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Transient expression of VPX gene of infectious bursal disease virus (IBDV) in tobacco plant (*Nicotiana tabacum cv. xanthi*)

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Abstract

Infectious bursal disease virus (IBDV) is a highly contagious, deadly and immunosuppressive disease in young chickens that has a negative impact on the economy of the poultry industry. It is appeared that the precursor VP2 protein (VPX) is the major host protective immunogen of infectious bursal disease virus (IBDV). Plants can be used to produce recombinant poultry vaccines economically.The VPX gene were cloned into a plant expression vector ; pCAMBIA1305.1 and introduced to *Agrobacterium tumefaciens* strain of LBA4404. Using vacuum agroinfiltration method the gene was transiently expressed and total protein leaves of tobacco was extracted and the presence of VPX proteins was detected using dotblot test. This investigation has been revealed that the expression of protein was higher in the wounded than unwounded leaves of whole plants. It was also, indicated that the rate of expression was higher at 6th day after vaccuminfiltration than 4th and 5th day post-infilteration and had showed a decline trend since 7th day. To our knowledge, this is the first report of VPX in plant expression system thus this research could make a field of producing vaccines for controlling IBDV in tobacco.

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Introduction

Infectious bursal disease (IBD), also called as a Gumboro disease, which is a highly contagious, acute and immunosuppressive disease in young chickens (Mcferran *et al*., 1980). This disease can rapidly develop B lymphocytes in the bursa of fabricius, which can lead to vaccination failures and susceptibility to other infections and diseases (Muller *et al*., 2003; Sharma *et al*., 2000). Pathogenic strains of IBDV have widely seen in Africa, Europe, Asia and South America. It is capable of causing mortality in 70% of the herd, thus it shows that the IBDV could cause important economic losses in the poultry industry worldwide. (Balamurugan and Kataria 2006, Van der Berg 2000 and, Muller *et al*., 2003). It has been documented that IBDV belongs to Avibirnavirus genus of the Birnaviridae family.

The genome of IBDV is bipartite double-strand RNAs, named segment A and B, which are packaged within a singleshell icosahedral capsid of 60 nm (Bruenn *et al*., 1991). Segment A encodes a polypeptide (approximately 115 kDa), which is proteolytically proceed to yield VPX, VP3 and VP4 proteins (Sanchez and Rodriguez, 1999). In the mature virions, VPX is preceded into VP2 through four VP4-target cleavage sites (Lejal *et al*., 2000). Segment B only can encode for VP1, the RNA dependent RNA polymerase of IBDV (Von *et al*., 2004). VPX as the precursor of VP2 protein is the major antigenic component that encodes at least two epitopes that are responsible for eliciting neutralizing antibody (Azad *et al*., 1987; Becht *et al*., 1988, Fahey *et al*., 1989)

A trend to control IBDV infection is currently achieved by vaccination with inactivated or attenuated live IBDV which does not complete its protection against IBDV infection. This approach also induces the risk of residual pathogenicity and the probability of recurrence of virulence after several cycles of cultivation (van den Berg, 2000; Muller *et al*., 2003). In recent years, researchers attempt to use the recombinant technology to generate vaccines for controlling IBD. So far, suitable expression VPX gene in insect cells (lee *et al*., 2004; Martinez – Torrecuadrada *et al*., 2000; Coston *et al*., 2001; Chevalier *et al*., 2002) and in prokaryotic systems (Hosseini *et al*., 2004) have been introduced for underlie a suitable response which can lead to other proteins of IBDV VPX as an alternative for recombinant vaccines against IBDV.

Plants are great resources that can be used to produce recombinant vaccines. Choosing plants for such purpose can have numerous advantages, such as low cost, high production of protein, stability, post-translational modification, safety against contamination of human and animal pathogens and scalability (Fischer *et al*., 2004; Wagner *et al*., 2004). Transient expression by Agrobacterium-mediated provides a rapid method for studying the functional characterization of genes and proteins than to the stable expression; transgenes can often be generated in a few days after of infiltration (Janssen and Gardner., 1989). So transient expression system can play an important role in the fast and sample study of recombinant proteins.

In the past reports VP2 gene had been expressed in several plant expression systems such as Arabidopsis thaliana (Wu *et al*., 2004) and rice (Wu *et al*., 2007), Tobacco(chen *et al*., 2012; porseidi *et al*., 2008), Alfalfa and Lettuce leaves(porseidi *et al*., 2008) but not for VPX . Given the importance of protein VPX for vaccinating poultry and also benefits of plant expression systems, in the present study VPX antigen gene was cloned in expression vector pCAMBIA1305.1 then *vacuum agro*infiltration *method were used to transform the gene into tobacco plant* and its expression was then assessed in the tobacco plant.

