effectiveness is restricted to virus strains with closely matched HA (Kilbourne *et al*., 1990). *Avian Influenza Viruses* (AIV) belong to the family of *Orthomyxoviridae*, a family consisting of five different genera including the *influenza virus A* genus. Virus subtypes are identified based on nucleotide sequence diversity and antigenic properties of the viral surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA) (Monto, 2006).

Recombinant subunit vaccines that incorporate antigenic viral membrane glycoproteins, such as Hemagglutinin (HA), are particularly attractive candidates, since these molecules can induce virus-neutralizing antibodies (Bardiya and Bae, 2005). The HA protein is a key antigen for generating protective immunity in response to *influenza virus* (Skehel and Wiley, 2000). Also, recombinant HA antigens produced in plants have been shown to be immunogenic and safe in various animal models (Mett *et al*., 2008; Kalthoff *et al*., 2010; Shoji *et al*., 2009). The HA1 antigenic domain of HA has been shown to induce an immune response equal to that of the full-size protein (Caton *et al*., 1982; Tonegawa *et al*., 2003). The *influenza virus* surface Haemagglutinin (HA) glycoprotein, which elicits the primary neutralizing immune response, is the main target for vaccine development (Caton *et al*., 1982).

Recently, good progress has been made in the production of plant-based antigens to overcome the problems associated with existing vaccine production systems. Plant-based expression system offers several advantages in terms of low capital investment, time efficiency, high yield, and a lower risk of contamination with human pathogens (Tonegawa *et al*., 2003).

Low level of gene expression and the time required for the generation of transfected lines in stable transformation experiments are the major obstacle for production of recombinant proteins in green plants (Bendahmane *et al*., 2000). Transient expression makes it possible to evaluate efficacy of a potential recombinant vaccine in a short time (Hashemi *et al*., 2005; Schillberg *et al*., 2005). Most of the works in the field of transient antigen expression in plant hosts have been conducted by means of plant viruses as vehicle for gene delivery and expression, in which the epitope of interest is usually inserted within the coat protein gene (Koprowski and Yusibov, 2001). This method has proved to be an efficient and rapid way for 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 (Sala *et al*., 2000). In contrary, genes with large size can be efficiently expressed in plants *via* Agrobacterium-mediated genetic transformation (Wroblewski *et al*., 2005). 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 (Hashemi *et al*., 2005; Pourseyedi *et al*., 2009). Furthermore, the method is a fast approach to test efficiency of novel vaccine candidates in inducing immunogenic response in animal models (Hashemi *et al*., 2005; Pourseyedi *et al*., 2009; Schillberg *et al*., 2005; Streatfield, 2005).

Many strategies have been proposed for the enhancement of recombinant protein expression including; chloroplast transformation (Daniell *et al*., 2011), use of strong promoters (Streatfield *et al*., 2001), untranslated leader sequences (Aziz *et al*., 2002), signal peptide (Kang *et al*., 2004), codon optimization (Floss *et al*., 2007). The Long time required for the generation of transfected plants expressing foreign antigens is another limitation for the production of recombinant proteins

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

(Simmons *et al*., 2009). 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 (Bhaskar *et al*., 2009). This method can be carried out in many ways including protoplast transformation (Sheen, 2011), vacuum infiltration (Sheen, 2011), agro-infiltration (Leckie and Stewart, 2011) and particle bombardment (Schweizer *et al*., 1999). Among the techniques, agro-infiltration takes advantages of a simple, cost effective and rapid procedure. This technique has been carried out in a variety of plants (Wroblewski *et al*., 2005; Zottini *et al*., 2008) with different experimental purposes (Zheng *et al*., 2012). Time course required for antigen production via agro-infiltration (and other types of transient gene expression) is significantly shorter than that of stable transformation (Leckie and Stewart, 2011).

Since 2008, many vaccine antigens for various subtypes and strains of *influenza A virus* have been expressed transiently in plants specifically in tobacco (*Nicotiana spp.*), including human H1N1 (Shoji *et al*., 2011), human H3N2 (Mett *et al*., 2008; Shoji *et al*., 2008) and avian H5N1 (Kalthoff *et al*., 2010; Shoji *et al*., 2009; Shoji *et al*., 2011; Madhun *et al*., 2011; Musiychuk *et al*., 2006).

Agrobacterium-mediated transient gene expression assay was carried out for expression of HA1 subunit of H5N1 *influenza virus* in tobacco plants. The present study was undertaken to investigate the possibility of producing biologically active recombinant hemagglutinin (HA1) from the A/Indonesia/05/05 strain of H5N1 *influenza virus* in *Nicotiana tabacum* as a subunit vaccine candidate against influenza in poultry and humans. The main goal of this investigation was to evaluate agro-infiltration as an effective and quick method for production of recombinant antigen of HA1 and the expressed antigen can be used as a potential recombinant vaccine. We described the construction of the recombinant vector and the expression of *HA1* gene at both RNA and protein levels. This recombinant H5 HA1 demonstrated specific antigenicity in vitro. So, the potential impact of transiently plant-made HA1 on influenza vaccine production is discussed.

