

CLONING AND TRANSFORMATION OF HEPATITIS B SURFACE ANTIGEN(HBsAg) GENE TO TOMATO (*Lycopersiconesculentum* Mill.)

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Abstract

Hepatitis B virus (HBV) is one of the most serious infectious diseases among human races worldwide. It is estimated that nearly 5% of world population are chronic carriers of HBV. Derivation of the hepatitis B vaccines from yeast and mammalian cells are tedious and expensive, so it can limit the use of these vaccines in developing countries. Biological studies have revealed information that plants are known to act as bioreactors that might successfully be able to offer economical ways for the production and boosting of recombinant proteins. Production of vaccines via plant expression system seems to be effective and promising process which can help to mitigate laborious and cost effects. In this study the appropriate primers were designed concerning highly plant expression sequence at the both sides of HBsAg gene and adequate restriction sites. HBsAg gene was cloned in a plant expression vector; pCAMBIA1304, to deploy constructs which they then were introduced into *Agrobacterium tumefaciens* strain LBA4404 using freeze-thaw procedure and leaf disk technique for transforming into cotyledonary explants of tomato. Transgenic lines were screened and regenerated from selection media containing cephotaxim and hygromycin and CTAB method used to isolated genomic DNA of the transgenic plant. The use of PCR technique and sequencing at the further examination could detect and made a proof of the presence of HBsAg gene in the transgenic plant.

Keywords:

Hepatitis B surface antigen, Vaccine, Gene transformation, Tomato, *Agrobacterium*.

Introduction

It has been well documented that several hundred million people are chronic carriers of HBV (1) which is accounted for 5 % of the world's population. Liver cirrhosis and hepatocellular carcinoma are cause of death in approximately 70-100 millions of them (2,3,4). Hepadenaviridae

is one of the major virus family that Hepatitis B virus is a member of this family (5,6,7). This virus has spherical double shelled particles with a diameter of 42 nm. These particles are known as Dane particles. The inner part of the particles called core antigen (HBcAg) and the outer

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protein of Dane particle is Hepatitis B surface antigen (HBsAg) (4).Molecular farming is a new approach in industry for producing valuable recombinant protein at the high level with low cost in transgenic organisms. In the past decades, many proteins with high therapeutic value including monoclonal antibodies, industrial and therapeutic enzymes, hormones, cytokines, interleukins and vaccines were successfully produced in transgenic plants (8).

Plants can be exploited for the production of recombinant vaccines. Plant cell has enzymatic systems that are necessary for post-translational modification (9). Utilizing the plants as host systems can have several advantages: The plants are capable of producing series of vaccine in large amount and with less expensive manner (10). Plant-based vaccines can offer safety against shared human-animal diseases compared with animal cells-based vaccines (2). These vaccines would be stable under temperature changes; on the other hand, they can be distributed with no need of cold storage (when they are carried in the form of plant tissue or seed materials) (4).

Tomato (*Lycopersicon esculentum* Mill.) is one the most important vegetable crop worldwide (10-13). This vegetable is rich in vitamin C and contains other vitamins, carotene, amino acids, natural sugar and mineral salts (9,13). In addition, tomato is a short day crop that can be grown in greenhouse (12), such characteristics make it a candidate for biopharmaceutical production. The first report of tomato transformation was introduced by McCormick *et al* (14). Since then tomato has been used for production of vaccines, and it was for the first time that rabies virus glycoprotein expressed in transgenic tomatoes (15). There are several antigens that have been successfully produced in tomato: chlorotoxin B subunit, F protein of respiratory syncytial virus (16), *E. coli*

heat-labile enterotoxin B subunit (17) and hepatitis E virus OFR2 region (18).

It must be mentioned that tomato is a model plant for improving other dicotyledonous crop plants and in yeast artificial chromosomes were developed a complete genomic library (11).

In this communication, we report the transformation of HBsAg to tomato and base on our knowledge this work is the first research report of transformation of an antigen (HBsAg) in tomato, in Iran.

