



Original article

Isolation, cloning and transgenic expression of hepatitis B surface antigen (HBsAg) in *Solanum lycopersicum L*

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ABSTRACT

The Hepatitis B virus (HBV) infection is one of the most widespread viral infections of humans. HBV causes acute and chronic hepatitis. Chronic hepatitis leads to hepatocellular carcinoma, which is a significant cause of death. DNA-based immunization programs to control the spread of Hepatitis B in developing countries are costly and require special storage and transportation. The alternative way is to express Hepatitis B surface antigen (HBsAg) in plants to develop oral vaccines. In this study, HBsAg gene was isolated, cloned, and then transformed in tomato plants. The transgenic tomato plants were confirmed through RT-qPCR. HBsAg expression was analysed in mature green and red stages of tomato fruit through quantitative real-time PCR. It was observed that expression of HBsAg was high in matured red tomato as compared to mature green. The present study is the first step to developing *Solanum lycopersicum* as an edible vaccine production system in this world region.

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1. Introduction

Hepatitis B virus (HBV) is a double-stranded DNA virus that belongs to Hepadnaviridae family (Rehermann and Nascimbeni, 2005). The genome of HBV virus is 3.2 kb in length, and it is the smallest among DNA viruses (Kim et al., 2016). There are ten genotypes, and these genotypes have different geographical distributions (Sunbul, 2014). The present HBV epidemic results from introducing and disseminating new subgenotypes from main A, D, and F genotypes over a long period. Genotypes A and D are mostly found in Africa, Europe, and India; B and C in Asia; genotype E is restricted to West Africa, and genotype F prevalence is in Cen-

tral and South America. The lipid envelope of infection virion contains hepatitis B surface antigen surrounded by an inner nucleocapsid composed of hepatitis B core antigen. (Gerlich and Robinson 1980).

Hepatitis B virus is the leading cause of acute and chronic liver disorders, and its infection is a serious global health issue (Madihi et al., 2020). About 40% of chronic hepatitis cases lead to hepatocellular carcinoma, the second major cause of death. Deaths due to liver cirrhosis and hepatocellular carcinoma are 1.34 million per year (WHO, 2018).

Although effective vaccines are available against hepatitis B infection, it is still a major health issue in the developing world (Kumar et al., 2007). The current immunization programs of HBV are based on recombinant vaccines derived from the costly microbial fermentation process and mammalian cell cultures. Furthermore, these vaccines require well-developed infrastructure to keep them in cold-chain throughout their manufacturing process, transportation, and storage which is not affordable for developing countries. In contrast, plant-based vaccines can be produced at a much lower cost and are independent of cold storage requirements (Penney et al., 2011). Molecular farming in plants has many advan-

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tages over existing expression systems as it does not require skilled labor and expensive equipment (Fischer & Emans, 2000; Giddings et al., 2000; Julian et al., 2003; Twyman 2003). The production cost of recombinant proteins in a plant expression system is 2–10% of the cost of microbial fermentation and 0.1% of the cost of mammalian cells or transgenic animals (Molowa et al., 2002). Plants-based vaccines prefer mammalian expression systems as no external carbon source is needed for plants because they are fueled by photosynthesis. Plants-based vaccines are free of contamination by a mammalian pathogen, another critical advantage of a plant vaccine. Because of these advantages, the production of antigens, vaccines, and other eukaryotic proteins in plants is more attractive (Gunasekaran and Gothandam 2020).

The production of edible vaccines based on transgenic plants is one of the positive directions in new types of vaccines. *HBsAg* gene has already been expressed in tobacco (Mason et al., 1992; Thanavala et al., 1995; Sunil Kumar et al., 2003), potato (Richter et al., 2000), banana (Elkholy et al., 2009), tomato (Salyaev et al., 2007; Srinivas et al., 2008) and other fruits and vegetables (Marcondes and Hansen 2008; Pniewski et al., 2006).

The above research works identified the potential use of *HbsAg* gene in plant systems to develop edible vaccines. In the present project, the same technology employed in the local registered variety of tomatoes will not pose any future intellectual property rights issues. At present, tomato is an important crop plant cultivated worldwide, and its production and consumption continue to increase. Tomato is also a major source of essential nutrients. Most importantly, the tomato's successful molecular farming model has been established (Srinivas et al., 2008, Fisher et al., 2004). In Pakistan, tomato cultivation and consumption have increased in fresh and processed forms, hence selected for the present study.