MATERIALS AND METHODS

Cloning and Expression of Influenza HA1

The HA1 sequence, encompassing amino acids 17–342 of the A/Indonesia/05/05 strain of *influenza virus* (NCBI accession number: AFM78567.1), was optimized for expression in plants and synthesized by GENSCRIPT (www.Genscript.com). Codon-optimization refers to the alteration of gene sequences, to make codon usage match the available tRNA pool within the cell/species of interest. Codon-optimization has emerged as a powerful tool to increase protein expression by genes from small RNA and DNA viruses, which commonly contain overlapping reading frames as well as structural elements that are embedded within coding regions (Floss *et al*., 2007). To obtain HA1 molecules in the plant expression system, the signal peptide (aa 1–16) were removed from the entire HA1 sequence and the Kozak sequence (GCAACA) was added to the N-terminus prior to the start codon (Kozak, 2007) and the endoplasmic reticulum retention signal (KDEL) which has been reported to increase recombinant protein accumulation in plant tissues (Haq *et al*., 1995) and a poly-histidine affinity purification tag (6His) were added to the *C*-terminus. Start codon (AUG) and stop codon (UGA) were also added into the 5' and 3' ends of the construct, respectively. Recognition sites of BamHI and SacI restriction enzymes were introduced into the 5' and 3' ends of the synthetic gene, respectively. The resulting sequence was inserted into the pGem-T Easy vector (Bioneer, South Korea). The synthetic

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

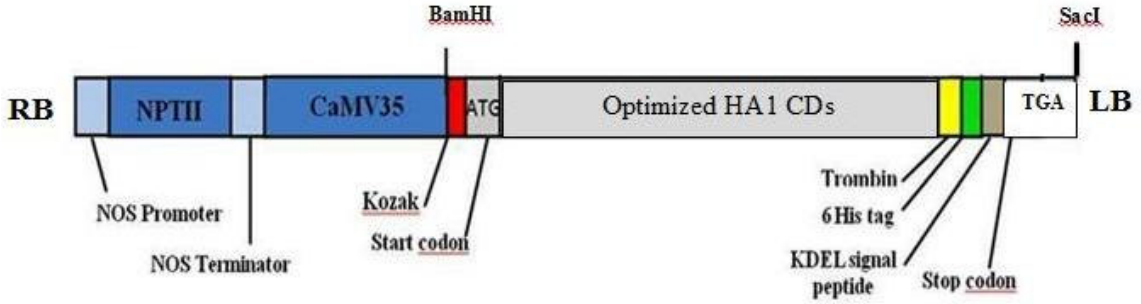

Figure 1. Schematic diagram of the plant expression system construct used for agro-infiltration.

HA1 gene was removed from pGem-T Easy vector by digestion with BamHI and SacI and was inserted into the binary vector pBI121, yielding 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 LB4404 by heat shock method. Transfected cells were screened by kanamycin-resistance and PCR. The resulting bacterial strain was grown in AB medium (18.7 mM NH4Cl, 2.5mM MgSO4, 2 mM KCl, 0.07 mM CaCl2, 2.7M FeSO4, 17.2 mM K2HPO4, 6.4 mM NaH2PO4, 0.2% glucose) supplemented with rifampicin 50 mg L^{-1} and kanamycin 50 mg L^{-1} overnight at 27°C. Agrobacterium was cultured to exponential phase (OD600= 1.5) and centrifuged at low speed (4,000×g). The pellet was resuspended in infiltration buffer (10 mM MES (Morpholino EthaneSulfonic acid) pH 5.5, 10 mM MgSO4 and 150 µM acetosyringone) to an OD600 of 0.5 and left at room temperature for three hours. The bacteria were introduced into the *Nicotiana tabacum* leaves by using needle-free syringe for leaves or vacuum infiltration for the whole plant at a cell density of *OD600*= 0.5 as described elsewhere (Habibi-Pirkoohi *et al*., 2014). Three days after vacuum infiltration, leaf tissue was harvested, and homogenized using a household blender. The extracts were clarified by centrifugation (78,000×g for 30 minutes) and plant-derived HA1 antigen was used for in vitro analysis.

Relative Gene Expression Using Quantitative Real-Time PCR Assay

Real Time PCR assay was performed to analyze gene expression at transcription level. In order to verify *HA1* gene expression and determine the relative transcript levels of genes, we harvested the infiltrated *N. tabacum* leaves and measured mRNA levels by qPCR analysis. Three samples of transfected plants were used for Real Time PCR. Total RNA was extracted from infiltrated leaf tissue using a commercially available High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The quality of RNA was determined by ethidium bromide-stained agarose gel electrophoresis and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Genomic DNA contamination was removed by DNase I digestion (Fermentas, Germany). First strand cDNA was synthesized via reverse transcription using oligo $(dT)_{20}$ primer (Hoffmann *et al*., 2001) with RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) as described by the manufacturer. The resulting cDNA mixtures were used as templates for Real Time PCR. Expression of the synthetic gene was quantitatively analyzed using a Real-Time

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

PCR system (Bio-Rad, USA). 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 HA1 F (5' TGGAGTTTCTTCTGCATGTCC 3') and HA1 R (5' GTTCTGCTGCATCATTTGGA 3') primers. One-step qPCR reactions were performed under the following conditions: 50ºC for 2 minutes, 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds, 60ºC for 1 minute and dissociation stage at 95ºC for 15 seconds, 60ºC for 1 minute, 95ºC for 15 seconds. Target gene amplifications were verified during the melting curve analysis step. Transcript levels of HA1 were expressed as relative values normalized to the transcript level of Actin F (5' TGAAGGTTACGCCCTTCCTC 3') and Actin R (5' TGCAACACAGCGAGCTTAACC 3'), also measured by qPCR and used as an internal references (Rotenberg *et al*., 2009). Homogeneity and specificity of amplified products were confirmed by melting curve analysis (data not shown). Based on Cycle threshold (Ct) values from qPCR analyses, the expression of genes evaluated by three technical replicates in two biological samples in the transcripts were quantified. Relative expression of the *HA1* gene in relation to reference gene were calculated using the REST 2009 software V. 2.0.13 (Qiagen, Hilden, Germany) (Pfaffl *et al*., 2002). Relative values were calculated using the gene with lowest expression as a reference.