Materials and methods

Bacteria

Escherichia coli strain DH5 α used as a host for maintaining and proliferating the construct and *Agrobacterium tumefaciens* strain LBA4404 for transformation of the HBsAg to tomato.

Primers

The appropriate primers were designed considering plant highly expression sequence (Kozzak sequence), both sides HBsAg gene, adequate restriction sites, His Tag sequence to detect expression and purification and factor Xa for removing His Tag sequence. These primers were used for amplification of this gene. The nucleotide sequences of primers were as follow: Forward primer 5'- CATGCC ATGGCACATCATCATCATCATC AACAAACATCAGGATTCCTAG-3' and reverse primer 5'- CATCAGGGT CACCCTATTAAATGTATACCCAGAG AC-3' (*NcoI* in forward and *BstEII* in backward has underlined).

Vectors

Two vectors were used in this study. One was yeast expression vector (pPIC) which carries HBsAg and ampicillin resistance genes and another one was plant expression vector pCAMBIA1304 (CAMBIA Co. Australia) which carries kanamycin resistance gene for selection of the colonies of the bacteria and hygromycin resistance gene for the

transgenic lines (Fig. 1). This vector carries LB and RB (Left and Right Borders for integration of foreign gene into host genome, CaMV35s, (Cauliflower Mosaic Virus promoter which induce high level of transcription), NcoI and BstEII restriction sites and NOS, (Nopaline Synthase terminator which induce

termination process rate), GFP and GUS gene (As reporter genes) and Histidine tag for purification steps (Fig. 2).

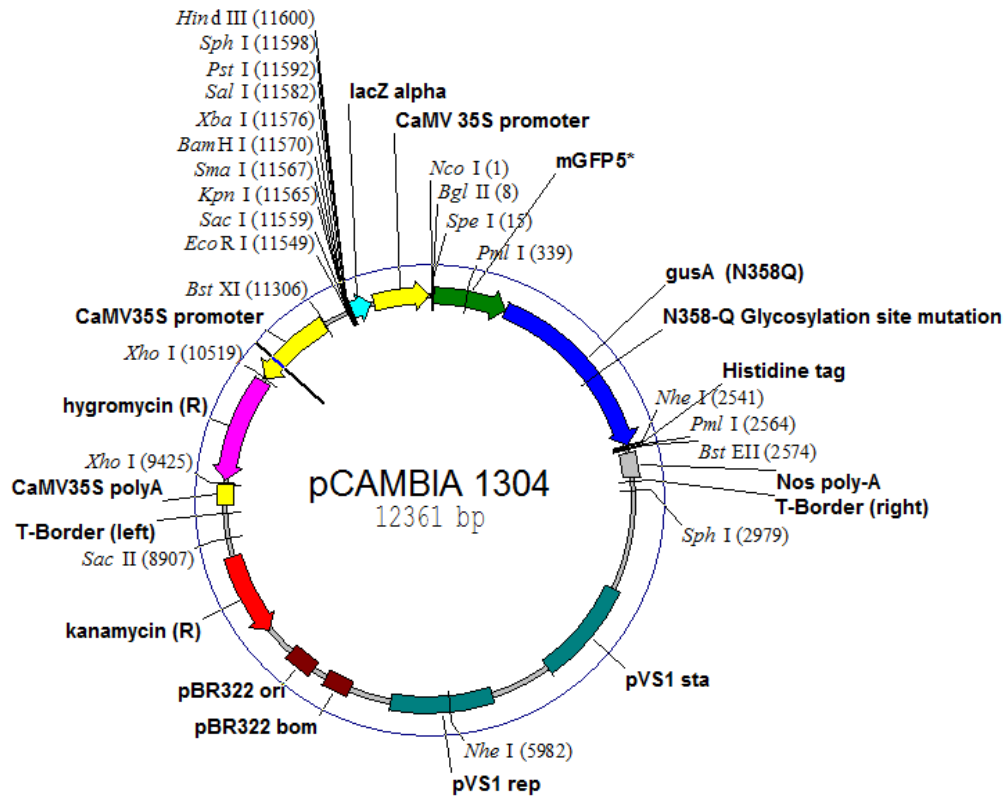


Fig. 1: The Schematic representation of plant expression vector pCambia1304.