2. Materials and methods

2.1. Isolation and cloning of *HBsAg* gene

HBsAg gene, complete cds having gene bank accession number AY738913.1 was retrieved from nucleotide database and was analyzed for restriction sites through BioEdit software. Unique *Bam*H1 and *Sac*1 enzymes were selected to clone the *HBsAg* gene under 35S promoter in pBI121. Full-length primers with the above unique restriction sites were designed. The forward primer was added with *Bam*H1 site beside 5'-CG-3' protective bases, while the reverse primer was added with *Sac*1 restriction site beside 5'-C-3' protective base. The sequence of forwarding primer was 5'-CGGGATC CATGGAGAACATCACATCAGGA-3', and the reverse primer was 5'-CGAGCTCTTAAATGTATACCCAAAGACA-3'. DNA from an infected person was isolated using DNA purification kit (Thermo Scientific, #K0721). *HBsAg* gene was amplified from 100 ng of DNA using phusion high fidelity DNA polymerase (Thermo Scientific, #F530L). The amplified product was purified using GeneJET PCR Purification Kit (Thermo Scientific, #K0701). A 1000 ng of the purified product was digested with *Bam*H1 (Thermo Scientific, #ER0051) and *Sac*1 (Thermo Scientific, #ER1131) enzymes. Similarly, pBI121 was also digested with *Bam*H1 and *Sac*1 enzymes. The restricted backbone of pBI121 was gel-eluted using GeneJET Gel Extraction Kit (Thermo Scientific, #K0691). A ligation reaction was performed using T4 DNA Ligase (New England Biolabs, #M0202). The ligated product was transformed into DH5 α through electroporation. PCR was carried out from plasmids extracted from transformed colonies for confirmation of clone. Further confirmation of clone was carried out through sequencing. The resultant pBI121:*HBsAg* was transformed and confirmed in *Agrobacterium tumefaciens* strain GV3101pMP90 (2nd Lab China #AC1001).

2.2. Genetic transformation of tomato

The Rio Grande tomato variety seeds were surface-sterilized initially with 70% ethanol for 1 min and then with Clorox (5.25% sodium hypochlorite) for 10 min. The seeds were then rinsed three times with distilled water to remove the traces of clorox and then sterilized. For seven days, the seeds were germinated on MS0 medium at 25 \pm 2 $^{\circ}$ C under a 16/8 light and dark. Cotyledonary leaves were cut from seven days old seedlings and were used for infection by transformed *Agrobacterium tumefaciens*. *Agrobacterium* suspension was prepared by inoculating a single colony in 25 ml of liquid LB media containing 50 mg/l kanamycin and 25 mg/l rifampicin and incubated for 36 h in the dark at 28 $^{\circ}$ C while shaking at 170 rpm. The 36 h grown culture was centrifuged at 4000 rpm for 5 min, and the supernatant was discarded. The pellet of the cell was suspended in inoculation media, and OD₆₆₀ was adjusted to 0.2. All media compositions are given in Table 1. This *Agrobacterium* suspension was added to cotyledonary leaves and placed on a shaker at 50 rpm for 20 min. The cotyledonary leaves were filter dried and placed on co-cultivation media for two days. The cotyledonary leaves were shifted to pre-selection media after rinsing three times with autoclaved distilled water and a final wash with inoculation media containing 500 mg/l cefotaxime for 15 min. After 07 days, cotyledonary leaves were shifted to selection media, allowing explants to regenerate shoots for 45 days in 16/8 light/dark at 25 $^{\circ}$ C \pm 2 $^{\circ}$ C. Passaging was regularly done after 15 days. Shoots having a length of 5 cm were placed on rooting media. Well-established roots were developed after 21 days. The plantlets with well-established roots were acclimatized with ambient temperature and humidity conditions in the growth room and then transplanted in pots containing soil.

2.3. Molecular analysis of transgenic plants

DNA was extracted from T0 transformed and non-transformed plants using GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific #K0791). T0 transformed plants were analyzed for transgene using PCR. The sequences of full-length primers used in PCR were 5'-ATGGAGAACATCACATCAGGA-3' and 5'-TTAAATGTATACC CAAAGACA-3'.