Total Soluble Protein Extraction and Purification

The Total Soluble Protein (TSP) was extracted from the Agrobacterium-infiltrated *N. tabacum* leaves as well as from non-infiltrated control leaves in liquid nitrogen with 1.5 w/v of extraction buffer containing 20 mM NaH2PO4, pH 7.5, 0.5M NaCl, 10% (v/v) glycerol and 0.5% PolyVinylPolyPyrrolidone (PVPP). The Total Soluble Protein (TSP) concentration in extracts was determined by using the Bradford protein assay using Bovine Serum Albumin (BSA) as standard in spectrophotometer (ND-1000, NanodropH). Extracts of TSP containing the HA1 expressed with a 6His tag at the C-terminal were purified using Immobilized Metal ion Adsorption Chromatography (IMAC) by applying the plant extract on QIAexpress® Ni-NTA (QIAGEN) charged with Ni2+. The concentration of purified HA1 in eluted fractions was quantified as % of TSP and mg kg^{-1} fresh weight of leaves using ELISA.

SDS-PAGE and Immunoassay Tests

Western blotting, protein dot blot, and ELISA analysis were used to determine expression efficiency of HA1. Sample proteins were resolved on reducing 12% SDS-PAGE and then visualized after Coomassie Brilliant Blue staining. For further characterization, the separated proteins on SDS-PAGE were transferred to nitrocellulose membrane by electroblotting (Bio-Rad, USA) for 1 hour room temperature at 12 V. The membrane was then blocked with blocking buffer [BSA in PBS] for 90 minutes at room temperature under agitating. The membrane was washed three times with washing buffer [0.5% (v/v) Tween 20, 1X TBS], each for 10 minutes at room temperature. The membrane was probed with the conjugated anti-6x His tag® mouse monoclonal antibody (Sigma-Aldrich) for overnight at 4ºC and washed again three times with washing buffer. Finally, the protein bounds were visualized by staining the membrane with DAB (Di-aminobenzidine) substrate. Detection of HA1 in protein extracts from the leaves of transfected tobacco was also analyzed by western blot using mouse monoclonal anti-*influenza A virus* (H5N1/HA1) [2B7] (ab135382) as a primary antibody at 1/3,000

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

dilution and the HRP-conjugated rabbit anti-mouse IgG as a secondary antibody.

Production of recombinant HA1 protein in transfected leaves was evaluated by standard protein dot blot assay. Briefly, 2 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 in PBS as blocking solution for 1 h. After incubation, the membrane was incubated with the conjugated anti-6x His tag® mouse monoclonal antibody (1:1,000 dilutions) for 1 hour at 37ºC. The membrane was washed three times with PBST/PBS and incubated with DAB (Di-AminoBenzidine) substrate. A small volume of bacterial HA1 antigen (about 2 µL) was used as positive control and the same volume of protein obtained from wild type plant was used as negative control.

Expression of the foreign gene was further evaluated by ELISA (Enzyme-Linked Immunosorbent Assay). ELISA plate was coated with total soluble proteins from the wild type and the transfected plants and bacterial HA1 antigen at 37°C for one hour; followed by incubation with 1% Bovine Serum Albumin (BSA) in PBS for 2 hours at 37°C to prevent non-specific binding. The wells were washed by PBST/PBS and incubated with conjugated anti-6x His tag® mouse monoclonal antibody (1:1,000 dilutions). Wells were developed with OPD (Ortho-PhenyleneDiamine) substrate for 10 minutes at 37 °C until the color became yellow; the color reaction was stopped by 2N H2SO4 and read at 405 nm of wavelength.

RESULTS

Transient Expression, Purification and Quantification of Recombinant Hemagglutinin

After agro-infiltration of *N. tabacum* leaves with the plasmid, containing codon optimized, artificially synthesized gene (HA1) encoding 325 amino acid protein, expression pattern of HA1 was evaluated at transcription level using Real Time PCR (qPCR). Results showed that the foreign gene was transcribed in infiltrated leaves (Figure 2). Amplification efficiencies of HA1 and the reference gene (Actin) were found to be 83 and 91%, respectively, indicating relevant amplification and accurate quantification of recombinant transcripts in the qPCR analyses. Relative gene expression was determined and the

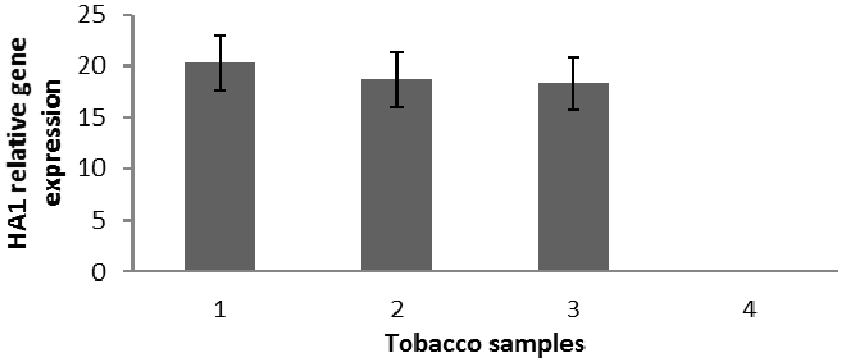

Figure 2. Quantitative measurement of *HA1* gene transcription in transfected leaves of tobacco via Real Time PCR. (1, 2 and 3): Three samples of transfected plants, (4): Negative control (non-transfected plant). The *Y*-axis is the samples of tobacco plants.