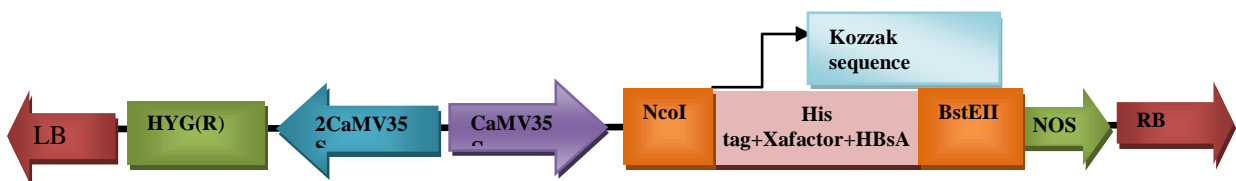


Fig. 2: T-DNA region of pCambia-HBsAg. LB and RB: Left and Right Borders, HYG(R): Hygromycin selectable marker, CaMV35s: Cauliflower Mosaic Virus promoter, NcoI and BstEII: restriction sites and NOS: Nopaline Synthase terminator.

Plant materials

Tomato (*L. esculentum* cv. Cal J), provided by Petoseed company (Branch of Bazargan Kala, Tehran, Iran). In order to setting the sterile and optimum growth conditions for plant tissue, after the seeds were sterilized, they transferred then into Murashige and Skoog (MS) media for seedling germination. When cotyledons were emerged, at the 9-12 days old of the growth, the proper cotyledons were selected for transformation.

Cloning of HBsAg gene in pCAMBIA1304

The Hepatitis surface Antigen fragment had been already cloned in the pPIC vector. This vector used as a template for PCR. The HBsAg (the gene) is 681bp (Including start and stop codons), however when it amplified by the primers, it reached to more than 720bp because of adding some extra sequences to the primers. The HBsAg gene was amplified using mentioned primers, then PCR product was extracted from the gel using BIONEER kit. So, this was digested with BstEII and NcoI in NEB buffer 3. The digested PCR product was extracted from gel using gel extraction kit again and then ligated with digested pCAMBIA1304 vector by T4 DNA ligase (Fermentase ligation kit) in 16°C overnight and the results were transformed to *E. coli*. Consequently, the HBsAg fragment was replaced in GUS-GFP region of pCAMBIA 1304 under the control of CaMV35s promoter and the NOS terminator by ligation process.

Transformation of E. coli using heat shock method

For transformation of recombinant vector to *E. coli*, 5µl of pCAMBIA-HBsAg

binary vector was mixed with 1.5 ml of competent cell of *E. coli* strain DH5α then transferred in a microcentrifuge tube and put it on the ice for 30 min. Vector was immediately submerged in bain-marie for 90 seconds at 42°C and then was put on ice for 2 min. Eight hundreds microliter of LB (Luria-Bertani Broth) was added to microcentrifuge tube. The culture was grown for 1 hour at 37°C shaker. Eventually, 100µl of transformed cells were cultured on the LB plates containing 50 mg/L kanamycin. Plate was incubated for 16-18 hours at 37°C (19). The pCAMBIA-HBsAg recombinant plasmid was verified by colony PCR (Fig. 3), digestion and sequencing.

Transformation of A. tumefaciens using freezing and thawing method

A. tumefaciens strain LBA4404 was grown overnight at 28°C in liquid LB medium with 80 mg/L streptomycin. Agrobacterium culture 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 bain-marie for 5min at 37°C. 1ml of LB was added and culture was grown 3 hours in a 28°C shaker. 100µl of transformed cells were cultured on the LB plates with a combination of 50 µg/ml kanamycin and 80 mg/L streptomycin. Plate was incubated for 2 days at 28°C (19).