For transcript analysis, total RNA was extracted from mature red tomato fruits of T1 plants using GeneJET RNA Purification Kit (Thermo Scientific, #K0731) as described previously (Ibrahim et al. 2021). Nanodrop was used to measure the quality and quantity of RNA. A260/A280 value was kept above 1.8 while A260/A230 value was maintained above 2. About 500 ng high quality of RNA was transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #K1622). 2 μ l of cDNA was used as a template to amplify the *HBsAg* gene using gene-specific 5'-G AACCTGCACGACTCTGCT-3' and 5'-GAGCCAGGA GAAACGGGCTGA-3' primers. The amplified products were analyzed by a gel documentation system (Bio-Rad, USA).

Quantitative Real-Time PCR was also carried out to check the relative expression of *HBsAg* gene in mature green and mature red stages of tomato fruits using FastStart Universal SYBR Green Master (Rox) (Roche, USA). Tomato LeEF-1 having gene bank accession number X14449.1 was used as an endogenous reference for normalization in the experiment. The sequence of forwarding primer of endogenous control was 5'-CTCTCAGGCTGACTGTGCTG-3', and of reverse, primer was 5'-GTTCACGGGCTGACCATCT-3'.

Gene-specific primers were designed using primer3 free online software to be used in quantitative PCR. A free edition of Beacon Designer software evaluated the integrity, specificity, and quality of primers. Cross-dimers, self-dimers, and hairpin structures were avoided. Matches of 3 bp at three ends of primers were also avoided. The quality of the amplicon structure was also evaluated

by mFold software. Following a previous protocol (Thornton and Basu, 2011), Real-Time PCR primers were designed. PCR master mix was prepared by mixing 12.5 μ l FastStart Universal SYBR Green Master (ROX), 0.5 μ l each of forward and reverse primer (15 μ M), 400 ng of template cDNA to the final volume of 25 μ l. The PCR profile was set at 95 °C for 10 min for initial activation of FastStart Taq DNA polymerase and then 40 cycles of 95 °C for 15 seconds, 60 °C for 1 min, and a final melt curve analysis of 60 to 95 °C for 1 min. The data was recorded as relative quantity.

3. Results

3.1. Isolation and cloning of HBsAg gene

The 696 bp fragment containing 681 bp HBsAg gene was amplified with primers containing *Bam*H1 and *Sac*1 restriction sites (Fig. 1a). The amplified product was purified, and 1 μ g of the purified product was digested with *Bam*H1 and *Sac*1 simultaneously. Similarly, pBI121 was also digested with *Bam*H1 and *Sac*1 enzymes, releasing a larger fragment containing the backbone of pBI121 (Fig. 1b). A ligation reaction was performed between pBI121 backbone and inserted containing HBsAg gene. The clone was confirmed through PCR using full-length primers, which produced 681 bp fragments, indicating that HBsAg gene has been successfully cloned into pBI121 under 35S promoter. The clone is named as pBI121:HBsAg. The physical map of pBI121:HBsAg was shown in Fig. 2.

3.2. Genetic transformation of tomato

About 302 cotyledonary leaves of *Solanum lycopersicum* L.cv. Rio Grande were transformed with *A. tumefaciens* having pBI121:

HBsAg in three different experiments. Of 302 cotyledonary leaves, only 26 cotyledonary leaves could survive on selection media and regenerated plants with an average transformation efficiency of 8.58%. The 39 well-rooted plants were produced on rooting media. Out of it twenty-one plants acclimatized to ambient temperature and humidity conditions (Fig. 3). Six plants went through all stages of growth and development and produced enough seeds for characterization (Table 2). The different steps involved in the transformation of tomato through *Agrobacterium* are shown in Fig. 4.

3.3. Molecular analysis of transgenic plants

The DNA template from transgenic plants gave amplification of 681 bp fragment while no such amplification was observed from DNA of control plant (Fig. 5a). Transgene expression was detected by Reverse transcriptase PCR (RT-PCR) analysis which amplified 155 bp fragments from transgenic plants (Fig. 5b).

3.4. Expression analysis of HBsAg in tomato fruits

The quantitative expression of the HBsAg gene was measured in mature green and mature red stages of tomato fruits. Mature red tomato showed higher HBsAg gene expression in all events than mature green (Fig. 6). Expression of HBsAg gene was 2.47 to 3.6 fold higher in mature red tomatoes compared to mature green. Maximum fold difference was observed in event 6, and minimum fold difference was observed in event 1 (Table 3).