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

results showed that the relative HA1 expression level was 17.9 to 29.2 (Figure 2). Transcripts of HA1 were not detected in the negative control (untransfected plant). Purified plant-produced HA1 protein by IMAC using Ni2+, at a yield of 160 mg kg^{-1} fresh weight of leaves, was eluted from the affinity column, and expression level was estimated to be 8.8% of TSP using ELISA. The purification of HA1 protein was resolved on 12% SDS-PAGE gel and then stained with Coomassie Briliant Blue (Figure 3-a). SDS-page analysis of the recombinant proteins showed a clear band with 45 KD weight, but this band was not shown in non-transfected plant. The weight of this protein was equal to positive control HA1 protein weight (Figure 3-a). A protein with estimated 45 KD molecular weight was detected in both western blot analysis using conjugated anti-6x His tag® mouse monoclonal antibody and specific mouse monoclonal anti-*influenza A virus* (H5N1/HA1) [2B7] (ab135382) antibody. No protein band was observed in protein samples of non-transfected plants (Figures 3-b and -c). Dot blot results confirmed expression of the foreign gene at translation level, whereas no signal was observed for wild type plants (Figure 4). Expression of the recombinant protein was further quantitatively measured by ELISA (Figure 5). Production of the recombinant protein was quite high in transfected leaves. In contrast, no strong signal was observed for non-transfected plants. ELISA analysis of transiently

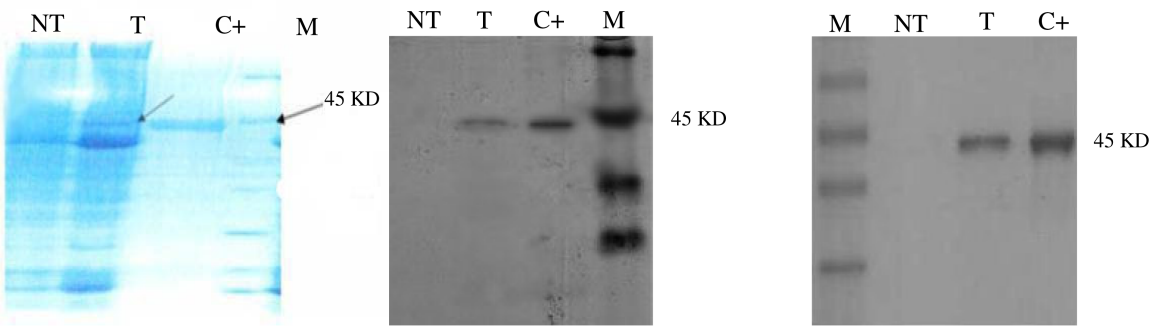

Figure 3. (A) SDS-PAGE analysis on transfected and non-transfected plants. Lane C+: Positive control (HA1); (B) Detection of HA1 in protein extracts from the leaves of transfected tobacco by western blot analysis using conjugated anti-6x His tag® mouse monoclonal antibody (Sigma-Aldrich). M: Protein marker (Fermentas), C+: HA1, NT: Non-Transfected plant, T: Transfected plant. (C) Detection of HA1 in protein extracts from the leaves of transfected tobacco by western blot analysis using anti-*influenza A virus* (H5N1/HA1) antibody [2B7] (ab135382) mouse monoclonal antibody [2B7] to *influenza A virus* (H5N1/HA1) as a primary antibody and the HRP-conjugated rabbit anti-mouse IgG antibody as a secondary antibody.

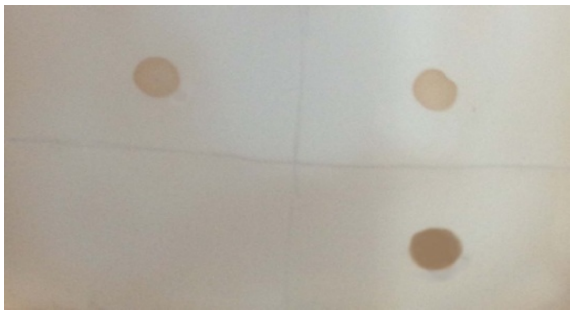

Figure 4. Protein dot blot for detection of recombinant protein in transfected leaves of tobacco. (1): Positive control; (2, 3): Protein sample of transfected plants, and (4): Protein sample of non-transfected plant.

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

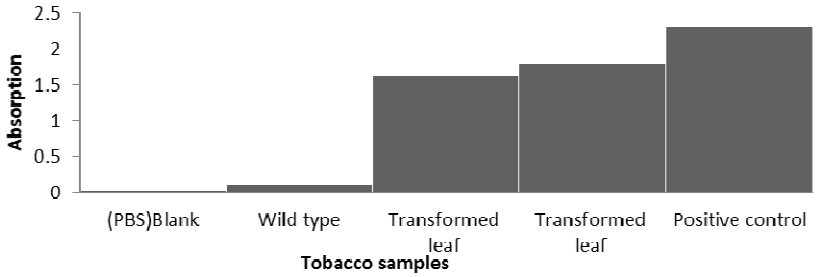

Figure 5. Quantification of recombinant HA1 expression in transfected plants by ELISA.

expressed HA1 from the TSP of infiltrated leaves and purified protein confirmed the expression of HA1 that was specifically recognized by mouse anti-His antibody.

DISCUSSION

The objective of our study was to explore the transient expression of HA1 antigen from A/Indonesia/05/05 strain of *influenza virus* (AFM78567.1), in *N. tabacum* amenable for the development of plant-produced vaccines. In recent decades, a large number of studies have been initiated to investigate the possibility to express recombinant vaccine antigens in plants as these antigens are considered as safe, low cost, easy to produce, rapid to upscale, and less vulnerable to contamination with animal pathogens compared with traditional inactivated or live attenuated egg-based vaccines. For example, immunogenicity associated to administration of H5 antigens produced in plants has been reported (Kalthoff *et al*., 2010; Shoji *et al*., 2009; Shoji *et al*., 2011; Madhun *et al*., 2011).