Materials and methods

Plant Materials

Seeds of tobacco (*Nicotiana tabacum cv. xanthi*) were obtained from Agricultural Biotechnology Laboratory of Tarbiat Modarres University, Tehran, Iran. In this study the candidate gene was transferred into the individual plant as well into young leaves, their performances then were compared for the expression

levels via Agrobacterium in vacuum infiltration system.

Bacteria

Escherichia coli strain *DH5α* and *Agrobacterium tumefaciens* strain *LBA4404* were used for cloning, proliferating the construct and transformation of the VPX gene into tobacco plant.

Primers

The primers, forward and reverse, were designed based on the VPX sequence. Specific primers containing *NheI* and *AflII* restriction sites, Kozak sequence- and His tag sequence was utilized to detect expression, purification and both sides of the gene.

The nucleotide sequences of primers were as following: Forward primer

5'ACACTACA GCTAGCACAATGACGAACCTGCAAGATCAAACC3′

reverse primer

5'ACATACTTAAGAAACTTTATTGCCAAATGTTTGAA CGTTAGTGATGGTGATGGTGATG ATAATCGTACC3'

Vector

Plant expression vector pCAMBIA13051 was used for this particular study. Such vector was able to carry kanamycin resistance gene for selection of recombinant strain of bacteria and a hygromycin resistance gene for selection in transgenic plants. The vector also carries catalase intron sequences to enhance expression of gene of interest. The CaMV35s (Cauliflower Mosaic Virus) promoter and NOS (Nopaline Synthase terminator) terminator can induce high levels of transcription, *AflII* and *SpeI* restriction sites can be used for cloning.

Cloning of the VPX gene in pCAMBIA1305.1

The mentioned primers at the above were used to amplify VPX gene and then, PCR product along with Bioneer Kit were applied for purification. The purified VPX gene and the pCAMBIA1305.1 vector were digested with , *AflII*, *NheI* and *SpeI* (BioLabs).

The pCAMBIA1305.1 vector was removed from the digested material using a gel extraction kit and the purified digested VPX gene were ligated using T4 DNA ligase (Takara) at 16 °C overnight and the final product then were transformed into *E. coli*. Eventually, the VPX gene was inserted into a GUSPlus gene region of pCAMBIA1305.1 between the catalase intron and NOS terminator by ligation process.

Transformation of Agrobacterium tumefaciens with the recombinant plasmid using freezing and thawing method

Agrobacterium tumefaciens strain *LBA4404* was grown overnight at 28°C in liquid LB medium containing 80 mg/L streptomycin (OD600 = 0.8-1). Agrobacterium culture was spun down at 3500 rpm for 10 min and resuspended in the 100µl of 20mM CaCl2. 5µl of vectors were added to suspension and mixed culture was submerged in liquid nitrogen and then in bainmarie for 5min at 37°C. 900 µl of LB was added to the culture and incubated for 3-4 h at the 28°C on the shaker. 100 µL of transformed cells were cultured on LB plates containing 50 µg/mL kanamycin and 80 mg/L streptomycin. Plates were incubated for 2 days at 28°C.

Plant transformation

Single colonies of *A. tumefaciens* which carrying the recombinant vector were grown in LB medium supplemented with 50 mg/L kanamycin and with 80 mg/L streptomycin (OD600 = 0.8) overnight at 28 °C. The cells were then harvested via centrifugation for 20 min at 4C at 3000 rpm and resuspended in MS induction liquid medium (4.4 g/L MS salts, %5 (w/v) Glucose, pH 5.5) containing 200 µM acetosyringone at 28C at 150rpm for 1-2 hours. Three Petri dishes containing leaf (The leaves were cut into 4-5 pieces) and suspension *Agrobacterium tumefaciens* were placed in a vacuum desiccator. Vacuum infiltration was performed using a vacuum pump under 300 mm Hg vacuum for 30 min. The leaves were placed on filter paper inside the plate and incubated in the dark for 48 hr in 25°C and then incubated at 25°C with a 16/8 (day/night) h photoperiod for specified duration The same applies to the transfer of the whole plant.

Protein extraction and immune blot analysis

Total soluble proteins were extracted from whole leaves according to guy (1992) method and dot blot technique was set to analyze the proteins. All the solution preparation and the methods were performed as described by Roche Company and Anti-His6Peroxidase was provided by the same company. Ten nanograms of protein samples were dotted on the nitrocellulose membranes and blocked with western blocking solution 1X for 1 hour at 25°C.Then the solution was washed out and replaced with Anti-His6Peroxidase and put into incubator for 90 min at 25°C.After incubation the solution was removed from the nitrocellulose membranes then washed it with TBST1x, washing was repeated three times for 5 min each time. Finally the membrane was incubated with the precipitating substrate solution of BM Blue POD substrate, precipitating (Roche) for 10 min.