Plant transformation

Tomato cotyledons were used for agrobacterium mediated transformation. Before inoculation explants were placed into pre-culture media and incubated for 48 hours. After

inoculation with *Agrobacterium*, the explants were placed into co-culture media and then incubated in dark place at 28°C for 48 hours. Finally, inoculated explants were transferred into selected media including co-culture media containing 6-benzylaminopurine (BAP 2 mg/L), 1-naphthaleneacetic acid (NAA 0.1 mg/L), cephotaxime (250 mg/L) and hygromycin (7.5 mg/L). Then they were placed in a growth chamber at a photoperiod of 16:8 (L:D) hours.

Polymerase chain reaction (PCR) analysis of transgenic plant

Genomic DNA of transgenic plant was extracted, using modified CTAB method (20). PCR amplification of genomic DNA for presence of HBsAg gene was carried out using primers described above and plants with HBsAg gene were specified.

Results

The nucleotide sequence of HBsAg gene was modified using designed primers via PCR technique. 720-bp-long fragment of this PCR product was ligated by T4 DNA ligase into the CaMV35S in upstream and

Nos terminator in downstream of pCAMBIA1304 vector between BstEII and NcoI restriction sites. Then these construct were transformed into *E. coli* strain DH5 α . Presence of HBsAg gene was proved by colony PCR technique (Fig. 3) and digestion reaction which was conducted by BstEII and NcoI restriction enzymes (Fig. 4). Finally, designed construct were transformed in to *Agrobacterium tumefaciens* strain LBA4404 using freeze-thaw method and using Colony PCR technique, it was confirmed that those recombinant constructs were transformed into the *Agrobacterium* (Fig. 5).

Plant transformation

After 3 weeks, the cotyledonary leaves which were inculcated with *Agrobacterium*, regenerated a new plants at the cutting edges (Fig. 6). The regenerated plants sub-culture on the same medium (MS medium, 2mg/L BAP, 0.1 mg/L NAA, 7.5 mg/L hygromycin and 250 mg/L cephotaxime) in tissue culture glass bottles (Fig. 7).

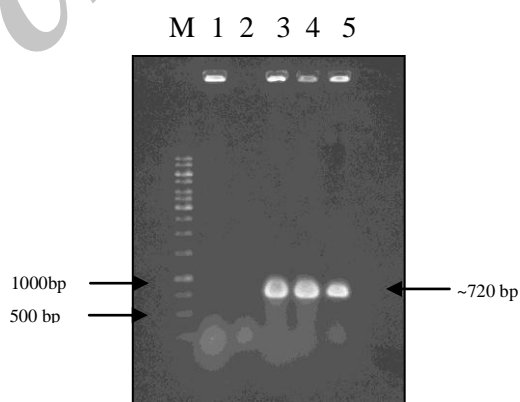


Fig. 3: Proof of the presence of HBsAg gene in pCAMBIA1304 by colony PCR technique. M: GeneRuler™ 1 kb DNA Ladder (Fermentase), Lane 1: Negative control (pCAMBIA1304 without HBsAg gene), Lane 2: ddH₂O, Lane 3: positive control (pPIC), Lane 4 and 5: Random colonies selection.