In this study, the potential of using *N. tabacum* for production of *influenza A virus HA1* surface antigen which is the major subunit of HA glycoprotein involved in attachment of the *influenza virus* to sialic acid-containing host cell receptors (Suzuki, 2005), was investigated. To achieve high expression levels of HA1 in *N. tabacum*, multiple criteria were considered in the construction of plant expression vector. Previous studies suggested that incorporating Kozak sequence in the upstream of the start codon can significantly increase the efficiency of translation in eukaryotic cells (Kozak, 1989; Kozak, 2007). Therefore, a Kozak (GCAACA) plant translation initiation sequence was included in front of the start codon of HA1 to ensure high expression levels. In addition, it has been reported that HA1 is an N-linked glycoprotein which is directed into the secretory pathway and retained in the ER for post-translational modifications (Kalthoff *et al*., 2010; Musiychuk *et al*., 2006). Furthermore, several researchers included an ER retention signal, SEKDEL in the C-terminal as it is expected to sequester the protein in the ER to fold correctly, as well as to increase protein stability and preventing the entry of protein into the Golgi apparatus, the site for plant specific glycosylation (Mett *et al*., 2008; Shoji *et al*., 2008), and C-terminal 6His tag to facilitate purification (Mett *et al*., 2008). Also, previous reports suggested that the synthetic codon adapted gene to tobacco with optimized GC content while removing sequence repeats, cryptic splice sites and RNA destabilizing sequence elements expression in tobacco resulted in

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

the high level accumulation of recombinant functional proteins (Holmberg *et al*., 2001; Perlak *et al*., 1991). Therefore, we synthesized HA1 artificially following the above mentioned parameters to achieve the high level expression in *N. tabacum*. Previously, it has been shown that transient Agrobacterium-mediated transformation of HA1 produces vaccine antigens in tobacco (Kalthoff *et al*., 2010; Musiychuk *et al*., 2006). Here, CaMV35S promoter, Kozak sequence, ER signal peptide were used and codons were optimized to enhance gene expression, similar to previous reports (Sala *et al*., 2000; Streatfield *et al*., 2001; Kang *et al*., 2004; Gil *et al*., 2001; Fischer *et al*., 1999). 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 HA1 antigen requires further investigation.

The relative expression of HA1 in the infiltrated leaves was measured by qPCR assays. The highest gene expression of recombinant plant-produced HA1, as measured by quantitative real-time PCR, was detected 29.2 at 3 days post infiltration (dpi). Kanagarajan *et al.* (2012) also transiently expressed the Hemagglutinin Antigen from Low Pathogenic Avian Influenza A (H7N7) in *N. benthamiana*. The highest relative expression of HA in the infiltrated leaves of *N. benthamiana* was 26.5, which is comparable to our results. As confirmed by Real Time PCR assay, transient expression level of the HA1 transgene was fairly high, similar to earlier reports (Leckie and Stewart, 2011; Wroblewski *et al.,* 2005; Janssen and Gardner, 1989). Recent studies of *N. tabacum*-produced purified HA vaccine antigens reported production level of 20 to 200 mg kg^{-1} FW (Mett *et al.,* 2008; Kalthoff *et al.,* 2010). Shoji *et al.* (2008) transiently expressed HA from H3N2 (A/Wyoming/03/03) which was targeted to the Endoplasmic Reticulum (ER), in *Nicotiana benthamiana*. The HA product yielded ~200 mg kg^{-1} Fresh leaf Weight (FW). Mett *et al.* (2008) also transiently expressed the A/Wyoming/03/03 strain HA (stem domain and globular domain) and Neuraminidase (NA) proteins fused to the enzyme lichenase (LicKM) in *N. benthamiana*. The antigens were also ER-targeted and yielded 100 mg HA kg^{-1} FW or 400 mg NA kg^{-1} FW. Shoji *et al.* (2009) expressed a truncated version of plant codon-optimized HA from H5N1 (A/Indonesia/05/05) in *N. benthamiana* plants. This HA protein lacked the transmembrane domain and native signal peptide, and accumulated in the ER at a level of approximately 60 mg kg^{-1} FW. Daoust *et al.* (2010) expressed HA from the A/Indonesia/5/05 (H5N1) and the A/New Caledonia/20/99 (H1N1) strains by means of agro-infiltration in *N. benthamiana*. The HA was successfully expressed and the yield of HA was about 50 mg kg^{-1} FW. All of the above studies confirmed that influenza HA can be expressed to high levels in plants. In our study, transiently expressed purified HA1 fusion protein attained expression levels of 160 mg kg^{-1} FW, which are approximately 8.8% of TSP in *N. tabacum*. These results indicate that HA1 expressed in *N. tabacum* are modified correctly in a way similar to HA1 proteins produced in bird's cells during virus infection. Also, these observations confirm that HA1 enters into ER secretory pathway of the plant and that it is extensively expressed in subcellular organs.

As can be seen from western blot using anti-His tag monoclonal antibody and mouse monoclonal anti-*influenza A virus* (H5N1/HA1) antibody, the HA1 molecular weight was 45 KD, which demonstrated that it was equal to positive control HA1 protein weight. In western and dot blot assay, the protein sample obtained from transfected leaves generated a strong signal comparable to that of positive control (HA1); whereas protein of wild type plant was not

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

detectable. Enhanced expression of HA1 in tobacco leaves was more obvious in ELISA assay which quantitatively measured expression of the recombinant vaccine. Slight absorbance observed in wild type plant was probably due to unwanted cross reactions between protein samples and specific antibodies. In the present study, the ELISA results demonstrated that HA1 expressed in *N. tabacum* was correctly translated, folded and fully functional. To our knowledge, this is one of the more promising approaches, examining the possibility of generating viral vaccine antigens from H5N1 in a plant-based expression system. Obtained results, including high level expression of biologically active *influenza A virus HA1* antigen, suggest that plants may be an important complement to traditional vaccine production methods, and thereby making vaccine more accessible worldwide.