4. Discussion

Hepatitis B virus (HBV) is becoming a major threat to the whole world. Current immunization programs are based on recombinant vaccines derived from microbial fermentation and mammalian cell cultures. Using mammalian and yeast expression systems for the production of HBV vaccines requires expert personnel and excellent infrastructure. These vaccines are expensive, require a specific purification system, cold storage, and a unique transportation facility. Developing countries have limited facilities and resources to develop and sustain such recombinant vaccines to immunize its population. An attractive alternative to these is plant-based edible vaccines which are cost-effective and most suitable for developing countries (Twyman et al., 2003). Plant-based vaccine production seems to be effective and less laborious (Baesi et al., 2011).

The surface protein of the hepatitis B (HBsAg) virus is immunogenic and varies from 22 nm to 42 nm in diameter. These surface antigens have been used to develop vaccines against the hepatitis B virus. Hepatitis B surface antigen proved itself as a candidate region of the virus to be efficiently used in vaccine development (Arakawa et al., 1998). HBsAg is the first viral antigen that has been expressed in potatoes and bananas (Mason et al., 1992). The HBsAg protein assembles in plant tissues similar to those found in the commercial vaccine and blood of infected humans (Mason et al., 1992).

In the present investigation, a gene encoding the same hepatitis B surface antigen was isolated and transformed in the tomato plant. Its expression level was analyzed in the mature green and mature red stages of tomato fruit. Ripe red tomato showed higher expression of HBsAg gene in all events compared to mature green. Transcript expression of the HBsAg gene was 2.47 to 3.6 fold higher in mature red tomatoes compared to mature green. Ming Lou et al., 2007 also found enhanced expression of the HBsAg gene in mature red tomato fruits compared to unripe fruits and leaf tissues. In the present study, the expression of HbsAg was carried out by CaMV35S promoter, and this promoter is considered suitable for

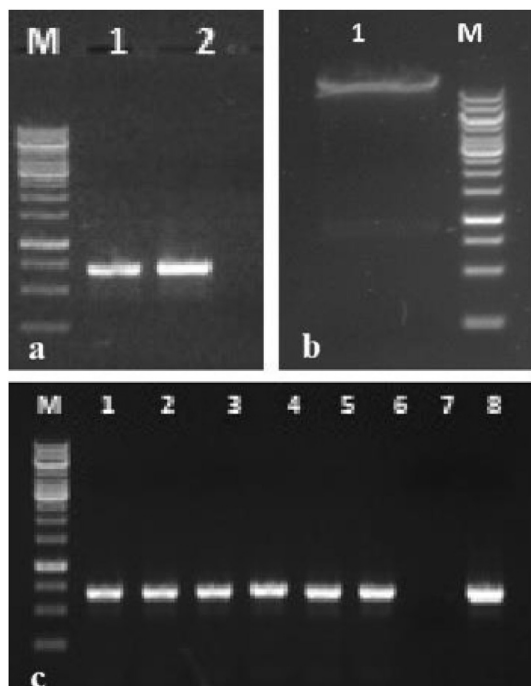


Fig. 1. Different steps in isolation and cloning of HBsAg gene. M, 1 kb DNA ladder; lane 1 and 2, 696 bp fragments amplified from DNA extracted from the blood of HBV patient. **a**, restriction of pBI121 with *Bam*H1 and *Sac*1 restriction enzymes. M, 1 kb DNA ladder; lane 1, pBI121 digestion produced two fragments, upper band is the backbone of pBI121, which was gel eluted. **c**, confirmation of pBI121:HBsAg through PCR. M, 1 kb DNA ladder; Lane 1–6, 681 bp fragment amplified from plasmids extracted from transformed colonies; lane 7, water control; lane 8, plasmid control.

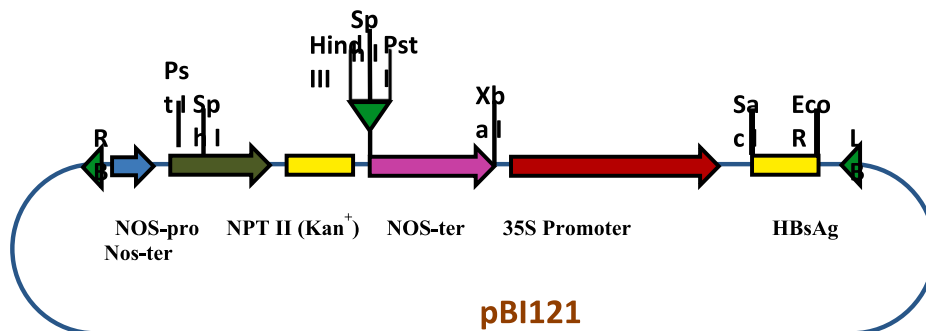


Fig. 2. Physical map of resultant pBI121:HBsAg .