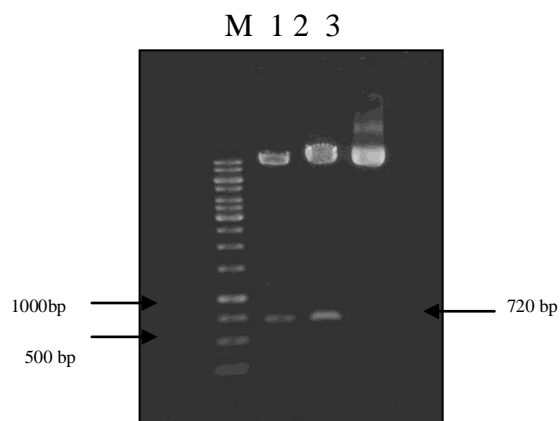


Fig. 4: Proof of the presence of HBsAg gene by digestion reaction. M: GeneRuler™ 1 kb DNA Ladder(Fermentase), Lane 2 and 3: recombinant vector after digestion by BstEII and NcoI enzymes, Lane3: Negative control (pCAMBIA1304 without HBsAg gene).

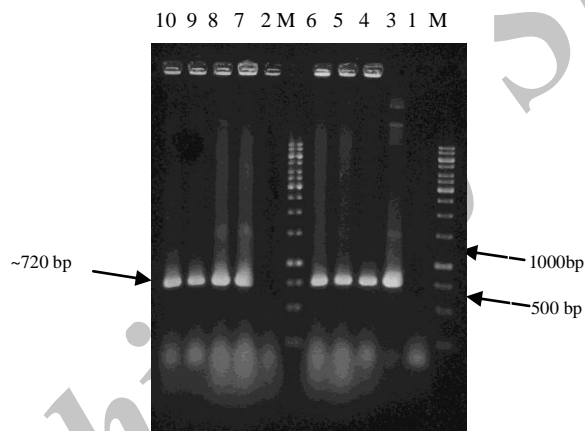


Fig. 5: Confirmation of HBsAg gene in *Agrobacterium* by colony PCR technique. M: GeneRuler™ 1 kb DNA Ladder(Fermentase), Lane1: ddH₂O (negative control), lane2: *Agrobacterium* without pCAMBIA-HbsAg (negative control). Lane: positive control (pPIC), Lane 4-10: random colonies selection.

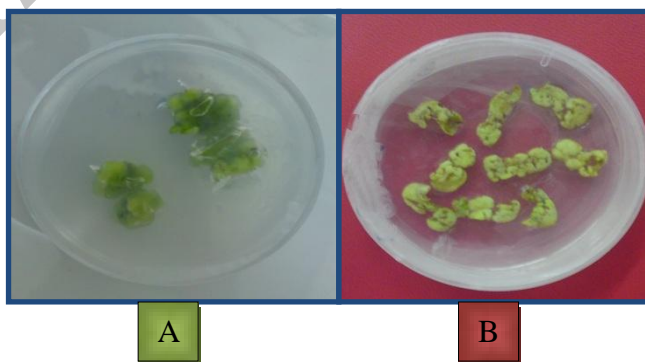


Fig. 6: Emergence of shoot from cotyledonary explants. A- regenerated explants after three weeks on the selection medium. B- negative Control (these explants were not inoculated with *Agrobacterium* on the same media).

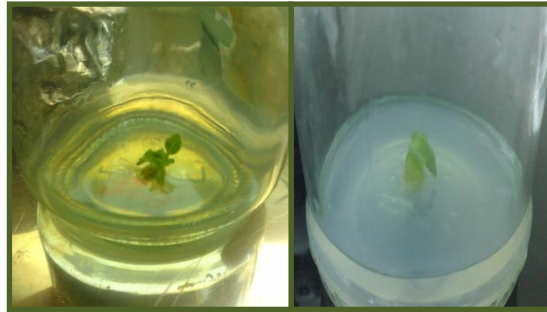


Fig. 7: Transforming the regenerated plants into the tissue culture glass bottles.