ACKNOWLEDGEMENTS

The authors would like to thank staff of Biotechnology and Plant Breeding Department, Faculty of Agriculture, Ferdowsi University of Mashhad.

REFERENCES

1. Aziz, M. A., Singh, S., Anand, K. and Bhatnagar, R. 2002. Expression of Protective Antigen in Transgenic Plants: A Step towards Edible Vaccine against Anthrax. *Biochem. Biophys. Res. Commun*., **299**: 345-351.
2. Bardiya, N. and Bae, J. H. 2005. Influenza Vaccines: Recent Advances in Production Technologies. *Appl. Microbiol. Biotechnol*., **67:** 299–305.
3. Bendahmane, A., Querci, M., Kanyuka, K. and Baulcombe, D. C. 2000. Agrobacterium Transient Expression System as a Tool for the Isolation of Disease Resistance Genes: Application to the Rx2 Locus in Potato. *Plant J*., **21**: 73-81.
4. Bhaskar, P. B., Venkateshwaran, M., Wu, L., Ane, J. M. and Jiang, J. 2009. Agrobacterium-mediated Transient Gene Expression and Silencing: A Rapid Tool for Functional Gene Assay in Potato. *PLoS ONE*, **4**: 581-592.
5. Caton, A. J., Brownlee, G. G., Yewdell, J. W., and Gerhard, W. 1982. The Antigenic Structure of the *Influenza Virus A/PR/8/34* Hemagglutinin (H1 Subtype). *Cell*, **31**: 417–427.
6. Daniell, H., Lee, S. B., Panchai, T. and Wiebe, P. O. 2011. Expression of the Native Cholera Toxin B Subunit Gene and Assembly as Functional Oligomers in Transgenic Tobacco Chloroplasts. *J. Molec. Biol*., **31**: 1001-1009.
7. Daoust, M. A., Couture, M. M. J., Charland, N., Trepanier, S., Landry, N. and Ors, F. 2010. The Production of Hemagglutinin-based Virus-like Particles in Plants: A Rapid, Efficient and Safe Response to Pandemic Influenza. *Plant Biotechnol. J*., **8**: 607–619.
8. Fischer, R., Vaquero, C., Sack, M., Drossard, J., Emans, N. and Commandeur, U. 1999. Toward Molecular Farming in the Future: Transient Protein Expression in Plants. *Biotechnol. Appl. Biochem*., **30**: 113-116.
9. Floss, D. M., Falkenburg, D. and Conrad, U. 2007. Production of Vaccines and Therapeutic Antibodies for Veterinary Applications in Transgenic Plants: An Overview. *Transgenic Res*., **16**: 315-332.
10. Gil, F., Brun, A., Wigdorovitz, A., Catala, R., Martinez-Torrecuadrada, J. L. and Casal, I. 2001. Highyield Expression of a Viral Peptide Vaccine in Transgenic Plants. *FEBS. Lett*., **488**: 13-17.
11. Habibi-Pirkoohi, M., Malekzadeh-Shafaroudi, S., Marashi, H., Moshtaghi, N., Nassiri, M. R. and Zibaee, S. 2014. Transient Expression of Foot and Mouth Disease Virus (FMDV) Coat Protein in Tobacco (*Nicotiana tabacom*) *via* Agroinfiltration. *Iran. J. Biotech*., **12**: 28-35.
12. Haq, T. A., Mason, H. S., Clements, J. D. and Arntzen, C. J. 1995. Oral Immunization with a Recombinant Bacterial Antigen Produced in Transgenic Plants. *Sci.*, **268**: 714-716.
13. Hashemi, H., Jourabchi, E. and Khodabandeh, A. 2005. Transient Expression of Human Growth Hormone in Potato (*Solanum tuberosum*), Tobacco (*Nicotiana tobacum*) and Lettuce (*Lactuca*