Results

The VPX gene was amplified via PCR technique using specific primers.1554bp-long fragment of this PCR product was subsequently cloned into the CaMV35S in upstream and Nos terminator in downstream between SpeI and AflII restriction sites. Then the recombinant pCAMBIA1305.1+VPX plasmid was transformed into *E. coli* strain DH5α. The presence of the VPX gene was detected and approved by colony PCR (Fig.1) and digestion reaction was performed by *SpeI* and *AflII* restriction enzymes (Fig. 2). Finally, the construct was transformed into Agrobacterium tumefaciens strain LBA4404 and applying colony PCR technique to illustrate that those recombinant plasmid was transformed into the Agrobactrium correctly (Fig. 3).

Immunodot blot analysis

The results of dot blot technique was approved the existence of VPX protein in inoculated plants. The findings also showed that protein expression was higher in the wounded leaves than intact leaves in whole plants. This result revealed information that wounding may prerequisite for the increases of gene expression after Agrobacterium-transient expression. The comparison of time between intact and wounded leaves at the vacuum infiltration has been revealed that the amount of expression was higher in 6 days after vaccum infiltration than 4 and 5 days post-infiltration. Also result had showed a decline trend at 7th day (Fig.4).

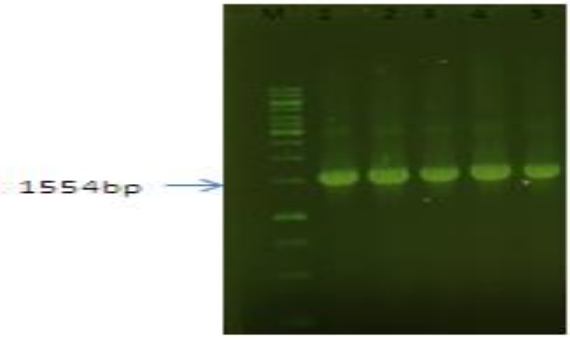

Fig. 1. Proof of the presence of VPX gene in pCAMBIA1305.1 by colony PCR technique.M: 1 kb DNA Ladder (Geneon), Lane 1: positive control, Lanes 2-5: Randomly selected colonies.

Discussion

Infectious bursal disease virus (IBDV) is the major threat due to its significant of negative impact on the poultry industry (Lemon *et al.*, 2000). It has been reported that the vaccination is essential way to control such infectious diseases. A great challenge that poultry industry is facing with, is a need to assemble method for production of vaccine that to be effective, unexpensive; stable and potentially capable of eradicating this pernicious disease. In recent years, various studies have demonstrated that the application of DNA vaccination can be a new signal to induce protective immunity against IBDV (Fordor *et al.*, 1999; Chang *et al.*, 2001; Heckert *et al.*, 2002).

Plant acts as a bioreactor in which can be utilized effectively for production of recombinant protein. As compared to other sources the plant has several merits including; large–scale and low-cost production potential, eukaryotic post-translational modifications, safety and stability (Schillberg *et al.*, 2002; Rajabi-Memari *et al.*, 2006 ;Rajabi-Memari *et al.*, 2010). Plant is a unique candidate by which both transient and stable expressions can be accomplished for recombinant proteins. So far, different plants were used to recombinant proteins productions (Ahangarzadeh *et al.*, 2012; Soltanmohammadi *et al.*,

2014). In plant the choice of transient expression over stable expression for recombinant proteins simply precede better because transient expression is facilitated easy, fast and cheap, and also expression of the gene can be analyzed without the needs to regenerate of a transformed cell into a transgenic plant (Kapila *et al.*, 1997; Srinivas *et al.*, 2008). Therefore, transient expression system can play a major role in the study of plant biology when dealing with vaccination production.

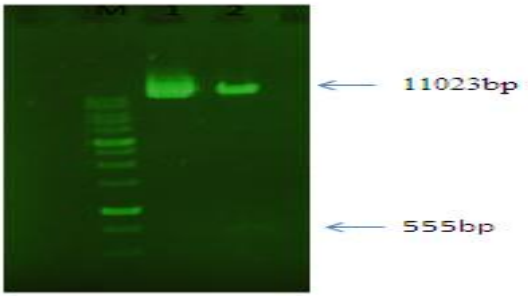

Fig. 2. Confirmation of the presence of VPX gene by digestion reaction. M: 1 kb DNA Ladder(Geneon), Lane1: control (pCAMBIA-VPX).Lane 2 recombinant vector after digestion by AflII and SpeI enzymes.