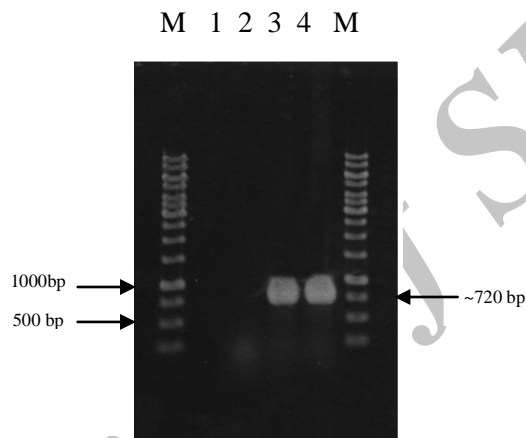


Fig. 8: PCR analysis of transgenic plants. M: GeneRuler™ 1 kb DNA Ladder. Lane 1: ddH₂O. Lane 2: wild type plant. Lane 3: transgenic plant. Lane 4: positive control (pPIC).

Extraction of genomic DNA and PCR analysis

Genomic DNA of transgenic and control plants were extracted using CTAB method(21). The presence of HBsAg gene into transgenic plant was proofed and detected using PCR technique, while control plants did not show any band on agarosegel electrophoresis (Fig. 8).

Discussion

The viruses, especially Hepatitis B virus, continue to challenge the human life and will become a major threat in our world. Because the recombinant vaccines are too expensive, their uses for immunization

propose have restricted in developed countries, so need to have simple solution, safe and inexpensive systems to produce these vaccines (10). One particular system which recently becomes significantly popular among biologists is use of the plants to produce therapeutic proteins and vaccines. It is estimated that the cost of recombinant proteins that produced by plants can be lower than those of cell-based production systems such as microbial fermentation systems (2-10% cost reduction) and mammalian cell cultures (0.1% cost reduction) (22). In the present study Hepatitis B surface antigen gene was cloned and transferred into

tomato. The binary vector, pCAMBIA1304 was used in plant transformation. Production of high yield DNA can be achieved by high copy number in E.coli through this vector. pCAMBIA 1304 induce high stability in Agrobacterium by pVS1 replicon. In addition it has small size which facilitate manipulation of the plasmid. This vector carries CaMV35S promoter, GUS and GFP coding regions, kanamycin and hygromycin resistance genes and NOS terminator. The GUS and GFP coding regions were replaced by the HBsAg gene between the BamHI and NcoI restriction sites. There are two factors which can promote the transcription. One is promoter and another is polyadenylation signal. In dicot plants CaMV35S is a suitable promoter because it is strong and constitutive, can cause high level transgene expression in leaves, fruits, tubers, roots and other organs (23). Strong polyadenylation signal is required for transcription stability (24). These views has been stated in other publications of authors in somewhere else (6, 7). In addition, forward primer was designed in concerning some sequences such as: Kozak sequence to boost the expression of HBsAg gene (23). His tag sequence is for recognition and purification of the protein from other plant proteins and factor Xa is for separation of the proteolytically cleaved His tag from the purified protein. The *Agrobacterium*-mediated transformation is a method for transferring target gene into plants. It also can cause constant transformation, and has a high potential of plant regeneration in comparison with other methods such as transient expression and cell suspension

culture (11). Transient expression is easier, faster and cheaper to use but the transgene can only express in infected tissues and would not transfer to the next generation (13). The cell suspension culture is faster but needs to clean rooms for cell proliferation and growth and consequently is more expensive than other methods (10). Tomato is one of the most important vegetable crop worldwide and has a high fruit biomass (13). Therefore this plant was selected as an expression platform for this research. In summary, this investigation has been shown that tomato plant can actually be used in molecular farming to elevate the production of recombinant protein such as vaccines(25). When compare the production of vaccine via transgenic plants over other vaccine production systems such as yeast and mammalian cells, it has been shown that the plants have unique advantages including, inexpensive, biosafety, large scale production and easy delivery. Therefore, tomato plant can become an excellent and robust candidate for production of hepatitis B vaccine. If one could minimize the certain limitations in which become main constraints, such as difficulty in regeneration procedure, time consuming and difficulty in expensive purification steps. It is likely that in future the development of new biotechnological techniques and advanced knowledge will become useful tools in offering new opportunities to overcome these barriers.

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