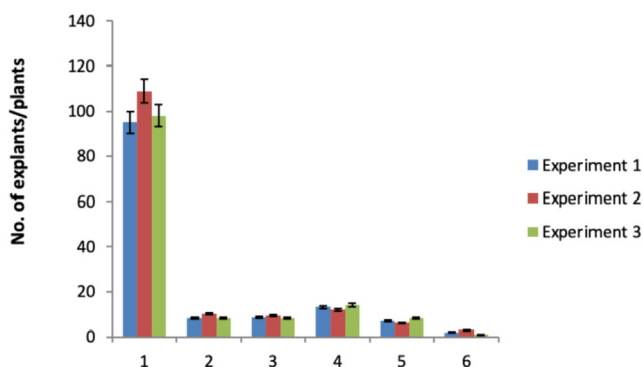


Fig. 3. Summary of transformation experiments. 1: Total explants used, 2: kanamycin-resistant explants, 3: Transformation efficiency (%), 4: Rooted plantlets, 5: Acclimatized 6: Plants produced seeds.

most dicot plants (Rajabi-Memari et al., 2006) while Lou et al., 2007 used fruit specific promoter to drive the expression of *HbsAg*. Thanavala et al. (2005) expressed hepatitis B surface antigen (*HBsAg*) in potatoes, and they recommended that plant-based

edible vaccine should be considered an essential part of global immunization. Elkholy et al. 2009 expressed *HBsAg* in bananas, and its expression was measured by western blot. Their study also concluded the feasibility of the expression of *HBsAg* in plants. Transgenic lettuce (*Lactuca sativa L.*) expressing hepatitis B virus surface antigen was found immunogenic in mice and human volunteers (Kapusta et al., 1999).

Different proteins, including immunogenic, have been expressed in the plant system and orally administrated to humans. Transgenic lettuce and potato expressing *HBsAg* have been found to elicit an immune response in humans (Chikwamba, 2002, mason et al., 1992). Plant selection should be made according to the eating habits of the targeted population and the agricultural infrastructure of that region. Keeping in view the production and consumption of tomatoes as fresh and processed food in Pakistan, it has been selected to express *HBsAg*. Six transgenic events showed successful expression of the *HBsAg* gene. It is proposed that in future studies, the immunogenicity and biosafety of the different transformed events will be compared using mice as an experimental organism. The present study was the first step in Pakistan to develop tomatoes as an edible vaccine production system in this world region.