So far, there is no report on VPX gene expression in plant, the present study was set to investigate the possibility of VPX gene transient expression in tobacco plant.

Currently, different transient expression systems have been deployed to study gene transformation in plant, such as vacuum infiltration (Simmons *et al.*, 2009), particle bombardment (Klein *et al.*, 1988) and agroinfection (Kapila *et al.*, 1997; Yang *et al.*, 2000; Goodin *et al.*, 2002). A method which used in this investigation was vacuum infiltration procedure. In this system, vacuuming the air from the spaces between the cells and subsequently reducing the pressure cause the transfer.

The binary vector, pCAMBIA1305.1 was used for plant transformation, this vector is harbouring CaMV35S promoter, catalase intron sequences and NOS terminator (which induces high level of experssion). Kanamycin and hygromycin antibiotics were applied to monitor the resistance gene for selection of recombinant bacteria and resistance gene for selection of transgenic plant respectively. Kozak sequence was used to enhance the expression of VPX gene and His tag sequence was set for identification and purification of targeted protein from total soluble protein. The results were obtained from this study indicates that agrobacterium vacuuminfilteration can be one of the best approach to evaluate level of VPX gene expression in plant (Fieler *et al.*, 1997, Gidding *et al.*, 2000).

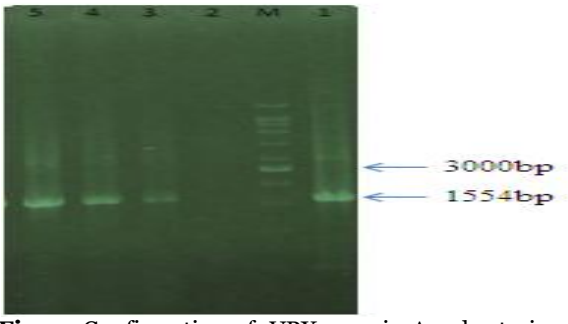

Fig. 3. Confirmation of VPX gene in Agrobacterium by colony PCR technique. M: 1 kb DNA Ladder(Geneon), lane2: Agrobacterium without pCAMBIA-VPX (negative control). Lane 1: positive control , Lane 3-5: random colonies selection.

The findings have demonstrated that the VPX gene expression was significantly higher in wounded leaves than the intact leaves and whole plant. This result was consistent to the similar result which was reported by Park (1997).

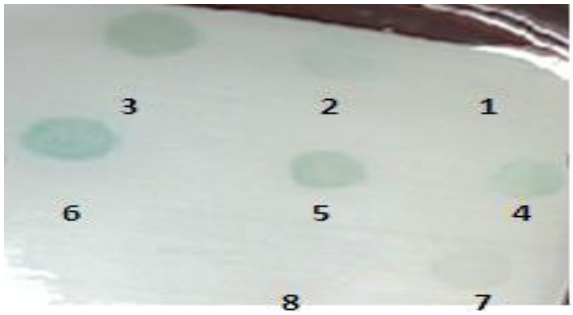

Fig.ure 4. Dot Blot Analysis of Protein Extraction of Tobacco. Lane 1:Intact leave after 4 days post vacuum, Lane 2:Wounded leave after 4 days post vacuum, Lane 3:Intact leave after 5 days post vacuum, Lane 4:Wounded leave after 5days post vacuum, Lane 5:Intact leave after 6 days post vacuum, Lane 6:Wounded leave after 6 days post vacuum, Lane 7:Intact leave after 7 days post vacuum ,Lane 8: Negative control plants.

It also has been observed that the co-cultivation days of post vacuum, generally can be effective for level of expression. The highest level of expression of VPX gene was generated at 6 days after vacuum, which is consistent with the similar results that obtained by Li *et al*., (2007). On the other hand, the expression of transgenic gene was often lasting for 10 days after infiltration and continues to decrease at 7th day (Kapila *et al*., 1997). It also appeared to be a decrease in expression VPX gene at 7 days after vacuum infiltration.

VPX acts as a key precursor of VP2 in which contains all the neutralizing domains and perhaps has the major protein that can offer protective responses in chickens against IBDV(Azad *et al*., 1987; Azad *et al*., 1990; Bayliss *et al*., 1991; Fahey *et al*.,1991). The findings in the present study revealed information which confirms the possibility of VPX gene expression in plant tobacco can be effectively, economically and environmentally safe and acceptable alternative to produce vaccines for controlling IBDV in poultry industry.

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