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

sativa) Leaves by Agroinfiltration. *I. J. Biotechnol*., **3:** 109-113.
14. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. and Perez, D. R. 2001. Universal Primer Set for the Full-length Amplification of All *Influenza A Viruses*. *Arch. Virol*., **146**: 2275–2289.
15. Holmberg, N., Farres, J., Bailey, J. E. and Kallio, P. T. 2001. Targeted Expression of a Synthetic Codon Optimized gene, Encoding the Spruce Budworm Antifreeze Protein, Leads to Accumulation of Antifreeze Activity in the Apoplasts of Transgenic Tobacco. *Gene*, **275**: 115–124.
16. Janssen, B. and Gardner, R. 1989. Localized Transient Expression of GUS in Leaf Discs Following Cocultivation with Agrobacterium. *Plant Mol. Biol*., **14**: 61-72.
17. Kalthoff, D., Giritch, A., Geisler, K., Bettmann, U. and Klimyuk, V. 2010. Immunization with Plant-expressed Hemagglutinin Protects Chickens from Lethal Highly Pathogenic *Avian Influenza Virus H5N1* Challenge Infection. *J. Virol*., **84:** 12002–12010.
18. Kanagarajan, S., Tolf, C., Lundgren, A., Waldenstrom, J. and Brodelius, P. E. 2012. Transient Expression of Hemagglutinin Antigen from Low Pathogenic *Avian Influenza A* (H7N7) in *Nicotiana benthamiana. PLoS ONE*, **7(3):** e33010.
19. Kang, T. J., Han, S. C., Jang, M. O., Kang, K. H., Jang, Y. S. and Yang, M. S. 2004. Enhanced Expression of B-Subunit of *Escherichia coli* Heat-labile Enterotoxin in Tobacco by Optimization of Coding Sequence. *Appl. Biochem. Biotechnol.,* **117**: 175-187.
20. Kilbourne, E. D., Johansson, B. E. and Grajower, B. 1990. Independent and Disparate Evolution in Nature of *Influenza A Virus* Hemagglutinin and Neuraminidase. *Proc. Natl. Acad. Sci. USA,* **87:** 786-790.
21. Koprowski, H. and Yusibov, V. 2001. The Green Revolution: Plants as Heterologous Expression Vectors. *Vaccine,* **19:** 2735-41.
22. Kozak, M. 1989. The Scanning Model for Translation: An Update. *J. Cell. Biol*., **108**: 229–241.
23. Kozak, M. 2007. The Scanning Model for Translation: An Update. *J. Cell Biol*., **108**: 229-241.
24. Leckie, B. M. and Stewart, C. N. 2011. Agroinfiltration as a Technique for Rapid Assays for Evaluating Candidate Insect Resistance Transgenes in Plants. *Plant Cell. Rep*., **30**: 325-334.
25. Madhun, A. S., Haaheim, L. R., Nostbakken, J. K., Ebensen, T. and Chichester, J. 2011. Intranasal c-di-GMP-Adjuvanted Plant-derived H5 Influenza Vaccine Induces Multifunctional Th1 CD4(+) Cells and Strong Mucosal and Systemic Antibody Responses in Mice. *Vaccine*, **29**: 4973–4982.
26. Mett, V., Musiychuk, K., Bi, H., Farrance, C. E. and Horsey, A. 2008. A Plantproduced Influenza Subunit Vaccine Protects Ferrets against Virus Challenge. *Influenza Other Resp*., **2:** 33–40.
27. Monto, A. S. 2006. Vaccines and Antiviral Drugs in Pandemic Preparedness. *Emerg. Infect. Dis.,* **12:** 55-61.
28. Musiychuk, K., Stephenson, N., Bi, H., Farrance, C. E. and Orozovic, G. 2006. A Launch Vector for the Production of vVaccine Antigens in Plants. *Influenza Other Resp*., **1**: 19–25.
29. Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L. and Fischhoff, D. A. 1991. Modification of the Coding Sequence Enhances Plant Expression of Insect Control Protein Genes. *Proc. Natl. Acad. Sci. USA,* **88**: 3324–3328.
30. Pfaffl, M. W., Horgan, G. W. and Dempfle, L. 2002. Relative Expression Software Tool (REST (c)) for Group-wise Comparison and Statistical Analysis of Relative Expression Results in Real-time PCR. *Nucleic. Acids Res.,* **30**: e36.
31. Pourseyedi, S., Hashemi, H., Omidi, M., Ali-Ghoreshi, S., ShahNejat-Boushehri, A. A. and Jourabchi, E. 2009. Transient Expression of *VP2* Gene of Very Virulent IBDV in Tobacco, Alfalfa and Lettuce Leaves by Agroinfiltration. *Veterin. J. (Pajouhesh and Sazandegi)*., **83**: 18-25.
32. Rotenberg, D., Thompson, T. S., German, T. L. and Willis, D. K. 2009. Methods for Effective Real-Time RT-PCR Analysis of Virus-induced Gene Silencing. *J. Virol. Methods,* **138**: 49–59.
33. Sala, F., Rigano, M., Barbante, A., Basso, B., Walmsley, A. M. and Castiglione, S. 2000. Vaccine Antigen Production in Transgenic Plants: Strategies, Gene Constructs and Perspectives. *Vaccine,* **21**: 803-808.
34. Schillberg, S., Twyman, R. M. and Fischer, R. 2005. Opportunities for Recombinant

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

Antigen and Antibody Expression in Transgenic Plants: Technology Assessment. *Vaccine*, **23**: 1764-1769.
35. Schweizer, P., Christoffel, A. and Dudler, R. 1999. Transient Expression of Members of the *Germin-like* Gene Family in Epidermal Cells of Wheat Confers Disease Resistance. *Plant J*., **20**: 541-552.
36. Sharma, M. K., Singh, N. K., Jani, D., Sisodia, R. and Thungapathra, M. 2008. Expression of Toxin Co-regulated Pilus Subunit A (TCPA) of *Vibrio cholerae* and Its Immunogenic Epitopes Fused to Cholera Toxin B Subunit in Transgenic Tomato (*Solanum lycopersicum*). *Plant Cell. Rep.,* **27**: 307–318.
37. Sheen, J. 2011. Signal Transduction in Maize and Arabidopsis Mesophyll Protoplasts. *Plant Physiol*., **127**: 1466-1475.
38. Shoji, Y., Chichester, J. A., Bi, H., Musiychuk, K. and dela-Rosa, P. 2008. Plant Expressed HA as a Seasonal Influenza Vaccine Candidate. *Vaccine*, **26**: 2930–2934.
39. Shoji, Y., Bi, H., Musiychuk, K., Rhee, A. and Horsey, A. 2009. Plant-derived Hemagglutinin Protects Ferrets against Challenge Infection with the A/Indonesia/05/05 Strain of Avian Influenza. *Vaccine,* **27:** 1087–1092.
40. Shoji, Y., Chichester, J. A., Jones, M., Manceva, S. D. and Damon, E. 2011. Plantbased Rapid Production of Recombinant Subunit Hemagglutinin Vaccine Targeting H1N1 and H5N1 Influenza. *Hum. Vaccine.,* **7:** 41–50.
41. Simmons, C. W., VanderGheynst, J. S. and Upadhyaya, S. K. 2009. A model of Agrobacterium Tumefaciens Vacuum Infiltration into Harvested Leaf Tissue and Subsequent in Planta Transgene Transient Expression. *Biotechnol. Bioeng*., **102**: 965-970.
42. Skehel, J. J. and Wiley, D. C. 2000. Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin. *Annu. Rev. Biochem.,* **69:** 531–569.
43. Streatfield, S. J., Jilka, J. M., Hood, E. E., Turner, D. D., Bailey, M. R. and Mayor, J. M. 2001. Plant-based Vaccines: Unique Advantages. *Vaccine,* **19**: 2742-2748.
44. Streatfield, S. J. 2005. Plant-based Vaccines for Animal Health. *Rev. Sci. Tech. Off. Int. Epiz.,* **24**: 189-199.
45. Suzuki, Y. 2005. Sialobiology of Influenza Molecular Mechanism of Host Range Variation of *Influenza Viruses*. *Biol. Pharm. Bull*., **28**: 399–408.
46. Tonegawa, K., Nobusawa, E., Nakajima, K., Kato, T., Kutsuna, T. and Kuroda, K. 2003. Analysis of Epitope Recognition of Antibodies Induced by DNA Immunization against Hemagglutinin Protein of *Influenza A Virus*. *Vaccine,* **21**: 3118–25.
47. Webster, R. G., Peiris, M., Chen, H. and Guan, Y. 2006. H5N1 Outbreaks and Enzootic Influenza. *Emerg. Infect. Dis.,* **12**: 3-8. (www.cdc.gov/eid)
48. Wroblewski, T., Tomczak, A. and Michelmore, R. 2005. Optimization of Agrobacterium Mediated Transient Assays of Gene Expression in Lettuce, Tomato and Arabidopsis. *Plant Biotech. J*., **3:** 259-273.
49. Zheng, L., Liu, G., Meng, X., Li, Y. and Wang, Y. 2012. A Versatile Agrobacterium-mediated Transient Gene Expression System for *Herbaceous* Plants and Trees. *Biochem. Genet*., **50**: 761-769.
50. Zottini, M., Barizza, E., Costa, A., Formentin, E., Ruberti, C. and Carimi, F. 2008. Agroinfiltration of Grapevine Leaves for Fast Transient Assays of Gene Expression and for Long-term Production of Stable Transfected Cells. *Plant Cell Rep*., **27**: 845-853.