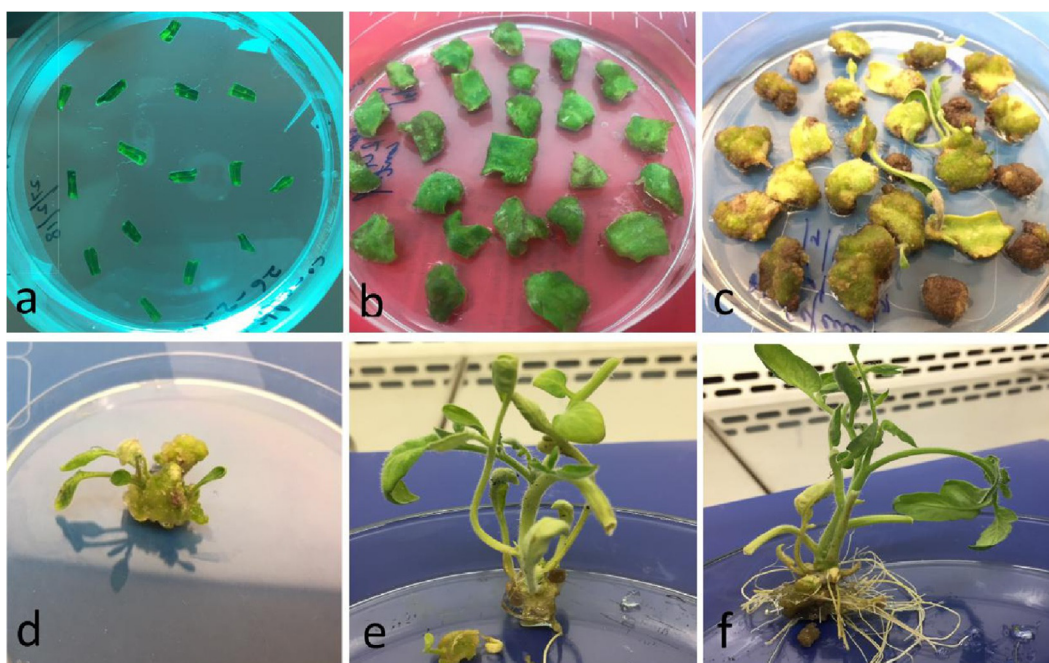


Fig. 4. Regeneration and shoot induction from transformed cotyledonary leaves. a, Transformed cotyledonary leaves; b, Shoot induction from transformed cotyledonary leaves on selection media; c, shoot elongation on selection media.

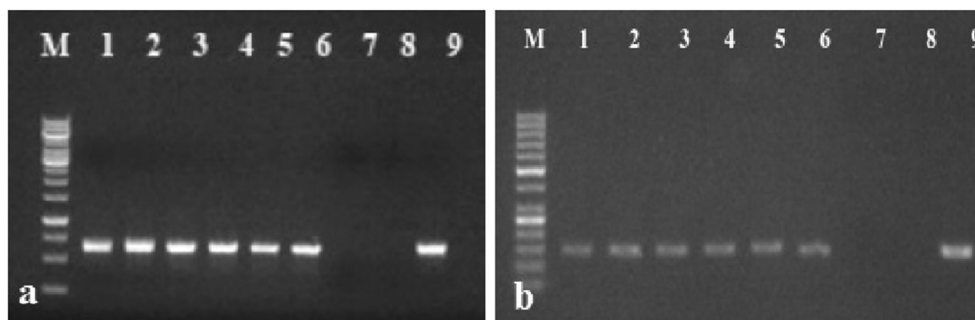


Fig 5. Confirmation and expression of the transgene in transgenic plants. a. PCR confirmation of transgene. M, 1 Kb DNA ladder; lane 1–6, amplification of 681 bp fragment from transgenic plants; lane 7, control plant; lane 8, water control; lane 9, plasmid control. b. expression of the transgene. M, 1 Kb DNA ladder; lane 1–6, amplification of 155 bp fragment from transgenic plants; lane 7, control plant; lane 8, water control; lane 9, plasmid control.

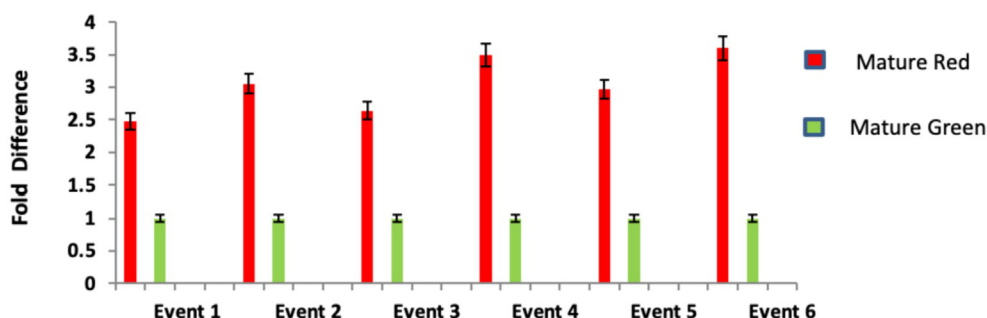


Fig. 6. Expression analysis of *HBsAg* gene in fruits of different tomato events. In every event, the expression is higher in the mature red stage.

5. Conclusions

An efficient transformation system was developed in tomatoes to produce edible vaccines against the hepatitis B virus. The results of this work recommended that plants can be used to produce immunogenic proteins against different diseases. This research concluded that tomato is a suitable candidate for the production of vaccines as it can be easily transformed, produced rapidly, and eaten raw. Transgenic plants expressing different antigens have been done successfully, but still, many questions remain unsolved. So further work needs to be done in the future, such as dose optimization and checking the antigen's immunogenicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2021.11.012>.

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