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

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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 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-
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.
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(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), agroinfiltration (Leckie and Stewart, 2011) and particle bombardment (Schweizer et al., 1999). Among the techniques, agroinfiltration 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 agroinfiltration (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; Musiyuchuk 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 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 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
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 acetylsyringone) 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...
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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' TGGAGTTTCTTCTGATGTCC 3') and HA1 R (5' GTTCTGCTGCATCATTTGGGA 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 was 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.
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 37°C. 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](image-url)

**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.
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 Brilliant 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](image-url)

**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](image-url)

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

elements expression in tobacco resulted in expressed HA1 from the TSP of infiltrated leaves and purified protein confirmed the expression ofHA1 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
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; Musiyuch 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
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.

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یبان موقت آنتیژن HA1 ویروس آنفلوآنزای H5N1 در Giaha توتون (N. tabacam) از طریق اگرواینترپاسیون

چکیده

هوش آنفلوآنزای A، یک خطر جهانی برای صنعت مکانی محصول می شود. به خصوص تحت تپی ه5 که توانایی باروری زایی شید در مکانی و انسان را دارد. اخیراً مسئولیت بیان گیاهی حاملانی را به عنوان جایگزین مناسب جهت بیان آنتیژن HA1 را به عنوان واکسن کسب کرده اند. هدف این تحقیق بررسی امکان بیان پروتئین HA1 در گیاه توتون (N. tabacam) وروش آنفلوآنزای شدید با باروری زایی پرندگان (H5N1) است. واکنش و بطور موثر در گیاه توتون بیان HA1 توتون، یک قطعه HA1 در کنده زن حشرات با تولید های کنکارگی، است. تغییرات HA1 موردنظر در کلن شد و با احتمال حاصل به درون گیاهان توتون با روش Real Time PCR هر توسط HA1 گردید. بیان نسبی پروتئین نورترکیب HA1 در روز پس از اگرواینترپاسیون استندریو شد و با استفاده از اگوئیدین های کتیلیز HA1 توسط آنتی بادی ضرب دیده هسته دند نشان دهنده در آزمون های دات بلات و سیستم زیستی سنجش قرار گرفته. به علت نتایج پایین، شکل تطبیقی می تواند ساختار آنتی جز Real آزمون وسیل باید آنتی بادی اختصاصی صورت گرفت. آنالیز HA1 نشان داد که زن در برگ های تاریخچه روتوپسی شده است. وسیل مولکولی پروتئین HA1 توسط وسیل پلات 3-45 کیلووات تشخص داده شد و در مقایسه با دسته قبلاً (HA1) چیه تاماز و تی نشان داد. نتایج الیا زناد داد که در برگ های تاریخچه ساخته در سطح بالایی باید بطوری که عملکردHA1 پروتئین نورترکیب 4/8 درصد از پروتئین کل محلول گزارش گردید و عملکرد HA1 پس از تخلیه با سطون کروماتوگرافی هیس تگ، 160 گرم پروتئین خالص به ازای هر کیلوگرم وزن گیاه بود. این مطالعه، اولین تحقیق در زمینه پروتئین بیان HA1 در توتون است. باید به نظر باید که از یک تولی استنبک عالی بیان این آنتیجن استفاده گردید. در این بحث، کارایی تکیه اگرواینترپاسیون در پیوند آنتی جز HA1 در توتون بوده بررسی قرار گرفت. کتیک اگرواینترپاسیون، پروپسی بیان آنتی جز HA1 نورترکیب را به دافع های گیاهی تسریع می یابد. از این روش، این نتایج فرضی برای بیان HA1 بیان شده بطور موثر در گیاه را به عنوان واکسن کاندید فراهم می کند.