[DOR: 20.1001.1.16807073.2017.19.2.2.9]

بیان موقت آنتی ژن HA1 ویروس آنفلوانزای H5N1 در گیاه توتون (*Nicotiana tabacum*) از طریق اگروایانفیلتراسیون

ا. س. فرساد، س. ملک زاده شفارودی، ن. مشتاقی، ف. فتوحی، س. زیبایی

چکیده

ویروس آنفلوانزای A، یک خطر جهانی برای صنعت ماکیان محسوب می شود، بخصوص تحت تیپ H5 که توانایی بیماری زایی شدید در ماکیان و انسان را دارد. اخیرا سیستم های بیان گیاهی حامیانی را به عنوان جایگزین مناسبی جهت بیان آنتی ژن های واکسن ها کسب کرده اند. هدف این تحقیق بررسی امکان بیان پروتئین HA1 در گیاه توتون (*N. tabacum*) از طریق اگروایانفیلتراسیون بود. در این مطالعه، زیر واحد بزرگ هماگلوتینین (HA1) ویروس آنفلوانزای شدیدا بیماری زای پرندگان (H5N1)، سنتز شد و بطور موقت در گیاه توتون بیان گردید. به منظور بررسی بیان HA1 در توتون، یک قطعه cDNA کد کننده ژن HA1 همراه با توالی های کداک، تگ هیستیدین (6His) و سیگنال پپتید KDEL طراحی و سنتز گردید. سازه مورد نظر در وکتور pBI121 کلون شد و پلاسمید حاصل به درون گیاهان توتون با روش اگروایانفیلتراسیون وارد گردید. بیان نسبی پروتئین نوترکیب HA1 نیز توسط Real Time PCR بررسی گردید. پروتئین HA1، ۳ روز پس از اگروایانفیلتراسیون استخراج شد و با استفاده از تگ هیستیدین تخلیص گردید. ایمنی زایی پروتئین نوترکیب HA1 توسط آنتی بادی ضد تگ هیستیدین در آزمون های دات بلات، وسترن بلات و الیزا مورد سنجش قرار گرفت. به منظور تایید شکل گیری صحیح ساختار آنتی ژن HA1، آزمون وسترن بلات با آنتی بادی اختصاصی ضد پروتئین HA1 نیز انجام گرفت. آنالیز Real Time PCR نشان داد که ژن HA1 در برگ های تراریخته رونویسی شده است. وزن مولکولی پروتئین توسط وسترن بلات، ۴۵ کیلودالتون تشخیص داده شد و در مقایسه با کنترل مثبت (HA1) هیچ تمایز وزنی نشان نداد. نتایج الیزا نشان داد که HA1 در برگ های تراریخته در سطح بالایی بیان شد بطوری که عملکرد پروتئین نوترکیب ۸/۸ درصد از پروتئین کل محلول گزارش گردید و عملکرد HA1 پس از تخلیص با ستون کروماتوگرافی هیس تگ، ۰/۱۶ گرم پروتئین خالص به ازای هر کیلوگرم وزن تر برگ بود. این مطالعه، اولین تحقیق در زمینه بررسی بیان موقت پروتئین HA1 در توتون است جایی که از یک توالی سنتتیک برای بیان این آنتی ژن استفاده گردید. در این پژوهش، کارایی تکنیک اگروایانفیلتراسیون در بیان آنتی ژن HA1 در توتون مورد بررسی قرار گرفت. کنیک اگروایانفیلتراسیون، پروسه بیان آنتی ژن های نوترکیب را در بافت های گیاهی تسریع می بخشد. از این رو، این نتایج فرصت بزرگی برای بررسی HA1 بیان شده بطور موقت در گیاه را به عنوان واکسن کاندید فراهم می کند.

[DOR: 20.1001.1.16807073.2017.19.2.2.9]